Role of p53, CstF-50, and BARD1 in the Regulation of mRNA Processing following DNA Damage

by

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

2009

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

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ABSTRACT

Following UV irradiation, cellular mRNA levels are transiently decreased due to the inhibition of transcription and mRNA polyadenylation. The UV-induced inhibition of 3' processing reflects the interaction of the polyadenylation factor CstF-50 with the tumor suppressors BRCA1/BARD1 as well as the BRCA1/BARD1-mediated degradation of RNA polymerase II (RNAP II). As CstF-50 can interact with BRCA1/BARD1 and RNAP II inhibiting or activating polyadenylation, respectively, and cells with reduced levels of CstF show an enhanced sensitivity to UV and reduced ability to ubiquitinate RNAP II and repair DNA, we propose a coordinating role for this factor in the DNA damage response.

Our studies add complexity to the cellular response to UV treatment by linking RNA processing to the p53 network. Here, we show that the C-terminal domain of the tumor suppressor p53 associates not only with BARD1 but also with CstF-50. Although the p53/CstF complex is detected independently of DNA damage, we found that the tumor suppressor BARD1 can coexist in complexes with CstF and p53 only after UV treatment. Consistent with this, we found that the C-terminal domain of p53 inhibits pre-mRNA cleavage in vitro. Significantly, siRNA-mediated knockdown of p53 has different effects on the UV-induced inhibition of polyadenylation in cells expressing different levels of p53. Supporting the physiological significance of these results, a previously identified mutation in p53 (Ser241Phe) decreases the p53-BARD1 interaction and abrogates inhibition of polyadenylation, which can be restored by the expression of wild-type p53. We also found that p53 participates in the post-transcriptional regulation of endogenous transcripts of housekeeping but not p53-regulated genes.

We also determined that the interaction between CstF-50 and BARD1 involves the ankyrin-BRCT linker but not the ankyrin or BRCT domains. The structural plasticity imparted

by the ANK-BRCT linker helps explain the regulated assembly of different proteins involved in DNA damage to BARD1, including BARD1-dependent stabilization of p53 and the induction of apoptosis. Furthermore, supporting the role of BARD1 in signaling in the DNA damage response, we found that UV-induced BARD1 phosphorylation is important not only for its functions associated with CstF-50 but also for its functions in regulating p53 phosphorylation and stability.

SIGNIFICANCE

The cellular response to DNA damage is a protective mechanism against disease. This cellular response could be either in the survival mode, where DNA repair occurs and gene expression is controlled along with cell cycle arrest, or in the death mode, where apoptosis is induced. Though the mechanistic connection between DNA repair/cell survival and apoptosis has not been established, several proteins are involved in both pathways, such as p53, BARD1 and BRCA1. Determining the connection between DNA repair, control of cell proliferation and apoptosis is critical in understanding how the breakdown of these pathways leads to various diseases, especially cancer.

Most cancer cells show mutations in various tumor suppressor proteins, such as p53, BARD1 and BRCA1. The p53 protein is especially significant in that it is mutated in more than 50% of known human cancers. Though the past several decades have provided an explosion of information on these tumor suppressor pathways most studies have usually focused on a single discrete pathway and its mechanism of action. This is also reflected in the therapies developed so far in treating cancer, where a single tumor suppressor pathway is targeted to combat the disease. However, more recent advances show that the functions of tumor suppressor pathways are far more interconnected than previously believed. Understanding the interconnections between these various tumor suppressor pathways will provide valuable insight into the field of cancer biology as a whole and most importantly to develop meaningful therapies in combating cancer.

This dissertation provides a broader understanding of the interplay between tumor suppressor and mRNA processing pathways following DNA damage. The studies presented in this dissertation focus on the functional interconnection between the p53 and BARD1 tumor suppressor pathways and the 3’ mRNA processing machinery, and their role in the cellular

response to DNA damage. We have identified that a functional connection between these pathways could control gene expression in a novel and unexpected manner: by controlling mRNA 3' end processing. In addition, a new regulatory role of phosphorylated BARD1 on p53 stability has been identified as well as structural insights into the interactions of BARD1 with p53 and CstF-50. Given that almost all eukaryotic mRNAs are polyadenylated, the identification of the 3' mRNA processing machinery as a p53 and BARD1-target expands the repertoire of p53/BARD1-regulated genes. These findings describe the potential of the 3' mRNA processing machinery to fill roles in signaling networks, which have persisted as a long-standing mystery. With accumulating evidence revealing the importance of mRNA 3' end processing in cell growth control (Chuvpilo et al. 1999; Takagaki and Manley 1998; Takagaki et al. 1996) and perhaps in disease, especially in tumor cells (Scorilas 2002), we postulate that regulation of mRNA 3' end processing may play an important role in tumor suppression.

Furthermore, the convergence of factors such as BARD1, BRCA1, CstF-50, RNAP II and p53, to inhibit mRNA processing during the DNA damage response makes this/these protein(s) complex(es) and its/their role in reducing cellular mRNA levels an ideal candidate to undergo regulation, promoting either cell survival or apoptosis. Although further studies are necessary to determine more details of this mechanism, these studies could provide valuable information for the development of targeted and synergistic therapies aimed at controlling gene expression, cell proliferation and apoptosis.

ACKNOWLEDGEMENTS

This work would not have been possible without the support of the many people around me for the past five years. I am indebted to my mentor – Dr. Frida Kleiman for exceptional support and generosity well beyond her scope as my advisor. She has been more than a supervisor of my scientific work and has been a mentor in a personal sense as a woman in science. Dr. Kleiman has been responsible not only for my scientific progress but emotional growth and stability as well during this period. This would not have been possible without her invaluable advice, patience and understanding, especially during challenging times and when I have been a difficult student. She has not only been a great scientific advisor but also a patient and caring mentor, both for which I am extremely grateful.

The Kleiman lab has provided a warm and stimulating environment to work in. I have to thank members of Dr. Kleiman's lab past and present for their support at all times and making it a pleasure to work in the lab. My Friends and Colleagues for almost all the past five years, Danae Fonseca, Nurit Mirkin, Murat Cevher and Samia Mohammed for assistance in performing experiments and helpful discussions and also for listening to my frustrations and the constant encouragement and support in so many ways. Especially Danae Fonseca and Samia Mohamed, for their invaluable contribution in developing some of the experiments presented in this dissertation. Students in the Kleiman lab who helped during various stages of this work, especially Bernadine Akukwe for her help and friendship in the early stages, , Xiaokan Zhang, Carmen Taveras Michael Ignat, Michael Szmyga, Sully Fernandez, Jorge Baquero, Navida Bholanauth, Xin Xin Zhao and Serge Kim for help at various stages and for enriching my experience in Dr. Kleiman's lab.

The members of my committee - Dr. Jill Bargonetti, Dr. Maria Figueiredo-Pereira, Dr. Serafin Pinol–Roma, Dr. Karen Hubbard and Dr. Susan Rotenberg for their support and constructive criticism, which has enhanced the quality of this work.

My friends and colleagues in Dr. Bargonetti's lab - Ernest Boamah and Angelika Brekman for technical assistance and helpful suggestions.

The faculty and staff of the Chemistry department for providing a warm atmosphere to work in, especially Dr. Klauss Grohmann for all the help in getting started and the encouragement.

My friends and colleagues in the Chemistry department – too numerous to mention but whose friendship and moral support has helped me on a daily basis.

My friends Ali and Sakina Yunus, Anna and Steve Nesbitt and Jayakrishnan Nandakumar (JK), for their generosity in many ways and never failing to support during tough times, while carrying out this work. Saumi Weerasinghe and Kanchana Bandara for their constant encouragement and support through the years, from many miles away.

My teachers through the years, especially my high school Biology teacher Mrs. Faith Wickramasuriya for inspiring a love of science and learning.

Members of my immediate and extended family for their advice, love and support at all times. My parents in law for their generosity, my siblings – Ishra and Nimaz for being understanding when it has been so hard to be away and patiently listening to my stories and frustrations, and my parents for making many sacrifices on my behalf, for believing that a woman is entitled to an education and career despite prevailing cultural norms and most of all for instilling in me the values which made this possible.

Finally, I am grateful to my husband Firaz and daughter Talia, the two people who have tolerated all my ups and downs and were my greatest source of comfort during this period. Firaz - for inspiring me as a scientist and the never ending love and support, including technical advice and assistance, Talia - for being the reminder that there is more to life than what we try to uncover through science.

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Chapter I

Background

Transcription stress response

The living cell requires thousands of proteins for its various functions and survival. The blueprint for these proteins resides in the genome containing millions of DNA base pairs. This DNA is subjected to constant insults, both by environmental factors as well as by products of normal cellular metabolism. The cellular response to DNA damage is a protective mechanism against disease and involves the coordinated control of many events beginning with signaling pathways that alert the cell about DNA damage and resulting in DNA repair, control of cell cycle or in some cases, apoptosis.

DNA damage could mainly affect two major nucleic acid transactions essential to the cell, DNA replication and transcription. While DNA replication is greatly diminished or altogether absent in most non proliferating cells, transcription must continue for the conversion of the blueprint DNA to an RNA intermediary before it is translated to protein. Therefore it is important to repair DNA damage in a timely manner, especially the ones present in actively transcribing genes. At the same time it is also important to control gene expression during this repair process to channel resources to the repair pathways as well as to prevent the formation of deleterious proteins.

Multiple strategies have evolved to minimize the genotoxic consequences of endogenous and environmental agents that damage the DNA. One example of this is the conserved nucleotide excision repair (NER) pathway that removes mainly helix-distorting lesions, including UV induced cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4PPs), as well as bulky chemical adducts (de Laat et al. 1999). The efficiency of NER is not equal in all parts of the genome and different kinds of lesions are removed from the DNA with different efficiencies. For instance, global genome repair (GGR) repairs lesions throughout the genome and

transcription-coupled repair (TCR) selectively removes DNA lesions from transcribed strands of active genes (Hanawalt 2002).

Accumulating evidence suggests that the blockage of the elongating RNA polymerase II (RNAP II) at sites of DNA damage is an early event that initiates TCR (reviewed by Muratani and Tansey 2003, van den Boom et al. 2002). The UV-induced turnover of RNAP II is one of the manifestations of this response. Levels of mRNA are transiently decreased, and normal recovery depends on TCR (Mullenders 1998, Ljungman et al. 1999, Hanawalt 2004, Derheimer et al. 2005). 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. A number of studies have shown that following UV irradiation the induction of the p53 tumor suppressor pathway and apoptosis depends upon the persistent lesions localized in the transcribed strand of active genes (Ljungman 2007, Ljungman et al. 1999, Arima et al. 2005). Recently, it has been described that TCR is part of a more complex response, the transcription-stress response (TSR), which includes not only the induction of TCR but also the accumulation of proteins involved in the DNA repair pathway and the induction of p53 and apoptosis (Figure 1, Ljungman 2007).

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 by the tumor suppressors BRCA1/BARD1 (Kleiman et al. 2005) 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 by the BRCA1/BARD1/CstF-50 complex or by RNAP II degradation would then function to prevent polyadenylation and stabilization of such RNAs (Kleiman and Manley 2001, Kleiman et al. 2005). Since 3' mRNA processing is required for mRNA stability, nuclear export and translation, the inhibition of this process not only would prevent the formation of deleterious proteins but also would inhibit nuclear export of mRNA following DNA damage, which has been linked to the accumulation of protein factors, such as p53, in the nucleus during TSR (Ljungman 2007).

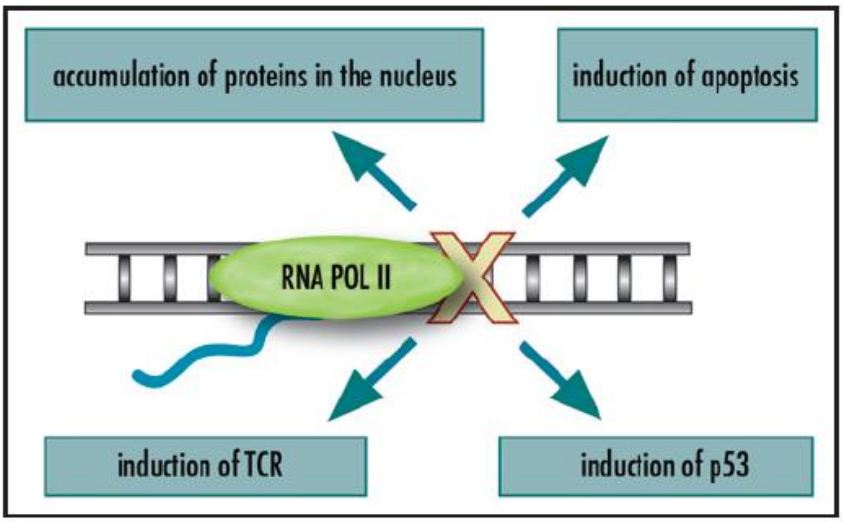

Figure 1 – The transcription-stress response. Inhibition of RNAP II-mediated transcription results in (1) accumulation of proteins such as p53 in the nucleus, (2) phosphorylation and activation of p53, (3) induction of apoptosis, (4) recruitment of DNA repair proteins to remove transcription blocking lesions via TCR. Modified from Ljungman 2007.

pre-mRNA 3' end processing

The production of mature mRNA requires the synthesis of a pre-mRNA by RNAP II followed by processing of this nascent precursor by 5' capping, splicing of introns and 3'cleavage and polyadenylation. In vivo mRNA processing occurs cotranscriptionally and is directed only to RNAs made by RNAP II (McCracken et al 1998, Bentley 2005). Capping of mRNA at the 5' end of the pre mRNA is required for mRNA splicing, export, stability and translation (Gu et al. 2005). Different splicing mechanisms remove intronic sequences of the pre-mRNA, regulating gene expression and generating the major source of protein diversity in the genome (Black 2003) The 3' end processing reaction consists of an endonucleolytic cleavage of the RNA followed by synthesis of the poly(A) tail at the 3' end. The poly(A) tail is important for mRNA stability, transport and translation (Zhao et al. 1999, Mandel et al. 2008). The assembly of the cleavage/polyadenylation machinery requires specific signal sequences in the mRNA precursor as well as interactions between multiple protein factors. The cis elements are found in almost all eukaryotic pre-mRNAs with the exception of histone mRNAs, which are cleaved but not polyadenylated (Gilmartin 2005). Although polyadenylation can occur at multiple sites, those containing the conserved cis elements are more efficiently cleaved (Mandel et al. 2008).

Mammalian pre-mRNAs contain three core sequence elements that define the polyadenylation site and two auxiliary sequence elements that enhance the 3' processing reaction. The core polyadenylation signal consists of a highly conserved hexanucleotide AAUAAA polyadenylation signal (PAS), the cleavage site and the G/U rich downstream element (DSE). The auxiliary sequences consist of an upstream element and a downstream element (Zhao et al. 1999). The PAS is located 10 to 30 nucleotides upstream of the cleavage site, and mutations in the PAS cause reduced levels of polyadenylated mRNA and increased

amounts of unprocessed pre mRNA. The DSE is a less conserved U-rich or GU-rich element generally located within 30 nucleotides downstream of the cleavage site. Although point mutations in the DSE have only a small effect on 3' cleavage activity, deletions of segments of the DSE have a more significant effect (Mandel et al. 2008). The cleavage site of the pre-mRNA is positioned between the PAS and the DSE, usually within a region of 13 nucleotides (Chen et al. 1995). Although the sequence around the cleavage site is not strictly conserved in pre-mRNA sequences examined, the optimal cleavage site seems to be CA. Then this cleavage site becomes the point of poly(A) addition. The auxiliary upstream element often consists of a U-rich element, and this element promotes binding of other polyadenylation factors to the cleavage site (Mandel et al. 2008). Auxiliary downstream elements are generally G-rich but lack a consensus sequence or distance from the cleavage site (Arhin et al. 2002; Dalziel et al. 2007).

Although the cleavage and polyadenylation signals seem to be relatively simple, a large number of protein factors are involved in these reactions. Recent studies have identified a large number of protein factors for the mammalian 3' end processing machinery (Figure 3, Mandel et al. 2008, Shi et al. 2009). In normal mammalian cells, cleavage-polyadenylation specificity factor (CPSF), cleavage stimulation factor (CstF), cleavage factors I and II, RNAP II and poly(A) polymerase (PAP) are involved in the cleavage step. CPSF, PAP and poly(A)-binding protein II (PABII) are involved in the polyadenylation step (Zhao et al. 1999).

Mammalian CPSF consists of five subunits, CPSF-30, CPSF-73, CPSF-100, CPSF-160 and hFip1. CPSF-160 is the largest subunit of CPSF and interacts directly with the PAS of pre-mRNA to direct the cleavage reaction, and is also required for polyadenylation. CPSF-160 also associates with the transcription factor TFIID and the C-terminal domain (CTD) of RNAPII (McCracken et al. 1997; Dantonel et al. 1997). Structural and biochemical evidence suggest that

CPSF-73 is the endoribonuclease for the 3' cleavage reaction. However, in the absence of other proteins of the 3'end processing machinery, it possesses only weak ribonuclease activity towards pre-mRNA substrates (Mandel et al. 2006). CPSF-100 not only shares sequence homology with CPSF-73 but also interacts with it. The exact function of CPSF-100 protein is currently unknown. CPSF-30 is required for both cleavage and polyadenylation and preferentially binds to the U rich sequences surrounding the cleavage site (Barabino et al. 1997). hFip1 mediates interactions with PAP and also interacts with CPSF-30. The primary function of hFip1 may be to bring PAP close to the polyadenylation site (Kaufmann et al. 2004).

CstF is a heterotrimer consisting of 77, 64 and 50 kDa subunits. The current evidence suggests that CstF may be dimeric in the 3' processing machinery (Mandel et al. 2008). CstF-64 contains an RNA recognition motif (RRM) and the RNA binding domain at its N-terminus, which preferentially binds to the G/U rich DSE (Takagaki and Manley 1997). The hinge region immediately following the RRM binds to CstF-77 and symplekin. The C-terminal domain of CstF-64 can interact with transcription factors and may play a role in regulating transcription (Proudfoot 2004). CstF-77 bridges CstF-64 and CstF-50 and also 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). CstF-50 is required for cleavage in vitro and contains seven WD-40 repeats, which can mediate protein-protein interactions and is a common feature of regulatory proteins (van der Voorn and Ploegh 1992). CstF-50 can bind to CstF-77 and can also self associate forming a dimer (Mandel et al. 2008). CstF-50 binds with a high affinity to the CTD of RNAP II (McCracken et al. 1997, Hirose and Manley 1998) and also the splicing

coactivator SRm160 (McCracken et al. 2003), establishing another link between transcription and 3' end processing. As mentioned before, CstF-50 associates with the tumor suppressor BARD1/BRCA1 following DNA damage and this interaction inhibits 3' end cleavage of pre-mRNA (Kleiman and Manley 1999).

Figure 2 – The 3' processing factor CstF-50. The CstF-50 subunit of the CstF trimer complex contains 7 WD-40 (tryptophan-aspartic acid) repeats and an N-terminal hydrophobic region. Modified from Mandel et al. 2008.

Symplekin functions as a scaffold protein bringing together a large number of proteins in the 3' end processing complex (Mandel et al. 2008). Symplekin interacts with the hinge region of CstF-64 and forms a stable complex with CPSF and CstF (Takagaki and Manley 2000). Although the role of symplekin in nuclear mRNA 3' processing has not been clearly identified yet, several studies show the involvement of symplekin as an essential factor in histone pre-mRNA 3' end processing (Kolev et al. 2005, Sullivan et al. 2009) and cytoplasmic polyadenylation (Barnard et al. 2004).

CF I is required for cleavage in vitro and functions as a heterodimer. The primary function of CF I may be to provide additional recognition of the pre-mRNA substrate and aid the definition of the proper polyadenylation site. CF II contains two subunits: hPcf11 and hClp1. hPcf11 contains a conserved RNAP II CTD binding domain, which binds preferentially to the

phosphorylated form of the CTD. Mutations in this domain can abolish binding to the CTD and result in cell death (Sadowski et al. 2003). However these mutations do not affect 3' end processing but causes incorrect transcription termination (Zhang et al. 2005). hClp1 interacts with CF I and CPSF. Recent studies show that hClp1 is a RNA 5' kinase that is important for tRNA splicing and activation of siRNA (Weitzer and Martinez 2007). Dephosphorylation of a protein in CF I or CF II by a Ser/Thr phosphatase abolishes the cleavage reaction (Ryan 2007).

In mammals, PAP is required for both cleavage and polyadenylation. PAP interacts with other members of the 3'processing complex but does not require these factors for in vitro polyadenylation reactions. However, these interactions are important for defining the proper length of the poly(A) tail. PAP binds hFip1 and CPSF-160 and also binds tightly to the last three nucleotides of the pre-mRNA substrate (Balbo et al. 2007). PABP is required for correct and efficient polyadenylation. PABP binds directly to stretches of 11-14 adenylate nucleotides as they become available and this binding continues until proper poly(A) tail length is achieved (200-300 bases in mammals, Keller et al. 2000). Direct binding of PABP to pre-mRNA adjacent to PAP, increases the efficiency of polyadenylation 80 fold (Kerwitz et al. 2003).

Initially, the CTD of RNAP II was identified as a 3' processing factor in cells transiently transfected with CTD truncations that exhibit inefficient polyadenylation (McCracken et al. 1997). The CTD is made up of 52 heptapeptide repeats with a consensus sequence of YSPTSPS for each repeat. The repeated serine residues within the CTD are susceptible for phosphorylation (Cramer et al. 2001) and both the phosphorylated and unphosphorylated forms activated cleavage in vitro (Hirose and Manley 1997). The CTD interacts with CPSF-160, CstF-50 and CstF-77, though with much less affinity than with CPSF-160 and CstF-50 (Shatkin and Manley

2000). Protein binding as well as 3' end processing increases upon phosphorylation of the CTD (Ryan et al. 2002)

The assembly of all these factors is believed to be sufficient for cleavage to occur. It is likely that the recognition of the upstream PAS by CPSF-160 and the G/U rich DSE by CstF-64 help position the 3'end processing machinery on the pre-mRNA, bringing the endoribonuclease, CPSF-73 close to the cleavage site. The correct placement may help to activate CPSF-73 ensuring the nuclease is functional only when it is in the correct location of pre-mRNA, preventing nonspecific activity (Mandel et al. 2008).

The addition of the poly(A) tail and subsequent binding of the PABP has been shown to prevent degradation of mRNAs in mammalian cells (Ford et al. 1997). In the cytoplasm, the poly(A) tail and PABP interact with the methyl cap at the 5' end to promote translation (Wickens et al. 1997). While the presence of the poly(A) tail is sufficient for translation, the presence of both poly (A) tail and 5' cap is optimal (Wilusz et al. 2001). In addition, 3' end processing is coupled to transcription and splicing machineries (Calvo and Manley 2003; Maniatis and Reed 2003). The 3' end processing complex interacts with transcription factors and the CTD of RNAP II to help control transcriptional initiation and a proper poly(A) signal is essential for transcriptional termination (Proudfoot 2004). Thus, the poly(A) tail is an essential element for proper control of gene expression at several different levels in eukaryotes. Indeed, regulation of 3' end formation can play significant roles in cell growth control (Chuvpilo et al. 1999; Takagaki and Manley 1998; Takagaki et al. 1996) and perhaps in disease, especially in tumor cells (Scorilas 2002).

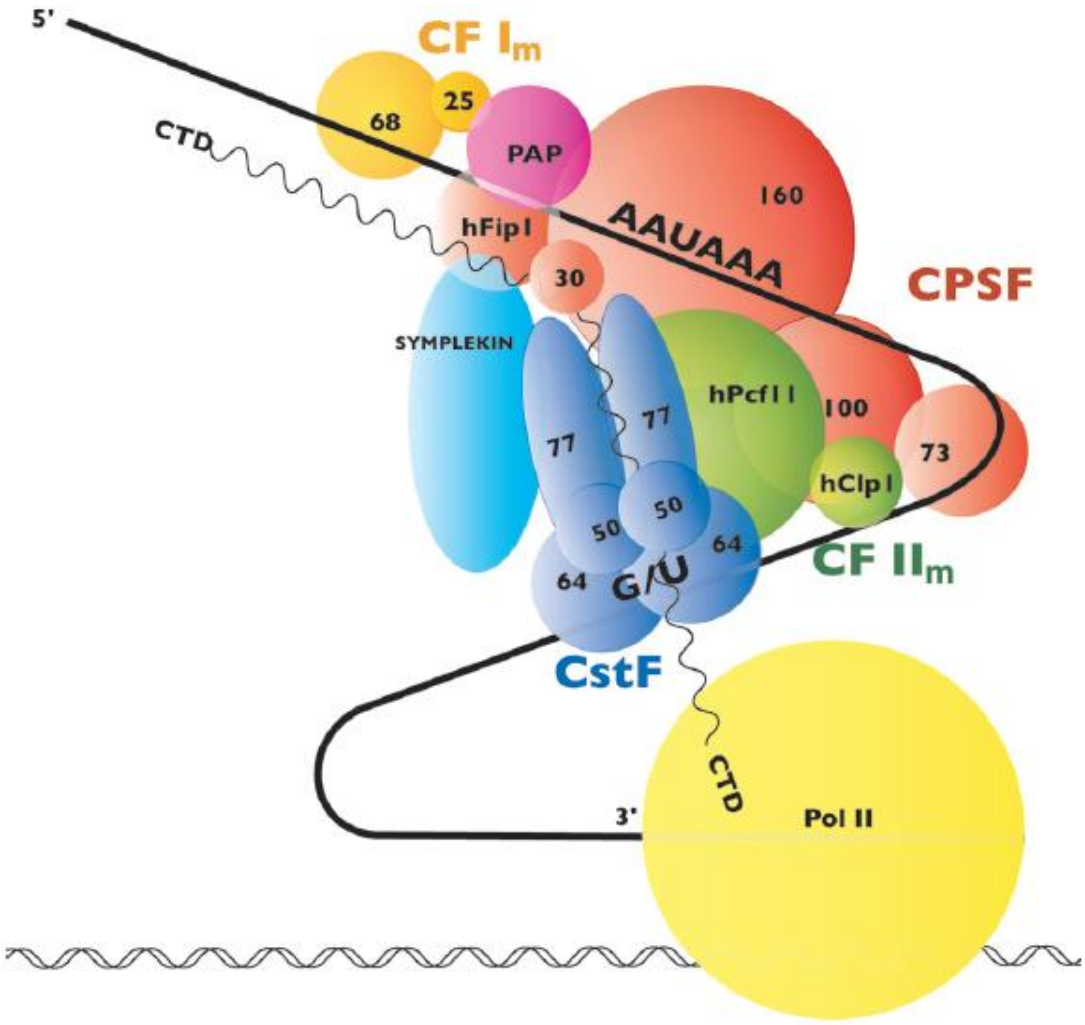

Figure 3 – A schematic model for the mammalian pre-mRNA 3'end processing machinery. The CstF complex is shown in its dimeric form based on current structural and biochemical information. Modified from Mandel et al. 2008.

The tumor supressor BARD1

The polyadenylation factor CstF-50 can interact with the tumor suppressor BARD1 following DNA damage and this interaction can inhibit the cleavage step of the polyadenylation reaction. BARD1 (BRCA1-associated RING domain 1) is a 97 kDa nuclear protein that associates with the breast cancer susceptibility gene product BRCA1 (Baer and Ludwig 2002). BARD1 and BRCA1 form a functional heterodimer via their respective RING finger domains. This interaction stabilizes both proteins and is required for several tumor suppressor functions of BRCA1. Although initially characterized as an accessory to BRCA1, the transforming effect of BARD1 inhibition in cultured cells (Irminger-Finger et al. 1998) and the existence of tumorigenic mutations in BARD1 (Ghimenti et al. 2002; Thai et al. 1998) support the view of BARD1 as a tumor suppressor by itself.

BARD1 shares many structural similarities with BRCA1 (Figure 4). Both proteins have a N terminal RING domain and two tandem BRCT domains in their C terminus. In addition to these RING and BRCT domains, BARD1 also has a three ankyrin repeat region, which may facilitate protein-protein interactions. Interestingly, the region between the ANK and BRCT domains is a highly conserved yet currently uncharacterized area. This complexity of structure indicates multiple functions of BARD1 (Figure 4, Irminger–Finger and Jefford 2006).The function and subcellular location of BARD1 and BRCA1 are regulated by nuclear-cytoplasmic shuttling (Henderson 2005). BARD1 contains six potential nuclear localization signal (NLS) sequences and one nuclear export signal (NES) sequence. The NLS sequences are located in tandem pairs, each adjacent to the conserved domains of BARD1. The NES is located adjacent to the NLS closest to the RING domain (Figure 4, Irminger–Finger and Jefford 2006). 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, cell cycle arrest and RNA processing (Schuchner et al. 2005). The excess of BARD over BRCA1 and BARD1 cytoplasmic localization is linked to the induction of apoptosis. (Jefford et al. 2004)

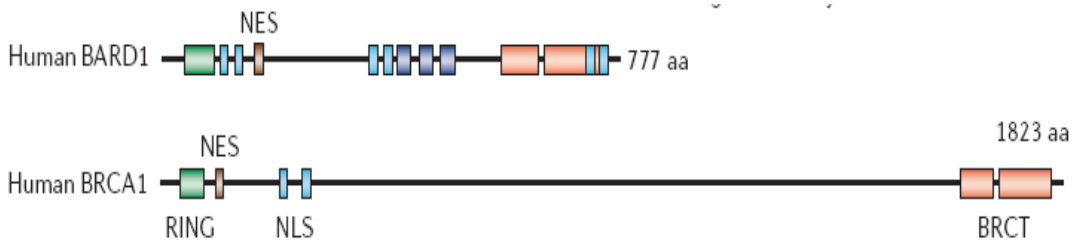

Figure 4– BARD1 domain structure compared to that of BRCA1. RING (green), ankyrin (blue), BRCT (red) domains and locations of potential NLS (light blue) and NES (brown) are indicated. Modified from Irminger-Finger and Jefford 2006.

BARD1, like BRCA1, is modified by phosphorylation in an ATM/ATR kinase dependent pathway upon DNA damage (Chapter IV, Figure 26, Kim et al. 2006). BARD1 has four ATM dependent phosphorylation sites; two of them T714 and T734 are evolutionary conserved. Mutations of either T714A or T734A significantly reduce DNA damage-induced phosphorylation of BARD1 (Chapter IV, Figure 27, Kim et al. 2006). BARD1 functions in the DNA damage response, such as inhibition of 3' mRNA processing (Kleiman and Manley 2001) and degradation of RNAP II (Kleiman et al. 2005) are both abrogated by mutations in these phosphorylation sites of the protein (Kim et al. 2006). Interestingly, cells expressing the T734A BARD1 mutant do not show the formation of the BARD1/CstF complex irrespective of UV

treatment, whereas cells expressing wild-type BARD1 are able to form the complex which increases significantly after UV damage (Kim et al. 2006).

Based on recent studies it has been suggested that BARD1 could have a dual mode of action: a survival mode, in which BARD1 is associated with BRCA1 and functions in nuclear DNA repair, control of cell cycle and inhibition of mRNA processing and a death mode, independent of BRCA1 associated with apoptosis (Figure 5, Irminger–Finger and Jefford 2006). These seemingly distinct functions will be discussed in detail in the following sections.

BRCA1-dependent functions of BARD1:

The DNA damage-induced inhibition of 3' end processing 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. Supporting the physiological significance of these results, the Gln564His germline mutation of BARD1, identified in an ovarian tumor, reduces binding to CstF-50 and abrogates inhibition of polyadenylation (Kleiman and Manley 2001). Additionally, both BARD1 and BRCA1 contain N-terminal RING domains that have been shown to exhibit significant E3 ubiquitin (Ub) ligase activity in the context of the BRCA1/BARD1 heterodimer (Chen et al. 2002; Hashizume et al. 2001; Morris and Solomon 2004; Xia et al. 2003). It has been shown that the RNAP IIO LS, the hyperphosphorylated form of the enzyme that functions in transcription elongation, but not RNAP IIA LS, the hypophosphorylated form that engages promoters, is a specific target of the BRCA1/BARD1 E3 Ub ligase activity (Kleiman et al. 2005; Starita et al. 2005). As part of those studies, it has been shown that siRNA-mediated knockdown of

BRCA1/BARD1 resulted in stabilization of RNAP II after DNA damage. It has been suggested that the UV-induced degradation of RNAP II is also another, possible redundant, mechanism to explain the inhibitory effect of UV irradiation on 3' mRNA processing (Kleiman et al. 2005).

In addition to its ubiquitin ligase activity towards RNAP II, BRCA1/BARD1 complexes exhibit auto ubiquitination through K6, which is an unconventional linkage (Sato et al., 2004). Autoubiquitinated BRCA1/BARD1 structures are found at sites of DNA repair, indicating that autoubiquitination might regulate BRCA1/BARD1 functions and induce DNA repair pathways (Chen et al. 2002). BRCA1/BARD1 also ubiquitinate gamma tubulin to regulate centrosome formation and control cell cycle progression through the G-M checkpoint (Starita et al. 2004). nucleophosmin, a nucleolar phosphoprotein involved in cell cycle regulation, is also a target of BRCA1/BARD1 ubiquitin ligase activity and co-expression of BARD1 and BRCA1 leads to stabilization of nucleophosmin, probably due to ubiquitination via K6 linkage (Sato et al. 2004). Besides BRCA1/BARD1 can monoubiquitinate histone 2AX (H2AX), implying a function for BRCA1/BARD1 in chromatin modification (Chen et al. 2002).

The involvement of BRCA1/BARD1 complexes in DNA repair has also been studied. It has been shown that BARD1 and BRCA1 locate to nuclear dots during S phase and relocate with RAD51 to proliferating cell nuclear antigen (PCNA) containing structures after UV or gamma irradiation (Scully et al. 1997). Furthermore, BRCA1/BARD1 complexes have also been described as the functional unit in double strand break repair, highlighting its significance in the DNA repair pathway (Jasin 2002).

Taken together these studies suggest that the BRCA1/BARD1 complex is involved in control of cell cycle and gene expression. However, BRCA1 independent functions of BARD1 have also been described, mostly relating to induction of apoptosis.

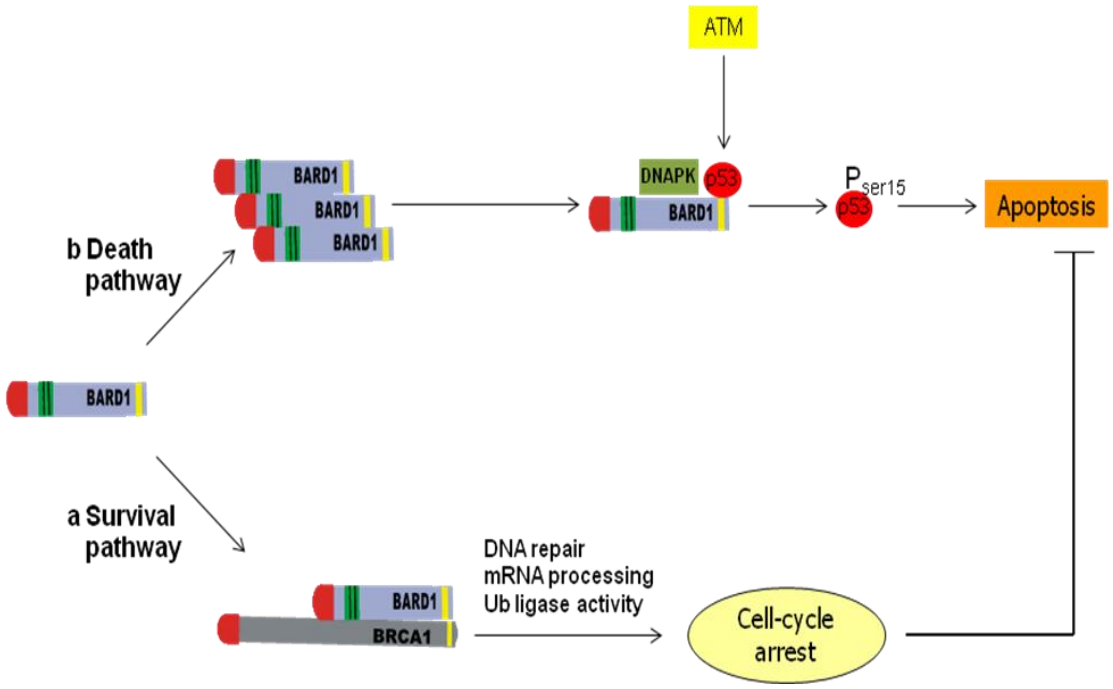

Figure 5– Hypothetical model of BARD1 pathways and function. BARD1 functions in two major pathways. The first is a cell survival pathway (a) mediated by the BRCA1-BARD1 heterodimer. The second (b) is a cell death pathway, which is independent of BRCA1. Modified from Irminger-Finger and Jefford 2006

BRCA1-independent functions of BARD1:

BARD1 expression can be transcriptionally upregulated in response to genotoxic stress (Irminger-Finger et al. 2001) and increases in BARD1 expression is also associated with apoptosis in the brain following hypoxia (Irminger-Finger et al. 1998). The excess of BARD1 over BRCA1 leads to both the cytoplasmic localization of BARD1 and the induction of apoptosis (Irminger-Finger and Jefford 2006). It has been described that BARD1 deficient cells are defective in the apoptotic response to genotoxic stress; this proapoptotic activity of BARD1 is stimulated by nuclear export (Rodriguez et al. 2004) as well as binding to the tumor suppressor p53 (Irminger-Finger et al. 2001). Furthermore, overexpression of BARD1 leads to apoptosis associated with stabilization of p53. Interestingly, the apoptotic function of BARD1 is dependent on functional p53 but inhibited by BRCA1.

It has been shown that BARD1 directs phosphorylation of p53 at Serine 15 by interacting with Ku-70, a subunit of the DNA-dependent protein kinase (DNA-PK, Feki et al. 2005). Serine 15 phosphorylation of p53 is lost in cancer cells deficient in full length BARD1, which are resistant to the induction of apoptosis. Ser 15 phosphorylation is essential for p53 stability (Saito et al. 2003), p53 transcriptional functions (Shieh et al. 1997) and in apoptosis (Khanna et al. 1998). Unexpectedly, a study characterizing the molecular architecture of the 3' processing complex has shown that Ku-70 is also part of the mRNA processing complex (Shi et al. 2009). The minimal region required for p53 binding to BARD1 spans the region between the ANK and BRCT domains (Irminger-Finger et al. 2001), which is also the minimum region required for binding CstF-50 (Kleiman and Manley 2001). Interestingly, the Gln564His germline mutation of BARD1 that reduces binding to CstF-50 and abrogates inhibition of polyadenylation (Kleiman and Manley 2001) also reduces the binding to p53 and the induction of apoptosis (Irminger-

Finger et al. 2001). Given that BARD1, is involved in the UV-induced inhibition of 3' end processing and could act as a mediator between genotoxic stress and apoptosis by binding to and stabilizing p53, the possibility arises of an interconnection between BARD1 functions in the mRNA processing pathway and the p53 pathway. The possible crosstalk between these two pathways supports the dual functions of BARD1 in the cell survival and death pathways following DNA damage discussed earlier.

The p53 pathway

The tumor suppressor p53 lies at the center of many stress response pathways that prevent growth and survival of potentially malignant cells. The p53 response is activated by many types of DNA damage and the antineoplastic effect of p53 is conferred by inhibiting propagation of cells with unrepaired DNA damage by enhancing DNA repair, promoting cell cycle arrest and/or by facilitating apoptosis in the case of extensive and unrepairable damage (Oren et al. 2002).

Active p53 exists in the cell as a tetramer consisting of four identical subunits. Each monomer consists of several well defined domains including a multipartite N-terminal transactivation domain (residues 1-73), a proline rich region (63-97), the centrally located DNA binding core domain (residues 94-312), the tetramization domain (residues 324-355) followed by the C terminal regulatory domain (360-393) (Figure 6, Latonen and Laiho 2005).

p53 accumulation and activation is regulated by post translational modifications, such as phosphorylation, acetylation and sumoylation (Bode and Dong 2004; Brooks and Gu 2003). At least 20 sites in the human p53 protein are modified depending on the type of damage and in response to the activation of different stress signaling pathways. Phosphorylation has been studied most intensively and has been found to play a critical role in the stabilization and activation of p53. Following DNA damage, p53 is phosphorylated at various sites, such as Ser 6, 9, 15, 20, 33, 37 and 46 and Thr18 and Thr81 in the amino terminal region; Thr 150 and 155 and Ser 149 in the central core; and Ser315 and Ser392 in the C-terminal domain (Bode and Dong 2004). These phosphorylation events selectively activate various functions of p53 in response to DNA damage. For instance, while p53 phosphorylation at Ser 15, 20 and 37 has been associated with the rapid response to DNA damage and leads to reduced interaction of p53 with its negative

regulator, MDM2 (Shieh et al. 1997), phosphorylation at Ser 46 has been associated with activation of apoptosis (Taira et al. 2007).

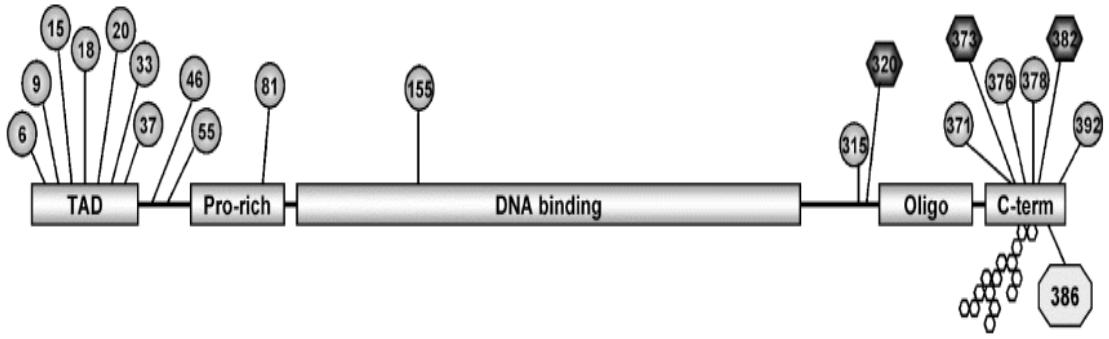

Figure 6 – Structure and posttranslational modification of the p53 protein. TAD, transactivation domain; Oligo, oligomerization domain: circles, Ser/Thr phosphorylation sites: hexagons, acetylation sites: octagons, sumoylation site. Modified from Latonen and Laiho 2005.

The induced phosphorylation of p53 is mediated by DNA damage response kinases, such as ATM following ionizing radiation-induced damage and ATR or DNA-PK following ionizing/UV irradiation (Bode and Dong 2004). As discussed earlier, BARD1, independently of BRCA1, also directs phosphorylation of p53 on Ser15 by binding to Ku-70, the regulatory subunit of DNA-PK (Feki et al. 2005).

Activation of p53 can induce several cellular responses but the best understood so far is the ability of p53 to act as a transcription factor, which can activate target genes involved in DNA repair, cell cycle arrest and/or apoptosis (Levine 1997, Vogelstein et al. 2000, Levine et al. 2006). For instance activation of transcription targets, such as Gadd45 (growth arrest and DNA damage inducible 45), promotes DNA repair by its association with the DNA replication and repair factor PCNA (Smith et al. 1994). The most important transcriptional target for p53 mediated cell cycle arrest is p21, a potent inhibitor of several cyclin-dependent kinase (CDK) complexes (el-Deiry et al. 1993). p53 transcriptional control of apoptosis is mediated mainly by the target genes such as PUMA and Noxa (Jeffers et al. 2003).

A growing number of studies have shown that transcription-independent activities of p53 also play a role in mediating some of the downstream effects following DNA damage. These functions are mostly related to either DNA repair or induction of apoptosis. One such transactivation independent function of p53 is its ability to participate in DNA repair by direct interaction with components of the repair and recombination machineries. p53 participates in nucleotide excision repair (NER) pathways by binding to NER factors XPB and XPD (Hanawalt 2002). An alternate mechanism is that the C-terminus of p53 could directly bind to altered DNA structures, which in turn could activate p53 as a transcription factor (Jayaraman and Prives 1995).

Another well studied transactivation independent functions of p53 is its ability to induce apoptosis by translocating to the mitochondria and binding to the anti-apoptotic Bcl-2 family of proteins (Derheimer et al. 2005) and causing oligomerization of the pro-apoptotic BAX protein and cytochrome C release in mitochondria.(Chipuk et al. 2004). Interestingly, a recent study shows BARD1 apoptotic function is also associated with its translocation to the mitochodria and

BAX oligomerization (Tembe and Henderson 2007). This suggests that the transcription independent apoptotic effect of p53 may be regulated in association with other interacting partners, such as BARD1.

The p53 tumor suppressor network has been intensively studied for over 25 years; however, genetic analysis long hinted at the existence of components that remained elusive (Vogelstein et al. 2000, Levine et al. 2006). For example, although p53 is clearly a transcriptional activator, numerous reports indicated that p53 also represses the expression of specific genes either directly or indirectly (Spurgers et al. 2006). The manner in which this occurred was obscure, with both transcriptional and posttranscriptional suppression as possible mechanisms. Interestingly, other transactivation independent functions of p53 have been recently described (Chang et al. 2007, He et al. 2007). For example, certain miRNAs, such as the miR-34 family, are transactivated by p53, offering an indirect p53-mediated control of gene expression at the posttranscriptional level and controlling a wide variety of pathways including cell cycle progression and apoptosis (Chang et al. 2007, He et al. 2007). The identification of the miR-34 family as p53 targets expands the repertoire of p53-regulated genes and provides evidence of a novel posttranslational mechanism to control target gene expression through the p53 network (Bommer et al. 2007).

CHAPTER II

Functional interaction of the tumor suppressor p53 with the 3' mRNA processing factor CstF-50 under DNA damaging conditions.

INTRODUCTION

The cellular response to DNA damage involves the coordinated control of many events, such as gene expression, DNA repair and apoptosis. During the DNA repair response, control of gene expression is important to allow the repair process and to prevent the formation of deleterious proteins. Gene expression could be controlled either by transcription or by mRNA processing, both resulting in changes in the cellular levels of mRNA. One example of this regulated response is provided by the transient decrease of the cellular levels of mRNA observed following UV irradiation (Hanawalt 1994, Ljungman et al. 1999).

mRNA processing is an essential step in mRNA maturation and involves posttranscriptional modifications such as capping, splicing and polyadenylation (Zhao et al. 1999). The poly(A) tail found on almost all eukaryotic mRNAs plays important roles in regulation of mRNA stability, translation and RNA transport (Neugebauer 2002, Mangus et la. 2005, Anderson 2005). The processing of the mRNA 3' end consists of an endonucleolytic cleavage of RNA followed by synthesis of the poly(A) tail. It has been shown that the regulation of 3' formation can play significant roles in cancer (Topalian et al. 2001, Kleiman and Manley 2001, Scorilas 2002, Rozenblatt-Rosen et al. 2009). The assembly of the cleavage/polyadenylation machinery requires interactions between multiple protein factors (reviewed in Zhao et al. 1999, Shatkin and Manley 2000, Mandel et al. 2008). Cleavage stimulation factor (CstF) is one of the essential 3' processing factors and 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 GU-rich element of the mRNA and genetically modified cells deficient in CstF-64 undergo cell cycle arrest and apoptotic death (Takagaki and Manley

1998). Both the CstF-50 and CstF-77 subunits interact specifically with the carboxy-terminal domain (CTD) of RNA polymerase II (RNAP II), likely facilitating the RNAP II-mediated activation of 3' end processing (McCracken et al. 1997, Hirose and Manley 1998). After DNA damage, 3' processing is inhibited as a result of CstF/BARD1/BRCA1 complex formation (Kleiman and Manley 2001) and of the proteasome-mediated degradation of RNAP II (Kleiman et al. 2005), suggesting the existence of possibly redundant mechanisms to explain the inhibitory effect of UV irradiation on 3' end processing. 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 the 3' processing factor CstF plays a direct role in the DNA damage response.

Another important manifestation of the DNA damage response is the accumulation of the tumor suppressor protein p53. Activation of p53 induces the expression of a large set of target genes, inducing several cellular responses such as DNA repair, cell cycle arrest and/or apoptosis (Vogelstein et al. 2000, Giono and Manfredi 2006). Although it is well established that p53 is a transcriptional regulator, other transactivation independent functions of p53 have also been described (He et al. 2007, Chang et al. 2007). For example, miRNAs are transactivated by p53 and the expression of those miRNAs causes dramatic changes in gene expression, offering an indirect p53-mediated control of gene expression at the posttranscriptional level (He et al. 2007, Chang et al. 2007). Interestingly, the induction of the p53 response upon DNA damaging conditions has been linked to the inhibition of RNAP II (Ljungman et al. 1999), resulting in a decrease in the levels of total poly(A)$^+$ mRNA. Although it has been implied that the DNA damage-induced inhibition of transcription is involved in this response (Ljungman et al. 1999), this p53-associated decrease in poly(A)$^+$ RNA levels might also be functionally associated with

the UV-induced inhibition of mRNA 3' processing (Kleiman and Manley 2001, Kleiman et al. 2005, Mirkin et al. 2008), offering another possible p53-mediated posttranscriptional mechanism to regulate gene expression.

In the current study, we present evidence that the C-terminal domain of p53 associates with CstF-50 and BARD1, connecting p53 to the RNA 3' processing machinery. Although the p53/CstF complex is detected independently of DNA damage, we found that the tumor suppressor BARD1 can coexist in complexes with CstF and p53 in nuclear extracts of UV–treated cells, suggesting that p53 may play a role in the UV-induced inhibition of mRNA 3' processing. Consistent with this, we show here that the C-terminal domain of p53 can inhibit the 3' cleavage reaction *in vitro* and that p53 siRNA-treatment has different effects on mRNA 3' processing in cells expressing different levels of p53. More importantly, cells expressing endogenous mutant p53 showed a decrease not only in the interaction of p53 with both BARD1 and CstF but also in the UV-induced inhibition of 3' processing, all of which can be restored by the expression of wild-type p53. We also provide evidence that mRNA precursors of housekeeping genes were enriched ~3 to 5 fold in the poly(A)$^+$ preparation over the total mRNA fraction in samples from cells expressing mutant p53 and low levels of p53. This enrichment in the poly(A)$^+$ preparation was significantly reduced following UV treatment or by the induced expression of wild-type p53. Interestingly, no change in the enrichment in the poly(A)$^+$ preparation was observed for mRNA precursors of p53-regulated genes. Our results provide new insights into p53 function and the mechanisms behind the inhibition of mRNA 3' processing following DNA damage. Together these results indicate a new link between mRNA 3' processing, tumor suppression, and the DNA repair machinery.

Results

The 3' mRNA processing factor CstF-50 binds to the tumor suppressor p53.

The 3' processing factor CstF-50 interacts with the BRCA1-associated RING domain protein BARD1, inhibiting mRNA 3' processing and linking it to the DNA damage response (Kleiman and Manley 1999). A germline mutation in BARD1 (Gln564His), which falls in the CstF-50 interaction domain, reduces the binding of CstF-50 to BARD1, interfering with the role of BARD1 in mRNA 3' processing (Kleiman and Manley 2001). Cells deficient in BARD1 expression are defective in the apoptotic response to genotoxic stress; this proapoptotic activity of BARD1 involves binding to p53 (Feki et al. 2005). Interestingly, the Gln564His germline mutation of BARD1 mentioned above also reduces its binding to p53 and induction of apoptosis (Irminger-Finger et al. 2001). The Gln564 was found to lie within the p53 interaction domain, which includes the three ankyrin domains and the region just downstream (Irminger-Finger et al. 2001). Taken together, those studies suggest a possible functional overlapping of BARD1, CstF-50 and p53 following DNA damage.

To further investigate this possibility, we first analyzed the physical association of p53 with BARD1 and CstF-50 using "pull-down" assays. We performed these assays using GST-tagged CstF-50 (GST-CstF-50), GST-tagged BARD1 (GST-BARD1), and full-length p53 (His-p53). To determine the p53 domain involved in these interactions, we also analyzed in the "pull-down" assays the His-tagged p53 derivatives described in Figure 7: a C-terminal truncated version (residues 1 to 293), a N-terminal truncated version (residues 94 to 393), and a C- and N-terminal truncated version (residues 94 to 293). Interestingly, the results showed that both full-length His-p53 and His-p53 (94-393) interacted directly *in vitro* with both GST-CstF-50 and GST-BARD1 (Figure 8, lanes 3 and 6). The p53/BARD1 interaction was stronger than the p53/CstF-50

interaction (compare lanes 3 to 6). However, neither CstF-50 nor BARD1 bound to either of the two derivatives that lacked the C-terminal region of p53 (lanes 3 and 6) or to the GST alone (lane 9). As samples were treated with RNase A, the observed p53/CstF-50 and p53/BARD1 interactions were not due to RNA tethering effect. Taken together, these data suggest that the C-terminal domain of p53, which has been suggested to have regulatory functions in the DNA repair response (Sauer et al. 2008), encompasses the CstF-50 and BARD1 interaction domain.

To test whether p53 might also be part of the previously described CstF-50/BARD1 complex, competition assays were carried out by incubating GST–BARD1 or GST-CstF-50 immobilized on glutathione-sepharose beads with nuclear extracts (NE) of non-UV-treated HeLa cells and increasing amounts of His-p53 (Figure 9). The effect of exogenously added recombinant p53 on the complex formation was easily assessed in these assays because HeLa cells, which express very low levels of p53 (Mantovani and Banks 1999; Wei 2005), were used as a source of the *in vivo* complex. The pulled-down proteins were analyzed by Western blot using antibodies against BARD1, CstF-50 and p53. Increasing amounts of His-p53 increased both the amount of CstF-50 "pulled-down" by GST-BARD1 (Figure 9, lanes 1-8) and the amount of BARD1 "pulled-down" by GST-CstF-50 (Figure 9, lanes 9-16), indicating that p53 stabilized the CstF-50/BARD1 complex. To confirm the simultaneous interaction of BARD1, p53 and CstF-50, we examined a mix of the three recombinant proteins described above by Superose-12 gel filtration followed by Western blot analysis (Figure 10). This produced two different fractions: one that contained BARD1, CstF-50 and p53 (fractions 14-15, M.W. ~250-200) and one that contains only CstF-50 and p53 (fractions 16-17, M.W. ~150-100).

These results indicated that the p53/CstF complex can be detected independently of BARD1 and that BARD1 can coexist in complexes with CstF-50 and p53.

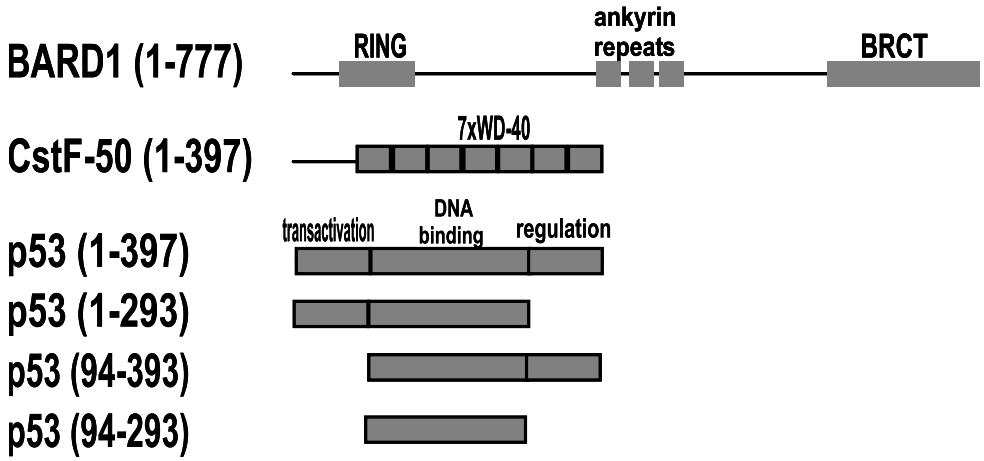

Figure 7: Diagram of CstF-50, p53 and BARD1 derivatives used in *in vitro* experiments. Features of the proteins are indicated.

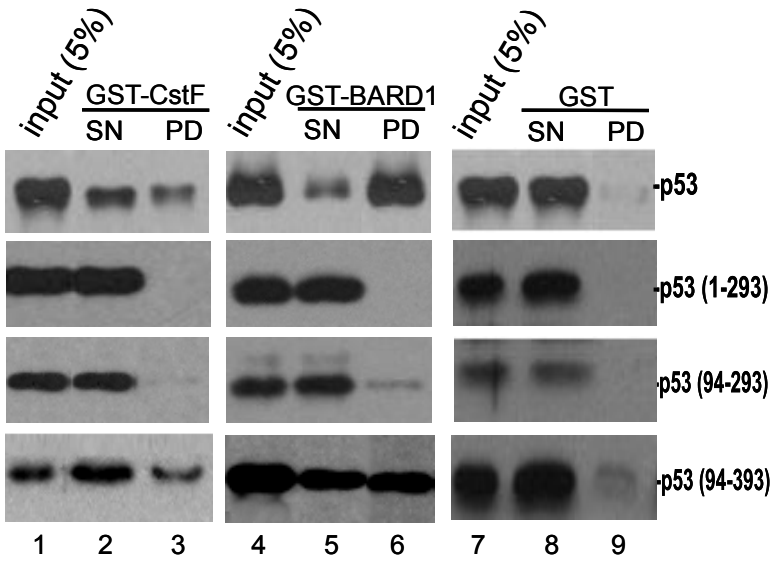

Figure 8: CstF-50 interacts with both BARD1 and p53 to form a protein complex. Requirement of p53 C-terminal domain for both CstF-50 and BARD1 interaction. Recombinant His-p53 or the indicated His-p53 derivatives were incubated with purified GST, GST-CstF-50 or GST-BARD1. Bound proteins were eluted and analyzed by Western blot with anti-p53 antibodies. 5% of His-p53 or His-p53 derivatives used in the reaction are shown as input.

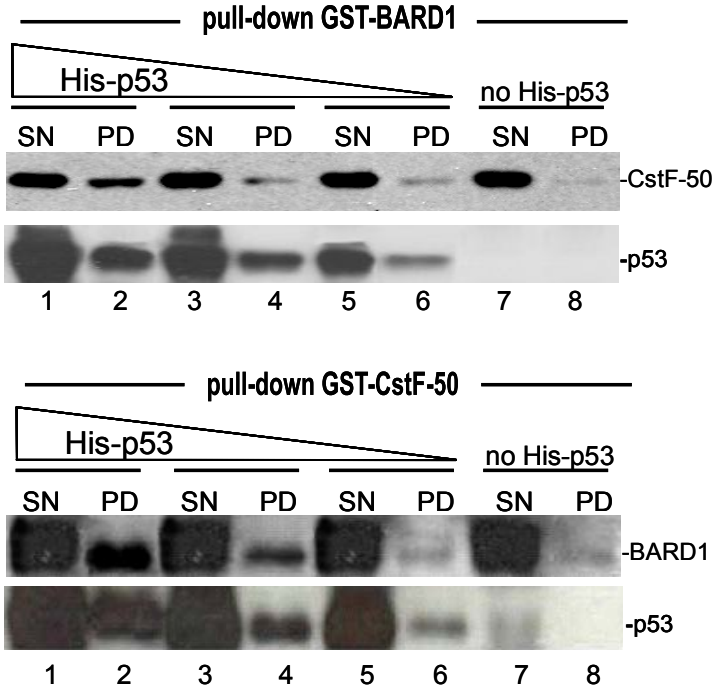

Figure 9: CstF-50 interacts with both BARD1 and p53 to form a protein complex. CstF-50, BARD1 and p53 can coexist in complexes. Either GST-BARD1 or GST-CstF-50 was immobilized in glutathione-Sepharose beads and tested for its ability to bind CstF-50/BARD1 and p53 from NE of HeLa cells, while increasing concentrations of recombinant His-p53 (5, 10 and 20 ng) were added. Bound proteins were detected by immunoblotting with CstF-50 and p53 antibodies. The pellets (PD) and the supernatant (SN) were analyzed.

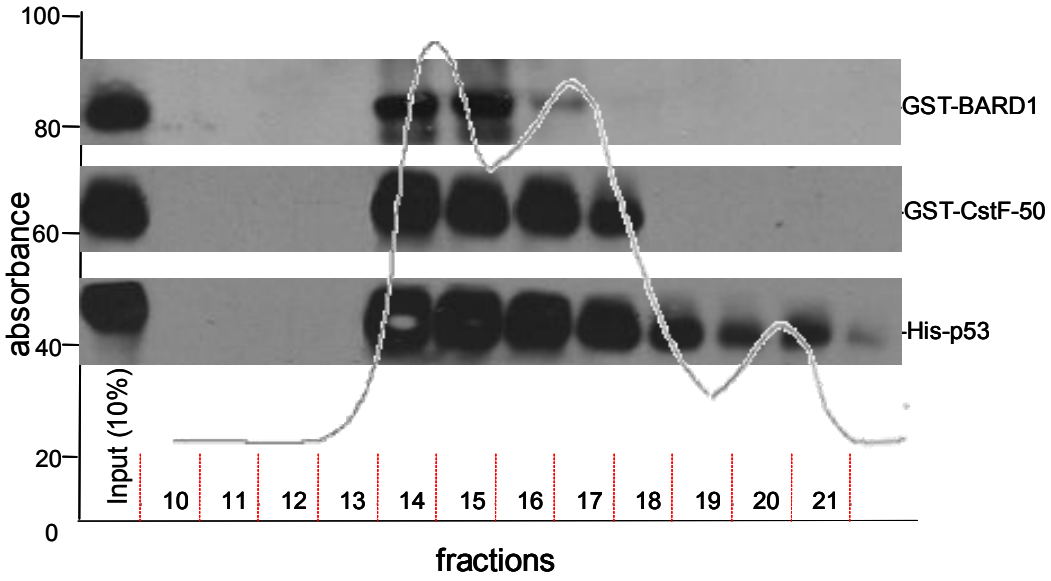

Figure 10: CstF-50 interacts with both BARD1 and p53 to form a protein complex. Cofractionation of BARD1, CstF-50 and His-p53. The p53/CstF-50/BARD1 complex in solution was analyzed by gel filtration using an analytical Superose-12 column. A mix of each recombinant protein was injected in the column and fractions were analyzed by Western blot. The UV-chromatogram is shown superimposed on Western blots.

While the p53/CstF complex is detected independently of DNA damage, BARD1 coexist in complexes with CstF and p53 only in NE of UV–treated HeLa and MCF-7 cells.

To further examine and characterize the binding of CstF-50, BARD1 and p53, we analyzed the ability of these proteins to interact in NEs of different cell lines by coimmunoprecipitation assays. As earlier studies had revealed a link between CstF and the UV-induced DNA damage response (Kleiman and Manley 2001), we decided to analyze the interaction of these factors under DNA damage conditions. Cells were treated with UV irradiation and allowed to recover for 2 hrs as described earlier (Kleiman and Manley 2001). NEs from these cells were analyzed by coimmunoprecipitation using antibodies directed against either p53 or the CstF subunit CstF-64, to ensure that any detected interactions were between p53 and intact CstF. Protein samples were treated with RNase A. Bound proteins were detected using antibodies against BARD1, CstF-64, p53 and topoisomerase II (Topo II). First, we used HeLa cells to study the complex formation because most of the functional properties of the 3' processing machinery (Takagaki et al. 1989, 1992; Takagaki and Manley 1992, 2000, Hirose and Manley 1998) and the UV-induced inhibition of 3' processing (Kleiman and Manley 2001; Kleiman et al. 2005) have been described in this biological system. It is important to highlight that HeLa cells are HPV-E6 transformed and the levels of p53 both before and after DNA damage are very low (Mantovani and Banks 1999; Wei 2005). Thus, we extended our studies to samples of MCF7 cells, which express normal levels of p53.

As described before, p53 accumulation and increase in the CstF-BARD1 complex formation were detected in the samples of both HeLa (Figure 11A, lane 5) and MCF7 (Figure 12A, lane 5) cells exposed to UV treatment. By way of comparison, lighter exposures of the p53 Western blot analysis of MCF7 samples were used. Interestingly, the results indicated that antibodies against CstF-64 also coprecipitated a significant amount of p53 independently of UV-

treatment (Figure 11A and 12A lanes 3 and 5). Similar results were obtained in the reciprocal coimmunoprecipitation analysis with p53 antibodies (Figure 11B and 12B, lanes 3 and 5). The results of the coimmunoprecipitation assays did not reflect the weak interaction between CstF-50 and p53 observed in the GST "pull down" assays (Figure 8, , suggesting that (a) factor(s) present in the NEs might stabilize the CstF-50/p53 complex. Although a significant amount of BARD1 co-precipitated with both CstF and p53, those interactions were observed only in the UV-treated samples. Similarly, we could detect p53 and CstF-64 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 p53 and BARD1, they clearly show that UV treatment induced the interaction between those factors, supporting the idea that p53 and CstF might be simultaneous binding partners of BARD1. These studies also showed that the coprecipitation of p53 with CstF was irrespective of DNA damage, indicating that the formation of the CstF/p53 complex might be independent of BARD1. Interestingly, the appearance of this/these complex/es coincided with the observed inhibition of 3' cleavage upon DNA damaging conditions (Kleiman and Manley 2001).

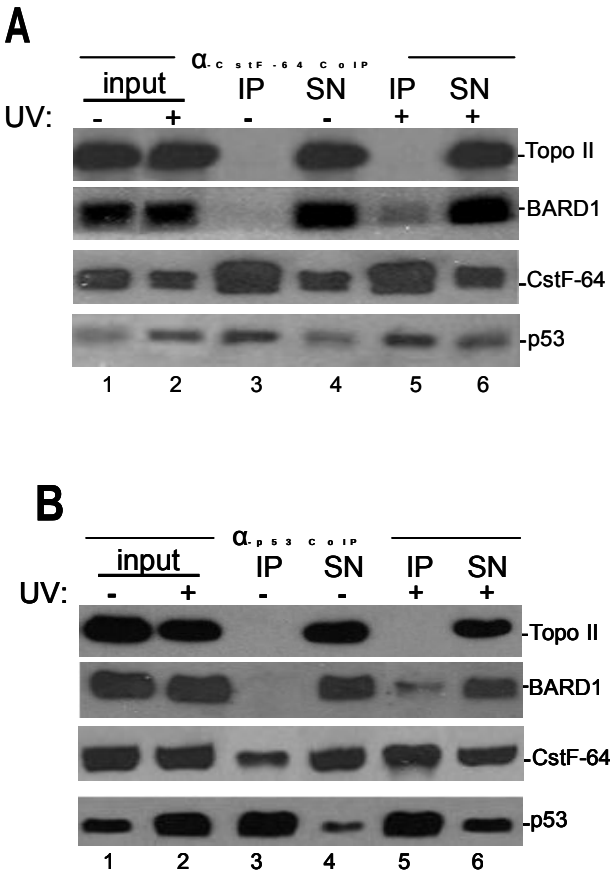

Figure 11: CstF-50 interacts not only with p53 and BARD1 to form a protein complex but also with p53 independently of BARD1 in NEs. CstF and p53 coimmunoprecipitate from NEs of HeLa cells irrespective of UV irradiation. Coimmunoprecipitation of BARD1 is observed only following UV irradiation. Nuclear extracts were immunoprecipitated with either anti-CstF-64 or anti-p53 antibodies. Equivalent amounts of the pellets (IP) and the supernatants (SN) were resolved by SDS-PAGE and proteins were detected by immunoblotting with antibodies against anti-BARD1, CstF-64, and p53. 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.

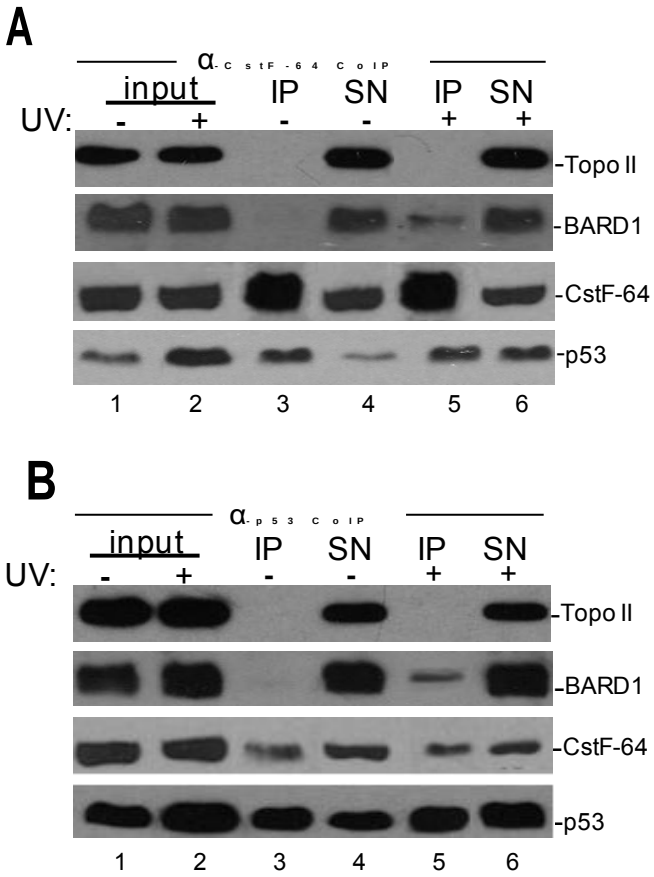

Figure 12: CstF-50 interacts not only with p53 and BARD1 to form a protein complex but also with p53 independently of BARD1 in NEs. CstF and p53 coimmunoprecipitate from NEs of MCF7 cells **(A-B)** irrespective of UV irradiation. Coimmunoprecipitation of BARD1 is observed only following UV irradiation. Nuclear extracts were immunoprecipitated with either anti-CstF-64 or anti-p53 antibodies. Equivalent amounts of the pellets (IP) and the supernatants (SN) were resolved by SDS-PAGE and proteins were detected by immunoblotting with antibodies against anti-BARD1, CstF-64, and p53. 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 C-terminal domain of p53 can inhibit mRNA 3' processing *in vitro*.

Given that a complex consisting of CstF, p53 and BARD1 was formed after UV treatment at times coincident with the observed inhibition of 3' cleavage (Kleiman and Manley 2001), we wanted to determine whether p53 plays a role in mRNA 3' processing. To address this possibility, we performed *in vitro* RNA cleavage assays with limiting amount of NE of HeLa cells and the addition of increasing amounts of recombinant His-p53 (Figure 13). We also examined the effect on 3' cleavage assays of the His-tagged p53 derivatives described in Figure 1A. As mentioned before, we used NE of HeLa cells because most of the functional studies on 3' processing have been done in these cells (Takagaki et al. 1989, 1992; Takagaki and Manley 1992, 2000, Hirose and Manley 1998; Kleiman and Manley 1999, 2001; Kleiman et al. 2005). As HeLa cells express low levels of p53 (Mantovani and Banks 1999; Wei 2005), the effect of increasing amounts of p53 on 3' processing was easily determined. Interestingly, only increasing concentrations of either full-length His-p53 (Figure 13, lanes 2-4) or His-p53 (94-393) (Figure 14, lanes 2-4) into the reaction mix significantly reduced the 3' cleavage of the radiolabeled adenoviral L3 pre-mRNA. However, neither of the two derivatives that lacked the C-terminal region of p53 (Figure 14, lanes 5-10) nor GST alone (Figure 14, lanes 5-7) had an effect on the cleavage reaction. These results correlate well with the binding data shown in Figure 1B. These results indicate that the same region of p53 required for binding CstF-50 and BARD1 is necessary for inhibiting 3' pre-mRNA cleavage.

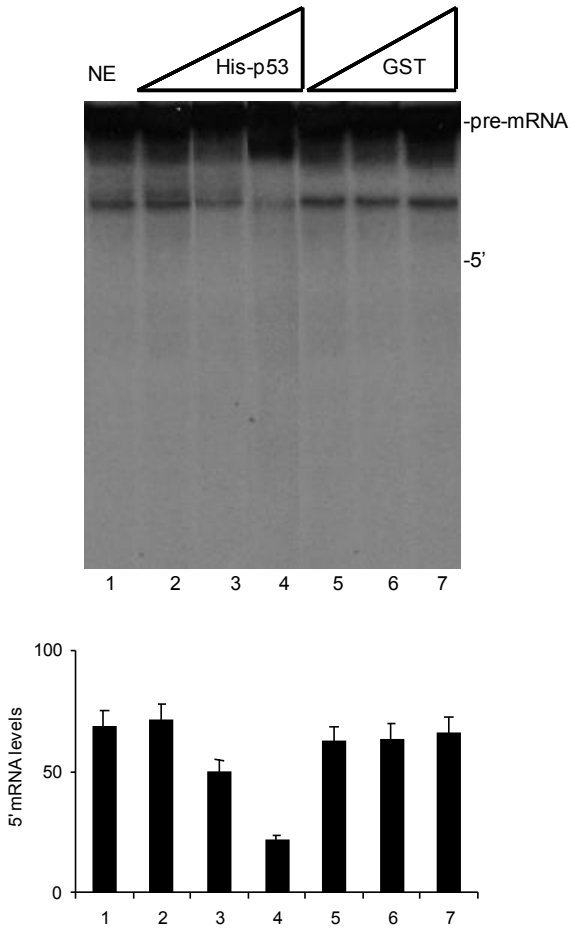

Figure 13: The C-terminal domain of p53 inhibits 3' cleavage *in vitro*. Inhibition of 3' cleavage by recombinant His-p53. NEs from HeLa cells were preincubated with no addition or increasing amounts of recombinant His-p53 or GST proteins (40, 80 and 120 ng). After 15 min, L3 pre-mRNA was added and incubation continued for 90 min. RNAs were purified and analyzed by denaturing PAGE. Positions of pre-mRNA and the 5' cleavage product are indicated. The images were analyzed by Image J program. The graph shows the mean values ± standard deviations for the quantification of the 5' cleavage product from three independent experiments.

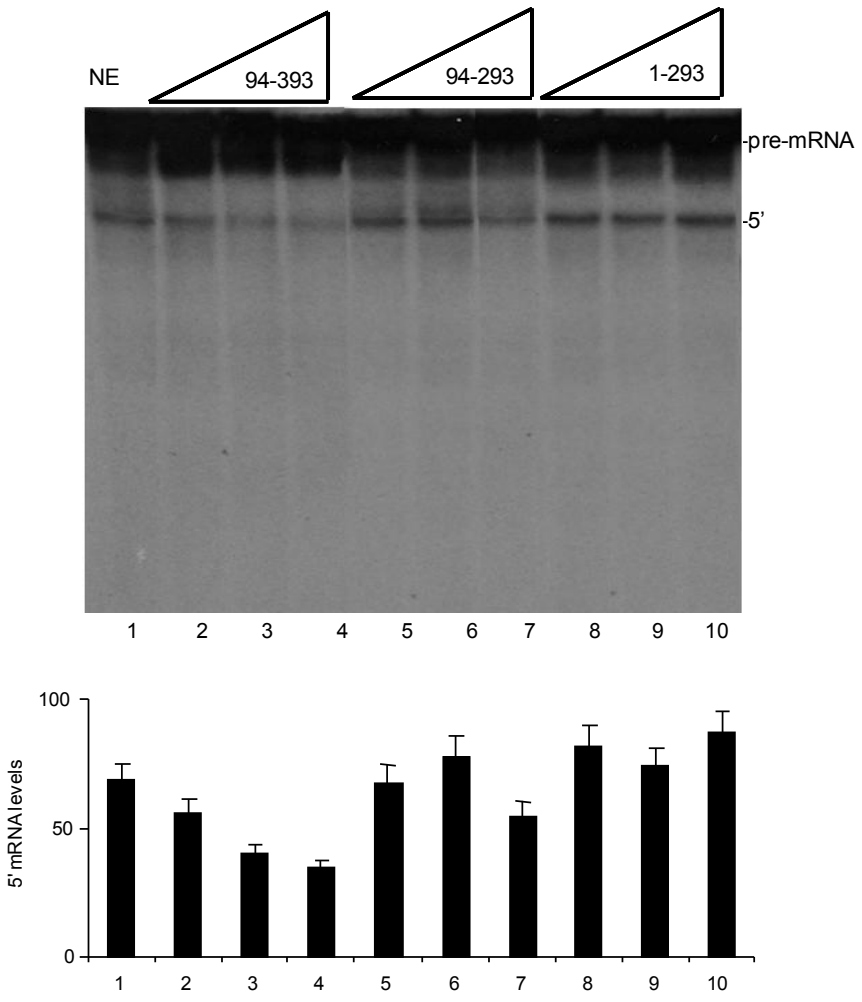

Figure 14: The C-terminal domain of p53 inhibits 3' cleavage *in vitro*. The COOH-terminal domain of p53 is necessary for inhibition of 3' cleavage. HeLa NEs were incubated with no addition or increasing amounts of the indicated His-p53 derivatives (40, 80 and 120 ng). 3' cleavage reactions were performed and analyzed as in Figure 13.

All together, these results indicate that p53 has an inhibitory effect on mRNA 3' cleavage. Initially, this inhibition was ascribed to the CstF/BARD1 complex formation (Kleiman and Manley 1999). However, these results suggest that other factors, such as p53, might also be involved in the regulation of 3' pre-mRNA processing either by the stabilization of the CstF-50/BARD1 complex or by its direct interaction with the essential 3' processing factor CstF-50.

Expression levels of p53 correlate with the levels of pre-mRNA 3' processing.

To study the significance of the interaction between the tumor suppressor p53 and the 3' processing factor CstF-50, we performed siRNA mediated knockdown of p53 in cells expressing low levels of p53, such as HeLa cells (Figure 15A), and normal levels of p53, such as MCF7 cells (Figure 15B). We also investigated the effect of siRNA mediated knockdown of p53 on the UV-induced inhibition of 3' cleavage described earlier (Kleiman and Manley 2001). Cells were treated with siRNAs for 48 hrs, exposed to UV light, and allowed to recover for 2 hrs. NEs were then prepared and analyzed by Western blotting. Figure 15A and B show that a 48 hrs p53 siRNA treatment resulted in a substantial depletion of p53 (≈90%) in NEs of both cell lines (lanes 5-6), independently of the UV treatment. Importantly, the expression levels of p53 in all the MCF7 samples were much higher than in the HeLa samples analyzed (Figure 16, compare lanes 1-6 to lanes 7-12). For comparison, lighter exposures of the Western blot analysis of MCF7 samples were used (Figure 15B). Consistent with previous results (Noda et al. 2000, Scherer et al. 2000), UV treatment significantly increased accumulation of p53 and this was not affected by control siRNAs.

Then we investigated the effect of siRNA mediated knockdown of p53 on the UV-induced inhibition of 3' cleavage described earlier (Kleiman and Manley 2001). Surprisingly, the depletion of p53 has different effects in cells expressing different levels of the protein. The depletion of p53 in HeLa cells abolished the UV-induced inhibition of mRNA 3' end cleavage (Figure 15A, compare lanes 4 and 6), indicating that p53 has an inhibitory effect on mRNA 3' cleavage under DNA damaging conditions. Consistent with earlier work from the Kleiman's lab, NEs from control siRNA-treated cells showed the UV-induced inhibition of 3' processing. As similar results were observed with the siRNA-mediated knockdown of BARD1 in HeLa cells (Kleiman et al. 2005), we propose that p53 together with the CstF/BARD1 complex might play a role in the UV-induced inhibition of 3' processing.

Strikingly, NEs from cells expressing normal levels of p53, such as MCF7 cells, showed no significant levels of 3' cleavage (Figure 15B, lanes 1-4). The lack of detectable levels of 3' processing in NEs of MCF7 cells was irrespective of control siRNA- (lanes 3-4) and UV-treatment (lanes 2 and 4). These results are consistent with the possibility that high levels of p53 inhibit 3' mRNA processing. Interestingly, and supporting this idea, siRNA-mediated knockdown of p53 in MCF7 cells resulted in extracts exhibiting significant levels of 3' cleavage (lane 5) and UV-induced inhibition of 3' cleavage (lane 6). It is important to highlight that p53 siRNA-treatment did not completely deplete p53 in UV-treated MCF7 cells (Figure 15B, lane 6). Interestingly, those samples from p53 siRNA-treated MCF7 cells showed similar levels of both p53 expression and 3' cleavage to samples from control siRNA -treated HeLa cells (Figure 16, compare lanes 3-4 to lane 11-12; and compare lanes 3-4 in Figure 4A to lane 5-6 in Figure 15B). These results indicate that p53 has an inhibitory effect on mRNA 3' cleavage and that this effect is dependent on the cellular levels of p53.

If p53 indeed inhibits 3' cleavage by interaction with CstF-50, then the addition of recombinant CstF-50 should enhance the levels of 3' processing in NEs prepared from MCF7 cells. Addition of increasing amounts of GST-CstF-50 to NEs from MCF-7 cells strongly increased L3 pre-mRNA 3' cleavage (Figure 17) allowing efficiencies similar to that observed in HeLa cells and in p53 siRNA-treated MCF7 cells. Addition of GST protein did not have any effect on 3' cleavage (lanes 4-6). These results provide evidence that the concentration of CstF appeared to be limiting for efficient cleavage in the MCF-7 extracts and that CstF is indeed the target of p53-mediated inhibition.

All together, these results indicate that mRNA 3' processing can be inhibited by p53 in a concentration-dependent manner. Our data revealed a dramatic difference in the inhibition of 3' processing in NEs from cells expressing normal and high levels of p53. A significant question is whether this reflects a global inhibition of 3' processing or a more selective inhibition. A prolonged inhibition of global mRNA processing would be expected to have deleterious effectson cell growth, which is not observed in MCF7 cells (data not shown). It is likely that the substantial reduction in 3' processing observed in this *in vitro* assays reflects the nature of the NEs. For example, these results indicate that the concentration of CstF is limiting for efficient cleavage in these extracts, and it may be that reduction in CstF activity (by interaction with p53 and/or BARD1) has a quantitatively more significant effect *in vitro* than *in vivo*. Consistent with this, previous studies showed that reduction in the cellular concentration of CstF-64 by a factor of 5-10 did not significantly affect growth or accumulation of most mRNAs (Takagaki and Manley 1998).

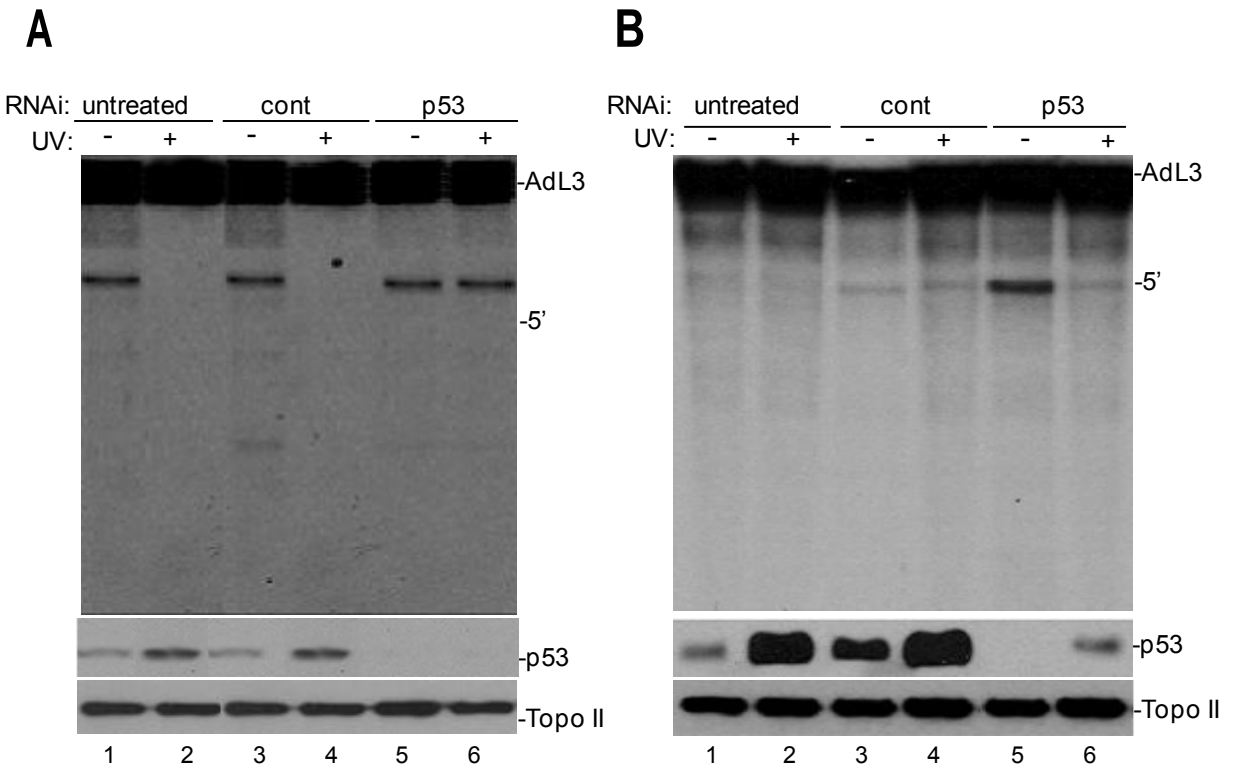

Figure 15: Levels of pre-mRNA 3' cleavage correlate with expression levels of p53. **(A)** siRNA mediated knockdown of p53 abolishes the UV-induced inhibition of 3' cleavage in HeLa cells. NEs from cells treated with p53/control siRNA and UV irradiation, and allowed to recover for 2 hours, were analyzed for L3 pre-mRNA 3' cleavage. Positions of pre-mRNA and the 5' cleavage product are indicated. Protein levels of p53 and Topo II were analyzed by Western blot. **(B)** siRNA mediated knockdown of p53 induces 3' cleavage in MCF7 cells. NEs from cells were analyzed as described in (A).

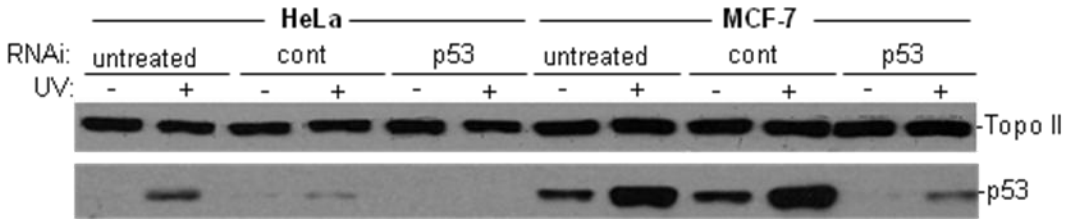

Figure 16: Levels of pre-mRNA 3' cleavage correlate with expression levels of p53. Comparison of p53 expression levels in NE from HeLa and MCF-7 cells analyzed in Figure 15. Protein concentrations in NE from siRNA treated/untreated cells were equalized by immunostaining with antibodies against Topo II.

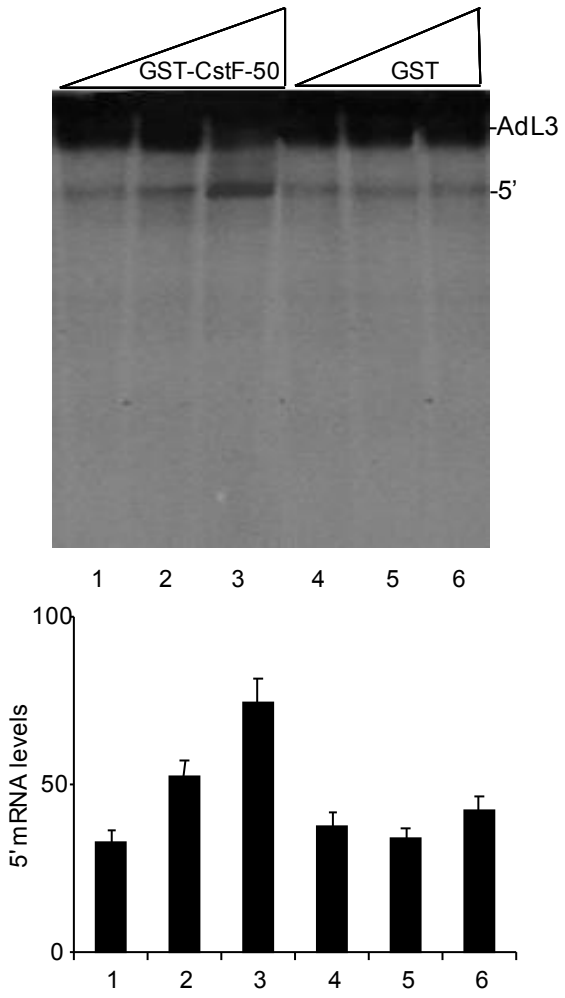

Figure 17: Levels of pre-mRNA 3' cleavage correlate with expression levels of p53. Addition of recombinant GST-CstF-50 enhances 3' cleavage in MCF-7 cells. MCF7 NEs were preincubated with increasing amounts of recombinant GST-CstF-50 or GST proteins (40, 80 and 120 ng). After 15 min, L3 pre-mRNA was added and incubation continued for 90 min. RNAs were purified and analyzed by denaturing PAGE. Positions of pre-mRNA and the 5' cleavage product are indicated. The images were analyzed by software Image J. The graph shows the mean values ± standard deviations for the quantification of the 5' cleavage product of three independent experiments.

A tumor-associated mutation in p53 disrupts the CstF-50, BARD1 and p53 interaction and is reversed by induction of WT-p53.

The above data provide considerable support for the existence of an inhibitory effect of p53 on pre-mRNA 3' end processing. To obtain genetic support for the significance of the CstF/p53 interaction, as well as to test the idea that it might be related to the function of p53 in tumor suppression, we took advantage of the isogenic colon cancer cell lines, DLD-1 and D-A2. DLD-1 cells carry the tumor-associated Ser241Phe mutation in p53 and they lack the expression of wild-type p53, which results in a defective p53 pathway (Yu et al. 1999). A two-step procedure was used to establish a tetracycline-off system for controlled wild-type p53 expression in D-A2 cells, leaving the expression of wild-type p53 under the regulation of doxycyclin (Dox, Yu et al. 1999). In order to investigate the possible effects of this mutation on 3' processing, we constructed a His-p53 recombinant protein containing the Ser241Phe mutation (His-p53 S241Q). The protein was first used in binding assays with recombinant GST-BARD1 and GST-CstF-50 (Figure 18,). The Ser241Phe mutation significantly reduced binding to BARD1 (compare lanes 4 to 5) but not to CstF-50 (compare lanes 2 to 3). These results indicate that this p53 residue is important for optimal interaction with BARD1.

To examine the effect of the Ser241Phe mutation on the p53, BARD1 and CstF interaction in more detail, we analyzed the complex formation in NEs from DLD-1 and D-A2 cells by coimmunoprecipitation. Cells were treated with UV irradiation and allowed to recover for 2 hrs as described earlier. D-A2 cells were grown in the absence of Dox to induce the expression of wild type-p53. NEs from these cells were analyzed by coimmunoprecipitation using antibodies directed against either p53 or CstF-64. Protein samples were treated with RNase

A. Bound proteins were detected using antibodies against BARD1, CstF-64, p53 and Topo II. As shown in Figure 19, UV treatment induced the accumulation of the mutant p53 in samples of DLD-1 cells (compare lanes 1 to 2). Interestingly, antibodies against p53 did not coimmunoprecipitate either CstF or BARD1 from NEs of DLD-1 cells irrespective of UV treatment (Figure 19A, lanes 3 and 5). However, the reciprocal coimmunoprecipitation analysis with CstF-64 antibodies showed the binding to BARD1 but not to p53 in samples from UV-treated DLD-1 cells (Figure 19B, lanes 3 and 5). Importantly, the coimmunoprecipitation of p53, CstF-50 and BARD1 with both p53 and CstF-64 antibodies was rescued in NEs from D-A2 cells following the induction of WT–p53 (Figure 20, lanes 3 and 5). The association of the three proteins in extracts from non-treated and UV-treated cells likely reflects the high levels of p53 expression in D-A2 cells growing in induction medium (without Dox, compare lanes 1 to 2).

All together, these results indicate that Ser241 or some structural feature around this residue is important for the p53/BARD1 interaction but not for the p53/CstF-50 interaction. Consistent with this, the Ser241Phe mutation in p53 prevented the formation of the BARD1/p53 complex in extracts from DLD-1 cells. Interestingly, this mutation also prevented p53 to bind CstF in those extracts, suggesting that the Ser241 might be important for binding (a) factor(s), such as BARD1 upon DNA damaging conditions, that might play a role in the stabilization of the CstF/p53 complex. The Ser241Phe mutation in p53 did not affect the CstF/BARD1 interaction, indicating once again that BARD1 can bind to CstF independently of p53.

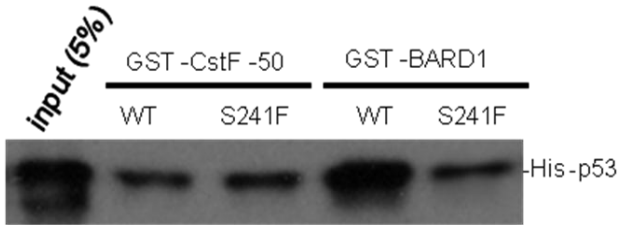

Figure 18: The Ser241Phe mutation in p53 disrupts the BARD1, CstF-50 and p53 interaction. Ser241Phe mutation on p53 diminishes binding to GST-BARD1 but not to GST-CstF-50. Recombinant His-p53 or His-p53 (Ser241Phe) derivative was incubated with purified GST-CstF-50 or GST-BARD1. Bound proteins were eluted and analyzed by Western blot with antibodies against p53. 5% of His-p53/His-p53 (Ser241Phe) used in the reaction is shown as input.

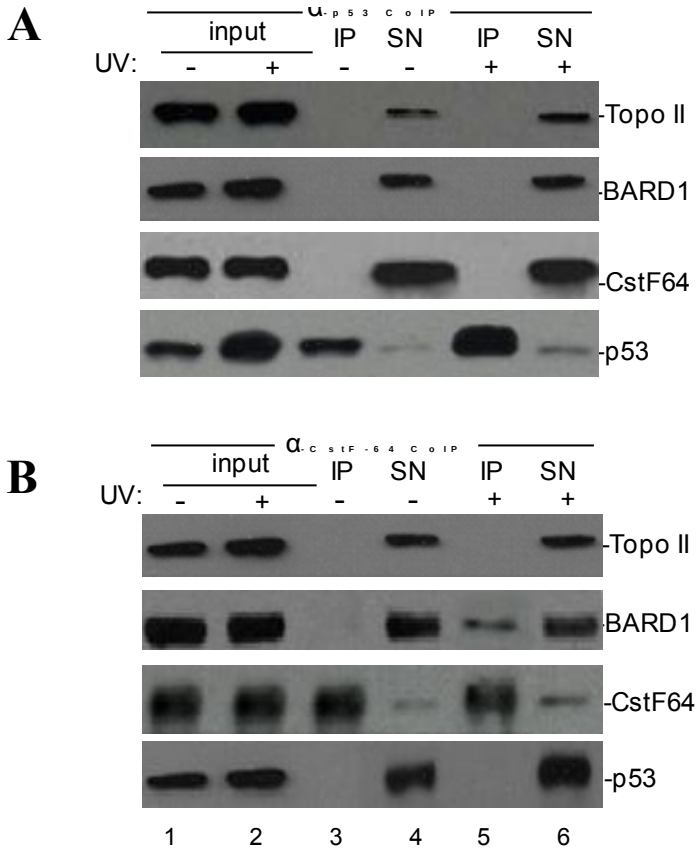

Figure 19: The Ser241Phe mutation in p53 disrupts the BARD1, CstFand p53 interaction and this is restored by WT-p53 expression. CstF, p53 and BARD1 do not coimmunoprecipitate from NE of DLD-1 cells that express Ser241Phe mutant p53. NEs were immunoprecipitated with either anti-p53 (A) or anti-CstF-64(B) antibodies. Equivalent amounts of the pellets (IP) and the supernatants (SN) were resolved by SDS-PAGE and proteins were detected by immunoblotting with antibodies against BARD1, CstF-64 and p53. Antibodies against Topo II were used as a control of specificity. Positions of Topo II, BARD1, CstF-64 and p53 are indicated. 20% of the NE used in the immunoprecipitation reaction is shown as input.

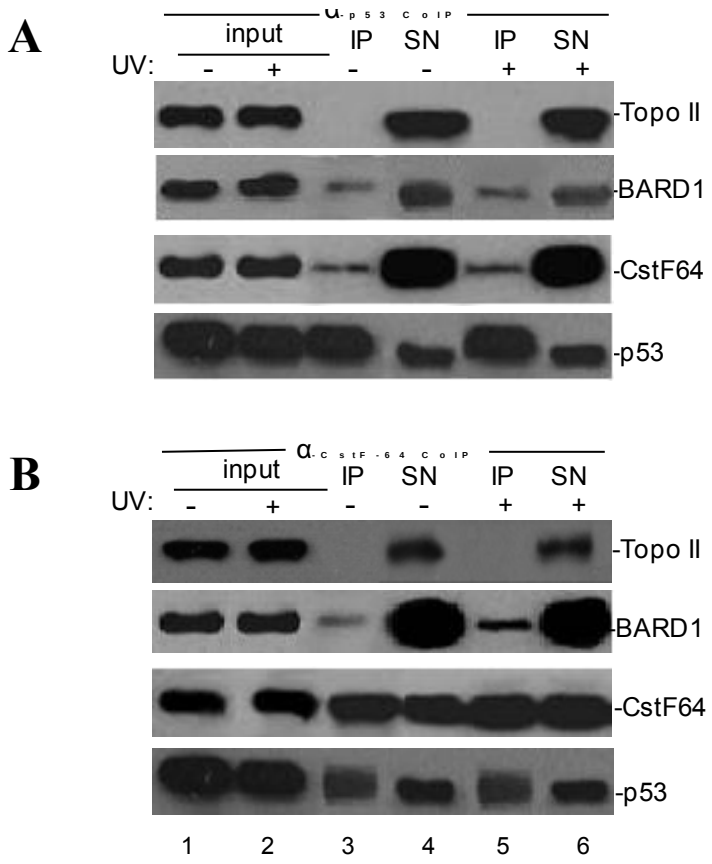

Figure 20: The Ser241Phe mutation in p53 disrupts the BARD1, CstF-50 and p53 interaction and this is restored by WT-p53 expression. CstF, BARD1 and p53 coimmunoprecipitate from NEs of D-A2 cells following induction of WT-p53 expression. While the CstF/p53 interaction is observed irrespective of UV irradiation, the coimmunoprecipitation of BARD1 with CstF and p53 is observed only following UV irradiation. Samples were analyzed as in Figure 19.

The Ser241Phe mutation in p53 abolishes p53-mediated inhibition of mRNA 3' cleavage and this is reversed by induction of WT-p53.

We next determined whether the Ser241Phe mutation influences the inhibitory effect of p53 on 3' end processing in NEs of DLD-1 and D-A2 cells upon DNA damaging conditions. To this end, DLD-1 and D-A2 cells were exposed to UV light and allowed to recover for the times indicated in figure 6A. The prepared NEs were then used to monitor p53 expression and 3' cleavage of the adenoviral L3 pre-mRNA as described above. As shown in Figure 19 (lanes 1-2), Western blot analysis indicated that the expression levels of mutant p53 increased in extracts from UV-treated DLD-1 cells (Figure 21A, lower panel). Interestingly, samples from DLD-1 cells showed high levels of 3' processing, showing efficiencies similar to that observed in non-treated HeLa cells (Figure 15A). UV treatment did not significantly affected 3'cleavage efficiencies in NEs from DLD-1 cells, suggesting that the Ser241Phe mutation reduces p53 inhibitory functions on 3' cleavage. Taken together, these results indicate that the decrease in the CstF/BARD1/p53 complex formation in DLD-1 cells (Figure 19) is associated with a decrease in UV-induced inhibition of 3' processing (Figure 21A). Furthermore, the data presented here suggest that serine 241 plays a critical role in the function of the p53 cleavage inhibitory domain.

To further confirm the involvement of p53 in this response**,** we used extracts from D-A2 cells grown with or without Dox in mRNA 3' cleavage assays as described above. While D-A2 cells grown in the presence of Dox showed p53 expression levels similar to that observed in DLD-1 cells (compare lanes 1 and 3 in Figure 21A to lanes 1-2 in Figure 21B), D-A2 cells grown in the absence of Dox showed significantly higher levels of p53 expression irrespective of UV treatment (Figure 21B, lanes 3-4). Like the samples from DLD-1 cells, D-A2 cells depleted of WT-p53 (plus Dox) showed high levels of 3' processing in samples from non-treated

and UV-treated cells (compare lanes 1 and 3 in Figure 21A to lanes 1-2 in Figure 21B). Strikingly, the induced expression of WT-p53 by the removal of Dox was sufficient to inhibit 3' cleavage in NEs from D-A2 cells irrespective of UV treatment (Figure 21B, lanes 3-4). Again, these results show a reverse correlation between the levels of pre-mRNA 3' processing and the levels of p53 expression. Moreover, the results indicate that the expression of WT-p53 and the UV-induced association of p53, BARD1 and CstF are necessary for 3' cleavage inhibition.

We next wanted to confirm whether the Ser241Phe mutation influences the inhibitory effect of p53 on 3' cleavage in NEs. We monitored 3' cleavage of the adenoviral L3 pre-mRNA in standard NEs from HeLa cells, which contain very low levels of p53 (Mantovani and Banks 1999; Wei 2005), with no addition or with increasing amounts of the His-p53 derivatives analyzed in Figure 18 (His-p53 WT and His-p53 S241F). As observed before (Figure 14), addition of increasing amounts of purified His-p53 WT to reaction mixtures effectively inhibited 3' cleavage, almost completely at the highest concentrations (Figure 22, lanes 2-4). Importantly, the His-p53 S241Q derivative was without detectable effect on 3' processing at all concentrations tested (lanes 5-7). As shown in Figure 19, the Ser241Phe mutation in p53 reduced binding to BARD1 but not to CstF-50, supporting the idea that serine 241 plays a critical role not only in binding BARD1 but also in the function of the p53 cleavage inhibitory domain.

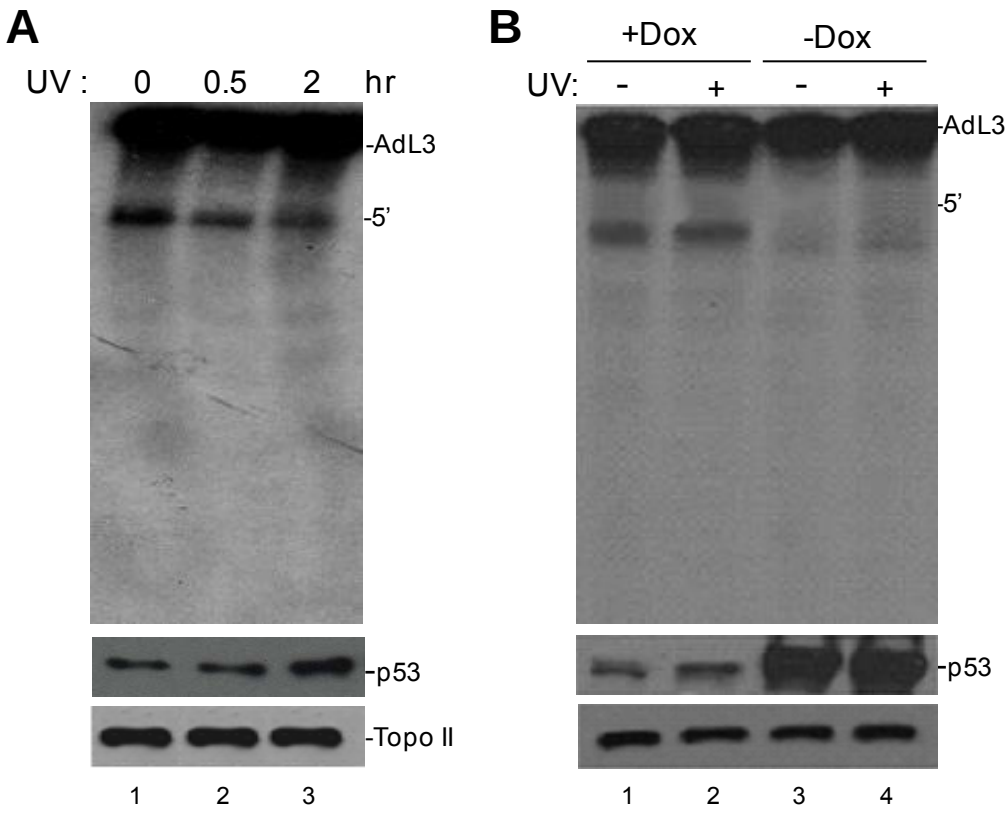

Figure 21: The Ser241Phe mutation abolishes the inhibitory effect of p53 on 3' cleavage and this is restored by WT-p53 expression. (A) Nuclear extracts from DLD-1 cells show no detectable inhibition of 3' cleavage following DNA damage. NEs from DLD-1 cells non-treated or UV-treated, and allowed to recover for the times indicated in the figure, were analyzed for L3 pre-mRNA 3' cleavage. Positions of pre-mRNA and the 5' cleavage product are indicated. Protein levels of p53 and Topo II were analyzed by Western blot. **(B)** The induced expression of WT-p53 in D-A2 cells inhibits pre-mRNA 3' cleavage. NEs from DA-2 cells non-treated or treated with Dox and/or UV irradiation were incubated in a reaction mix containing L3 pre-mRNA and analyzed as in (A).

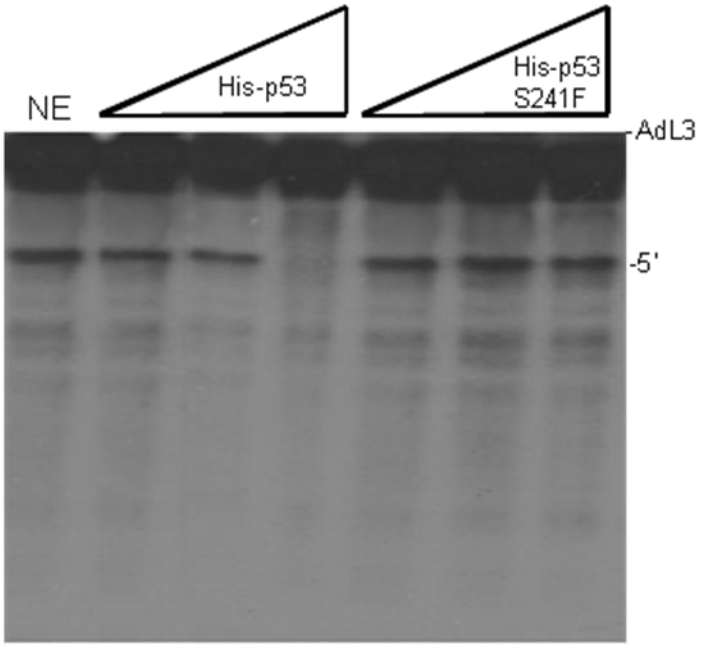

Figure 22: The Ser241Phe mutation abolishes the inhibitory effect of p53 on 3' cleavage and this is restored by WT-p53 expression. The Ser241Phe mutation in p53 abolishes the inhibition of 3' cleavage. NEs from HeLa cells were preincubated with no addition or increasing amounts of recombinant His-p53 or His-p53 (Ser241Phe) derivative (40, 80 and 120 ng). After 15 min, L3 pre-mRNA was added and incubation continued for 90 min. RNAs were purified and analyzed by denaturing PAGE. Positions of pre-mRNA and the 5' cleavage product are indicated.

p53 is involved in the UV-induced decrease of polyadenylated mRNAs.

The data presented above provided evidence that p53 together with BARD1 and CstF can inhibit mRNA 3' processing when conditions are such that p53 expression levels are high. To further investigate this, we wanted to determine whether p53 has an effect on the polyadenylation state of different endogenous mRNAs in different cellular conditions. We analyzed the enrichment of different mRNAs in the poly(A)$^+$ RNA over the total RNA population after p53 siRNA- and UV-treatment of MCF-7 cells (Figure 23A). We also extended these studies to the isogenic cell lines DLD-1 and D-A2 (Figure 23B). We analyzed housekeeping genes, such as β-actin and GAPDH, as well as a p53 regulated gene, such as p21.

Real time PCR quantification revealed that the three studied mRNAs did not show a significant change in the enrichment in the poly(A)$^+$ preparation over the total RNA fraction in samples from control siRNA- and UV-treated MCF-7 cells (Figure 23A) and from UV-treated D-A2 cells minus Dox (Figure 23B). As shown in Figure 20 (lanes 3-4) and in Figure 21B (lanes 3-4), those samples showed high levels of p53 expression and no detectable mRNA 3' cleavage. Interestingly, our results showed that GAPDH and actin mRNAs were enriched ~5 fold in the poly(A)$^+$ preparation in samples from p53 depleted MCF-7 cells (Figure 23A) and DLD-1 cells (Figure 23B). Both the p53 depleted MCF-7 cells (Figure 15B, lane 5) and DLD-1 cells (Figure 21A), which express only the p53 mutant, showed detectable levels of mRNA 3' cleavage. After UV treatment, the p53 siRNA depletion in MCF-7 was not complete , generating samples with no detectable 3' cleavage (Figure 15B, lane 6) and with a strong decrease in the enrichment in the poly(A)$^+$ preparation to the levels observed for control siRNA (Figure 23A). In contrast, UV-treated DLD-1 cells showed not only a slight decrease in the enrichment in the poly(A)$^+$ preparation to the levels observed for untreated DLD-1 cells (Figure 23B) but also a slight

decrease in levels of 3' cleavage (Figure 21A). D-A2 cells expressing WT-p53 did not show either detectable levels of mRNA 3' cleavage (Figure 21B) or enrichment of housekeeping genes in the poly(A)$^+$ preparation, and this was irrespective of UV treatment (Figure 23B). Taken together these experiments indicate that expression of WT-p53 is sufficient for inhibition of mRNA cleavage and reducing levels of polyadenylated mRNA of housekeeping genes. Interestingly, neither the knockdown of p53, the expression of WT or mutant p53, nor UV treatment had an effect on p21 poly(A)$^+$ mRNA enrichment (Figure 23 A-B), suggesting that the steady-state levels of p21 mRNA are regulated transcriptionally and not by changes in polyadenylation. This data is consistent with others previous observations that p53 target genes have different requirements for not only transcription but also 3' end processing (Gomes et al. 2006)

Taken together, these results show an inverse correlation between the levels of p53 expression and not only the levels of 3' cleavage but also the enrichment of housekeeping mRNAs in the poly(A)$^+$ preparation over the total RNA fraction, suggesting that p53 might decrease the levels of polyadenylated mRNAs by inhibition of mRNA 3' cleavage. As we proposed before, it is possible that the association of p53, BARD1 and CstF in different cellular conditions could play a role regulating mRNA 3' processing and, therefore, mRNA levels of different genes.

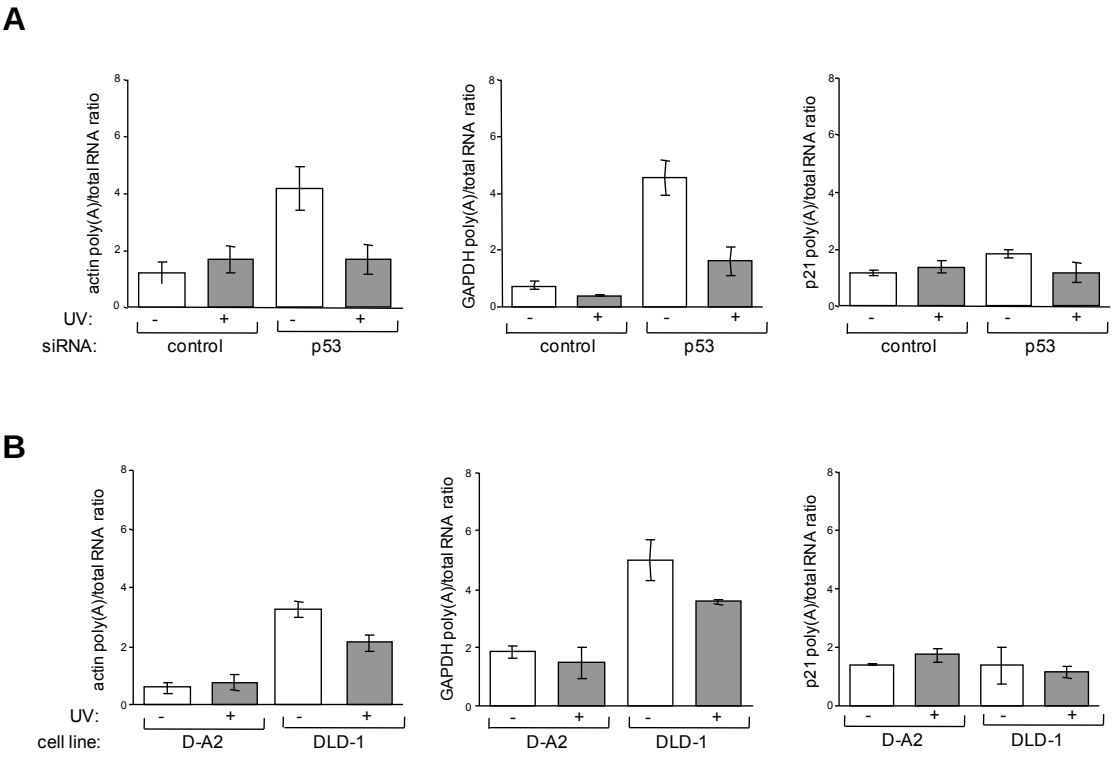

Figure 23: p53 expression induces the enrichment in the poly(A)$^+$ preparation over the total RNA fraction for β-actin and GAPDH mRNAs but not p21 mRNAs. Real time PCR analysis of β-actin, GAPDH and p21 mRNAs polyadenylation. Total and poly(A)$^+$ RNA were prepared from **(A)** MCF-7 cells treated with control/p53 siRNA and UV irradiation or **(B)** DLD-1/D-A2 cells following removal of Dox and UV treatment. Equal mass of total or poly(A)$^+$ RNA was used as a template in the RT-PCR reactions with primers specific to β-actin, GAPDH or p21 mRNAs. The results shown are the average of three PCRs from two different RNA extractions.

Discussion

We have proposed in previous work that the 3' processing factor 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 extended those studies and discovered that CstF-50 interacts directly with the C-terminal domain of the tumor suppressor p53 (Figure 8), connecting p53 to the RNA 3' processing machinery. Although the p53/CstF complex is detected independently of DNA damage, we found that the tumor suppressor BARD1, which is required for the UV-induced inhibition of mRNA 3' end formation (Kleiman and Manley 2001, Kleiman et al. 2005), can coexist in complexes with CstF and p53 in NEs of UV–treated cells (Figure 11-12), suggesting that p53 may play a role in the UV-induced inhibition of mRNA 3' processing. Several other observations support this idea. First, p53 can inhibit the 3' cleavage reaction *in vitro* (Figure 13). Second, the siRNA mediated depletion of either p53 (Figure 15 A-B) or BARD1/BRCA1 (Kleiman et al. 2005) shows a similar effect rescuing UV-induced inhibition of mRNA 3' processing. Third, the tumor-associated Ser241Phe mutation in p53 reduces binding to BARD1 and disrupts the CstF/p53/BARD1 complex association, and this complex formation is restored by the induced expression of wild type-p53 (Figure 19-20). Furthermore cells expressing this mutant p53 show reduced inhibition of mRNA 3' cleavage, which is restored by the induced expression of wild type-p53 (Figure 21). Finally, we determined that the expression of wild type-p53 has an effect on the polyadenylation state of different genes in different cellular conditions (Figure 23). Our results provide new insights into

p53 function and the mechanisms behind the inhibition of mRNA 3' processing following DNA damage.

Together these results indicate a new link between mRNA 3' processing, tumor suppression, and the DNA repair machinery, showing that the UV-induced inhibition of 3' processing might occur due to the activation of the CstF/p53/BARD1 complex. This is consistent with the possibility that the p53-associated reduction of mRNA levels observed in cells following DNA damage (Ljungman et al. 1999) may be not only due to reduced mRNA synthesis but also due to inhibition of mRNA 3' end processing. Our results suggest a novel function of p53 as an inhibitor of the 3' processing machinery. Considering that the p53 pathway is tightly controlled in cells following DNA damage (Vousden and Lu. 2002, Vousden 2006), the p53-associated control of mRNA 3' processing could be an effective mechanism employed to control gene expression in cells upon DNA damaging conditions.

Supporting the idea that the 3' processing machinery is interconnected with the p53 pathway, it has been shown that Rbbp6, a p53-binding protein, is part of the pre-mRNA 3' processing complex (Shi et al. 2009). Rbbp6 can also bind Rb and plays important roles in apoptosis, cell cycle and p53 regulation (Sakai et al. 1995, Simons et al. 1997). In the same study, Shi and colleagues (2009) showed that the Ku-70 subunit of DNA-PK is also part of the 3' processing complex. Interestingly, it has been shown that BARD1 stabilizes p53 by stimulating p53 phosphorylation at Ser15 by the DNA damage response kinase DNA-PK (Feki et al. 2005). As BARD1 is involved in the UV-induced inhibition of mRNA 3' processing and acts as a mediator between genotoxic stress and apoptosis by binding to and stabilizing p53 (Irminger-Finger et al. 2001, Fabbro et al. 2004, Feki et al. 2005), this further supports the functional link between both tumor suppressors and the mRNA processing pathways following

DNA damage. Consistent with our data, other links between mRNA 3' processing and tumor suppression have been described (Topalian et al. 2001, Kleiman and Manley 2001, Scorilas 2002, Rozenblatt-Rosen et al. 2009).

A recent study has shown that the interaction of BARD1 with CstF-50 occurs on the flexible linker region between the ankyrin repeats and the BRCT domain of BARD1 (Edwards et al. 2009). The CstF-50 binding domain of BARD1 lies in a region that shows structural plasticity, which might be important to the regulated assembly of different protein complexes. It has been suggested that this interaction between CstF-50 and the BARD1 linker could rigidify and, thereby, control placement of the BARD1 C-terminal region, facilitating interactions with other factors, such as p53, to regulate different aspects of the DNA damage response. Consistent with this, the tumor-associated mutation in BARD1 (Gln564His), which lies within the CstF-50 interaction domain, not only reduces binding to CstF-50 and abrogates inhibition of mRNA 3' processing (Kleiman and Manley 2001) but also reduces the binding to p53 and the induction of apoptosis (Irminger-Finger et al. 2001).

The ability of p53 to regulate cell fate depends on its ability to selectively activate cell cycle arrest genes or apoptotic genes. Although it is well established that p53 is a transcriptional regulator, it is also possible that p53 plays a direct role in activating target genes by regulating 3' processing, and therefore mRNA stability, by its interaction with CstF under different cellular conditions. It has been shown that cells can tolerate several-fold variation in the concentration of CstF without affecting cell growth (Martincic et al. 1998, Takagaki and Manley 1998, Chuvpilo et al. 1999, Chennathukuzhi et al. 2001, Shell et al. 2005). Takagaki and Manley (1998) showed that CstF plays critical roles in progression of the cell cycle and in cell survival, and that there is a threshold CstF level below which an irreversible process starts leading to apoptotic cell death.

Intracellular levels of CstF can modulate both alternative mRNA 3' processing and mRNA accumulation, reflecting a general mechanism of gene control. It is possible that, depending on the levels of p53, the amounts of different sets of mRNAs may be selectively controlled by this transactivation independent function of p53. Consistent with this, we show in the current study that p53 has different effects on the polyadenylation state of endogenous housekeeping genes but not of p53 regulated genes such as p21 (Figure 23).

Taken together, our studies identify a novel 3' RNA processing inhibitory function of p53, suggesting that the p53/CstF-50 interaction contributes to UV-induced inhibition of pre-mRNA 3' processing and provides evidence of a new link between mRNA 3' processing and tumor suppression.

Chapter III

BARD1 C-terminal domain interacts with the 3' mRNA processing factor CstF-50.

Introduction

The breast and ovarian cancer associated protein, BRCA1, together with its binding partner BARD1, control the cell cycle in response to DNA damage (Irminger-Finger and Leung 2002, Scully et al. 2004). Both proteins interact through N-terminal RING and adjacent helical domains to form a heterodimeric E3 ubiquitin ligase that constitutes the major catalytic activity of the BRCA1/BARD1 complex (Wu et al. 1996, Brzovic et al. 2001, 2003). While the direct targets of BRCA1-BARD1 ubiquitination are unclear, targeting likely involves conserved protein−protein interaction domains in both BRCA1 and BARD1.

Critical protein−protein interactions are mediated by a pair of sequence repeats at the C-terminus of BRCA1 called BRCT repeats (for BRCA1 C-terminal repeats, Bork et al. 1997, Callebaut and Mornon 1997). Similar repeats are found in a number of proteins involved in the cellular response to DNA damage (Glover et al. 2004). In BRCA1, the BRCT repeats mediate interactions with several proteins such as BACH1/BRIP (Cantor et al. 2001), CtIP (Yu et al. 1998, Li et al. 1999), and Abraxas (Kim et al. 2007, Liu et al. 2007). In each of these cases, the BRCA1 BRCT recognizes a phosphopeptide motif in the target protein, pSer-X-X-Phe (Manke et al. 2003, Yu et al. 2003). A series of structural studies have revealed that the N-terminal BRCT repeat contains a pocket which recognizes the phosphoserine, while the phenylalanine residue is recognized by an adjacent hydrophobic pocket formed at the interface between the N- and C-terminal BRCT repeats (Glover et al. 2004, Clapperton et al. 2004, Shiozaki et al. 2004). Cancer-associated mutations have been uncovered which specifically perturb the integrity of this phosphopeptide binding surface, demonstrating the critical importance of these interactions for the tumor suppression function of BRCA1 (Williams et al. 2004, Tischkowitz et al. 2008).

BARD1 also contains tandem BRCT repeats at its C-terminus, as well as a set of ankyrin repeats immediately N-terminal to the BRCT region. *In vitro* peptide binding studies suggest the BARD1 BRCT repeats may bind serine-phosphorylated peptides (Rodriguez et al. 2003), although attempts to isolate phosphorylation-dependent protein binding partners from human cells for the BARD1 BRCT region have been unsuccessful (Kim et al. 2007). Ankyrin repeats are also well-known protein−protein interaction modules (Mosavi et al. 2004), strongly suggesting that this region could also function to recognize targets of the BRCA1/BARD1 complex. Individual ankyrin repeats consist of a helix−turn−helix followed by a β-hairpin. Multiple repeats stack together such that the loops protrude from one face of the structure to constitute the protein interaction surface. Both the ankyrin and BRCT repeat regions of BARD1 have been demonstrated to be required for chromosomal stability and homology-directed repair of DNA damage in mammalian cells (Laufer et al. 2007). A number of missense variants within the BARD1 C-terminal regions have been isolated from breast and ovarian cancer patients, further highlighting the importance of this region for BRCA1/BARD1 function (Thai et al. 1998, Ghimenti et al. 2002, Sauer and Andrulis 2005).

A series of studies implicate the BARD1 C-terminus in the regulation of mRNA 3′ processing in response to DNA damage (Kleiman and Manley 1999, 2001, Kleiman et al. 2005, Mirkin et al. 2008). DNA damage triggers interactions between BRCA1/BARD1 and the 3' mRNA processing factor CstF (cleavage stimulation factor) at the sites of stalled transcription (Kleiman et al. 2005, Mirkin 2008). These interactions may regulate the inhibition of transcription through the targeted degradation of RNAP II (Kleiman et al. 2005, Mirkin et al. 2008), as well as the transient inhibition of 3' mRNA processing. Interactions between the BRCA1/BARD1 heterodimer and the CstF complexes depend on direct interaction of the

BARD1 C-terminus and the 50 kDa component of the CstF complex, CstF-50 (Kleiman and Manley 1999).

Dr. Mark Glover and colleagues (University of Alberta, Canada) studied the structures and CstF-50 binding characteristics of BARD1 C-terminal regions. They showed by limited proteolysis that the BARD1 ankyrin and BRCT repeats constitute independent folded modules linked by a flexible tether. They performed small angle X-ray scattering (SAXS) and those studies indicated that the ankyrin and BRCT repeats do not adopt a fixed orientation with respect to one another and imply that these protein−protein interaction modules do not form a contiguous rigid surface for interaction with binding partners. They also described that the crystal structure of the BARD1 BRCT repeat shows a degenerate BARD1 BRCT phosphopeptide binding pocket with intact pSer interacting motifs but which lacks binding determinants for the pSer + 3 hydrophobic specificity pocket at the inter-BRCT repeat interface (Figure 24).

As part of those studies, the CstF-50 binding properties of a series of BARD1 deletion mutants was determined. Previously, it was shown that the two C-terminal ankyrin repeats, the region just downstream, or both constitute the CstF-50 binding domain in BARD1 (Kleiman and Manley 1999). It was also shown that a tumor-associated mutation in BARD1 (Gln564His), which lies within the CstF-50 interaction domain, reduces binding to CstF and abrogates inhibition of mRNA 3' processing (Kleiman and Manley 2001). Consistent with that, our results map the principal CstF-50 interaction site to the ankyrin-BRCT linker. The protein pull-down experiments utilizing a series of purified BARD1 deletion mutants indicate that interactions between the CstF-50 WD-40 domain and BARD1 involve the ankyrin-BRCT linker but do not require ankyrin or BRCT domains. Taken together, these studies indicate that the structural plasticity imparted by the ANK-BRCT linker helps to explain the regulated assembly of different

protein BARD1 complexes with distinct functions in DNA damage signaling, including BARD1-dependent induction of apoptosis plus p53 stabilization and interactions. BARD1 architecture and plasticity imparted by the ANK-BRCT linker are suitable to allow the BARD1 C-terminus to act as a hub with multiple binding sites to integrate diverse DNA damage signals directly to RNA polymerase.

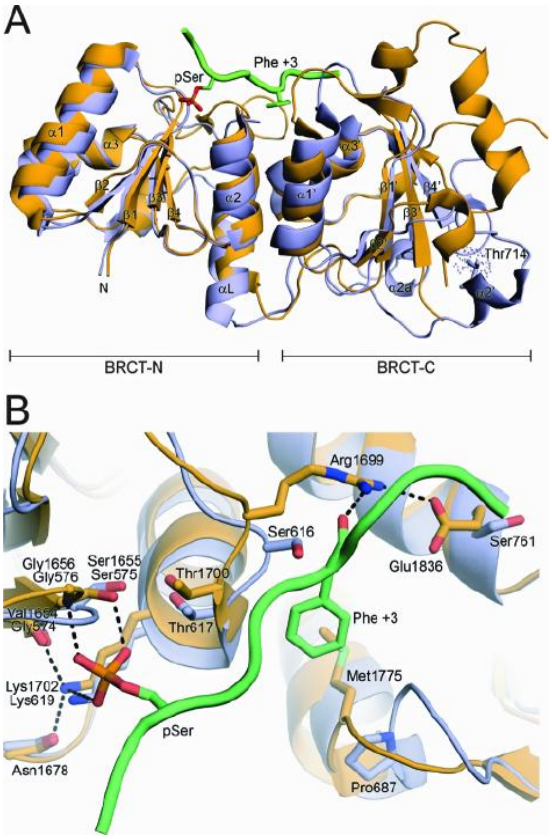

Figure 24: Structure of the tandem BRCT repeats of BARD1. (A) Overview of the BARD1 BRCT structure (orange) aligned with the structure of the BRCA1 BRCT (blue) bound to an optimized phosphopeptide target (green) (PDB accession code 1T2V). (B) Details of the phosphopeptide recognition surfaces of BRCA1 and BARD1, colored as in (A). Residues involved in peptide binding are labeled and shown as sticks. Modified from Edwards et al. 2009.

Results

The BARD1 Ankyrin-BRCT Linker Is Critical for Interactions with CstF-50

To probe the functional role of BARD1 C-terminal domain flexibility in interactions with its protein partners, we mapped the regions of BARD1 required for physical interactions with CstF-50. CstF-50 (92−431), lacking the 91 amino acid tail N-terminal to its WD-40 domain, was previously shown to be necessary and sufficient for BARD1 interactions by two-hybrid studies (Kleiman and Manley 1999). To confirm this interaction, GST-CstF-50 (92−431) and BARD1 (423−777) were purified separately from *E. coli* and used in GST pull-down assays (Figure 18A). BARD1 (423−777) bound to GST-CstF-50 (92−431), but not to GST alone, revealing a specific interaction between the two proteins. To map the region of BARD1 required for interaction with CstF-50, we compared binding of CstF-50 to the isolated ankyrin domain (BARD1 (423−553)), the linker-BRCT construct (BARD1 (554−777)), the BRCT repeat alone (BARD1 (569−777)), and the ankyrin-linker-BRCT construct (BARD1 (423−777)). The isolated BRCT repeat exhibited weak CstF-50 binding, and the ankyrin domain in isolation did not bind to CstF-50, suggesting these regions make minimal contributions to high-affinity CstF-50 interactions (Figure 25C). In contrast, the ANK-linker-BRCT and linker-BRCT displayed robust CstF-50 binding (Figure 25A-B), implicating the flexible ankyrin-BRCT linker as the core CstF-50 interaction region.

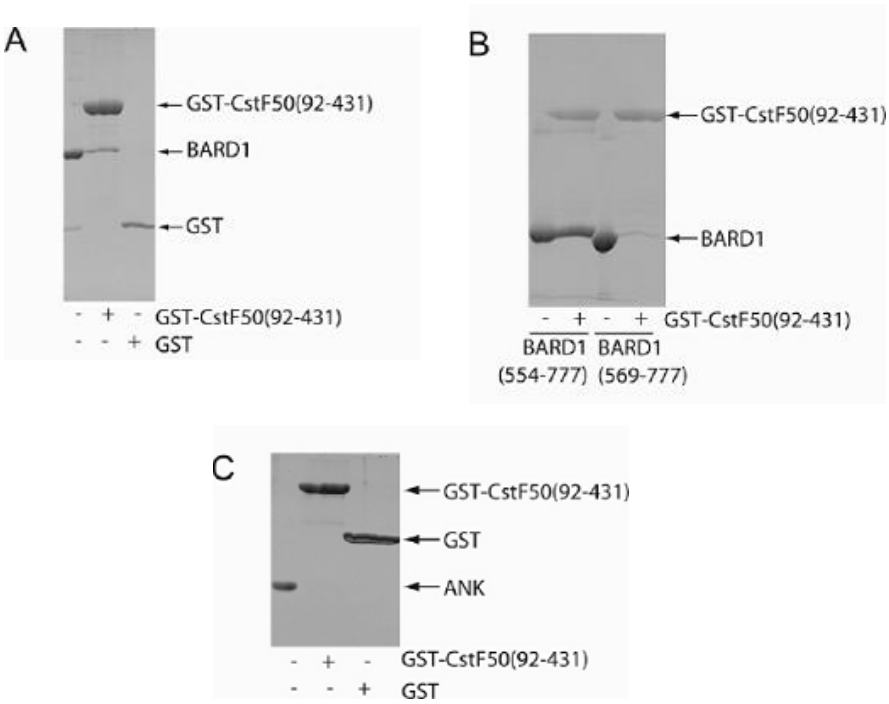

Figure 25: The BARD1 ankyrin-BRCT linker is critical for interactions with CstF-50. (A) Interaction of GST-CstF50 (92−431) and BARD1 (423−777). The BARD1 construct containing both ankyrin repeats and BRCT repeats was incubated with purified GST or GST-CstF50 (92−431). Bound proteins were eluted and resolved by SDS−PAGE. Five percent of the GST-CstF50 (92−431) used in binding reactions is shown. (B) Requirement of the BARD1 linker for CstF-50 interaction. BARD1-BRCT derivatives with or without the BARD1 linker were used in binding reactions with GST-CstF50 (92−431). Five percent of each of the GST-CstF50 (92−431) used in binding reactions is shown. (C) Lack of interaction between BARD1-ankyrin and GST-CstF50 (92−431). BARD1-ankyrin was incubated with purified GST or GST-CstF50 (92−431). Five percent of the BARD1-ankyrin used in binding reactions is shown.

Discussion

Functionally important conformational flexibility in the interactions with multiple protein partners has recently been characterized as critical in proteins that act as hubs for the integration of information for DNA repair and DNA replication pathways (Pascal et al. 2006, Shell et al. 2007). Here we find that BARD1, which acts in the control of the cell cycle in response to DNA damage (Irminger-Finger et al. 2002, Scully et al. 2004), unexpectedly employs a flexible linker region for functionally important interactions, suggesting how BARD1's structure may facilitate its ability to act as a hub for multiple partners and thereby provide a means to integrate information for control of transcription and RNAP II in response to DNA damage.

The crystallographic and small angle X-ray scattering data from Dr. Glover's laboratory and my biochemical data characterize the structural biochemistry for BARD1 interactions with CstF-50. The combined results indicate that the C-terminal region of BARD1 consists of a two-domain structure connected by a flexible peptide linker. SAXS data show that the solution structure is most consistent with an ensemble of ankyrin-BRCT models suggesting that the two domains sample a wide range of orientations with respect to one another reminiscent of those seen for mismatch repair interaction (Shell et al. 2007). In addition, a recent NMR spectroscopic study failed to uncover any interactions between the BARD1 ankyrin and BRCT domains (Fox et al. 2008). Thus, the protein interaction surfaces on the ankyrin and BRCT domains are not prealigned to form a contiguous recognition surface as might be expected but instead provide a means to alter BARD1 architecture depending upon its partner interactions with the linker region.

CstF-50 is the best characterized binding partner for the BARD1 C-terminal region. Our pull-down data demonstrate that specific interactions between the CstF-50 WD-40 domain and

BARD1 critically depend on the BARD1 interdomain linker (Figure 25). Structural studies of WD-40-containing protein complexes indicate that proteins of this family interact with protein partners at the depression formed at the center of the WD-40 β-propeller structure. These interactions can then form an anchor for assembly of larger protein complexes. Such interactions were first structurally characterized for heterotrimeric G-proteins where the β-subunit is a WD-40 protein (Wall et al. 1995, Gaudet et al. 1996). Interestingly, interactions between CstF-50 and the BARD1 linker could rigidify and thereby control architectural placement of the BARD1 C-terminal region, thereby reducing the relative flexibility of the ankyrin and BRCT domains and facilitating interactions with other factors, as seen for the Rad51 polymerization domain interaction with BRCA2 (Shin et al. 2003).

CstF-50 is implicated in the direct recognition of the CTD of RNAP II, potentially providing a critical interaction to recruit the CstF complex to the transcribing RNA polymerase holoenzyme (McCracken et al. 1997, Hirose and Manley 1998, Kleiman and Manley 1999). This interaction does not require the WD-40 domain of CstF-50 but relies on an N-terminal region that we have shown is not required for interactions with BARD1 (Fong and Bentley 2001). Significantly, since the minimal binding regions do not overlap, the RNAP II CTD, CstF-50, and the BARD1 C-terminal domains could potentially all interact within the elongating transcriptional complex. The proximity of the BARD1 BRCT to the CTD would allow for additional interactions between the phosphorylated CTD and the BARD1 BRCT. Since the BARD1 BRCT preferentially binds phosphopeptides with negatively charged side chains at the +2 and +3 positions *in vitro*, CTD binding by the BARD1 BRCT might be facilitated by phosphorylation at both the position 2 and 5 serines of the CTD.

Besides interactions with CstF-50, the BARD1 C-terminal region is implicated in interactions with p53. These interactions lead to the phosphorylation and stabilization of p53 and are furthermore implicated in facilitating apoptosis in response to DNA damage (Fabbro et al. 2004, Feki et al. 2005). The ankyrin repeats, linker, and a portion of the N-terminal BRCT repeat contain the minimal region of BARD1 required for p53 interactions (Feki et al. 2005). The Q564H BARD1 mutation attenuates BARD1-dependent induction of apoptosis, p53 stabilization, and interactions with p53, suggesting that the BARD1 linker region is critical for a functional interaction of BARD1 with p53 (Irminger-Finger et al. 2001). Thus, the C-terminal region of BARD1 can interact with diverse partner proteins to regulate different aspects of the DNA damage response. Such proteins that act as hubs in integrating DNA repair responses are potential master keys to understanding and eventual therapeutic interventions for cancer, highlighting the value of understanding the detailed structural biochemistry underlying pathway control by multiple structural interactions (Perry et al. 2007). For BARD1, the structural plasticity of this region, imparted by the ANK-BRCT linker, is likely key to the regulated assembly of different protein complexes with distinct functions in DNA damage signaling.

Chapter IV

UV-induced phosphorylation of BARD1 is required for the Ser-15 phosphorylation of p53 following DNA damage.

Introduction

p53 is a central factor in the cellular stress response and is tightly regulated to ensure its rapid activation as well as deactivation and degradation. Distinct activation pathways exist for p53 and they show specificity depending on the type of DNA damage. The p53 protein level is constantly balanced between synthesis and degradation by its negative regulator Murine/Human double minute 2 (Mdm2/Hdm2, Scheffner et al. 1993). Posttranslational modifications on p53, such as phosphorylation at Ser 15 and Ser 20/Thr18, can interfere with the interaction between p53 and Mdm2 affecting p53 stability (Saito et al. 2003, Dumaz et al. 1999). Ser 15 phosphorylation is also essential for the functions of p53 as a transcription factor (Shieh et al. 1997) as well as in apoptosis (Khanna et al. 1998). Although phosphorylation at Ser 20 and Thr18 mostly affect p53 stability by preventing its binding to Mdm2, these phosphorylation events are dependent upon initial phosphorylation at Ser 15 (Saito et al. 2003, Dumaz et al. 1999), underscoring the importance of phosphorylation at these residues in the regulation of p53. Following IR- and UV-induced DNA damage, p53 is phosphorylated by various kinases such as ATM (ataxia-telangiectasia-mutated), ATR (ATM and Rad3-related), Chk1 (checkpoint kinase 1), Chk2, JNK, HIPK2, DNA-PK depending upon the type and severity of the damage (reviewed by Oren et al. 2002).

BARD1 directs phosphorylation of p53 at Ser 15 by interacting with Ku-70, a subunit of the DNA dependent protein kinase (DNA-PK, Feki et al. 2005). It has been described that BARD1 expression can be transcriptionally upregulated in response to genotoxic stress (Irminger-Finger et al. 2001). Increases in BARD1 expression are also associated with apoptosis in the brain following hypoxia (Irminger-Finger et al. 1998). Consistent with this, BARD1 deficient cells are defective in the apoptotic response to genotoxic stress; and this proapoptotic

activity of BARD1 is stimulated by nuclear export (Rodriguez et al. 2004) as well as binding to the tumor suppressor p53 (Irminger-Finger et al. 2001). Furthermore, overexpression of BARD1 leads to apoptosis associated with stabilization of p53 (Feki et al. 2005). Interestingly, the apoptotic function of BARD1 is dependent on functional p53 but inhibited by BRCA1. Unexpectedly, a study characterizing the molecular architecture of the 3' processing complex has shown that Ku-70, which is part of the DNA-PK kinase that phosphorylates p53, is also part of the mRNA processing complex (Shi et al. 2009). The minimal region required for p53 binding to BARD1 spans the region between the ANK and BRCT domains (Feki et al. 2005), which is also the minimum region required for binding CstF-50 (Kleiman and Manley 1999). Interestingly, the Gln564His germline mutation of BARD1 that reduces binding to CstF-50 and abrogates inhibition of polyadenylation (Kleiman and Manley 2001) also reduces the binding to p53 and the induction of apoptosis (Irminger-Finger et al. 2001).

As part of a collaboration between Dr. Kleiman's and Dr. Lee's laboratories (Genetics of Development and Disease Branch, National Institute of Diabetes & Digestive & Kidney Diseases, NIH), the possible phosphorylation of BARD1 in response to DNA damage was studied. As part of those studies, they found that BARD1 undergoes phosphorylation upon ionizing radiation (IR) or UV radiation (Figure 26) 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 human BARD1 (Thr165, Ser244, Thr714 and Thr734). 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 27A). 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 27B). 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 27B, 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 Thr734 to Ala almost completely abolished BARD1 phosphorylation to a similar extent to Quad BARD1, even after IR treatment (Figure 27B). This result demonstrated the specificity of Thr734 phosphorylation of BARD1 in the cellular response to DNA damage.

As part of those studies, it was 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 (Figure 28). As mentioned before, the tumor suppressors BARD1-associated BRCA1 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). Importantly, Kim et al. (2006) have shown that DNA damage-associated functions of BARD1, such as degradation of RNAP II (Figure 29, Kleiman et al. 2005) and inhibition of pre-mRNA 3' processing (Figure 28A, Kleiman and Manley 2001), are abrogated in T714A and T734A mutants. These cells expressing the T734A BARD1 mutant do not show the formation of the BARD1/CstF complex irrespective of UV treatment, whereas cells expressing wild-type BARD1 are able to form the complex which increased significantly after the UV damage (Figure 28B, Kim et al. 2006). Taken together all these findings suggest that phosphorylation of T714 and T734 BARD1 is critical for the DNA damage functions of BRCA1/BARD1 complex.

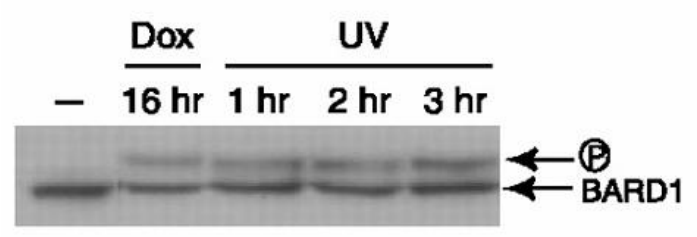

Figure 26 - BARD1 phosphorylation in response to DNA damage.

U2OS cells were either treated with 1μg/ml of doxorubicin or with UV and nuclear extracts prepared at indicated times were immunoblotted with anti-BARD1 antibody. Modified from Kim et al. 2006.

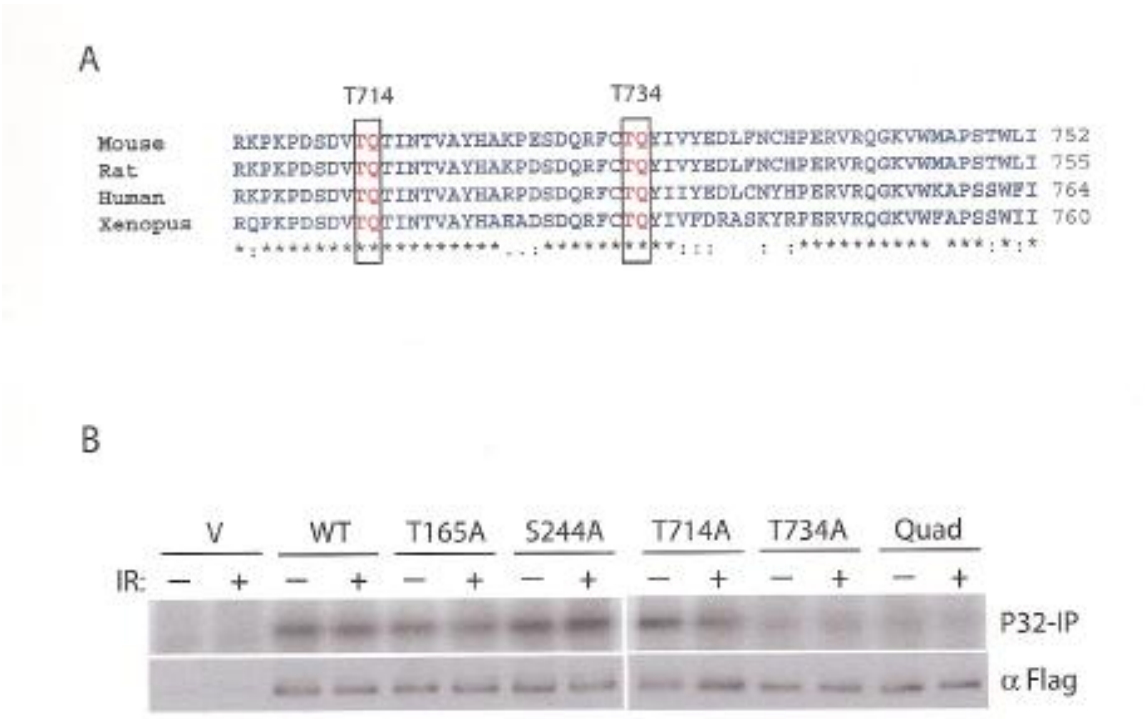

Figure 27 - BARD1 Thr714 and Thr734 are evolutionarily conserved and phosphorylated in vivo. (**A**) Amino acid sequence alignment near the C-terminal BRCT domain of different BARD1 orthologs. Boxes indicate conserved PIKK-phosphorylation (TQ) sites. T714 and T734 refer to the human BARD1 residues. (**B**) U2OS cells stably transfected with different BARD1 expression constructs were either untreated or IR-treated 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). Modified from Kim et al. 2006.

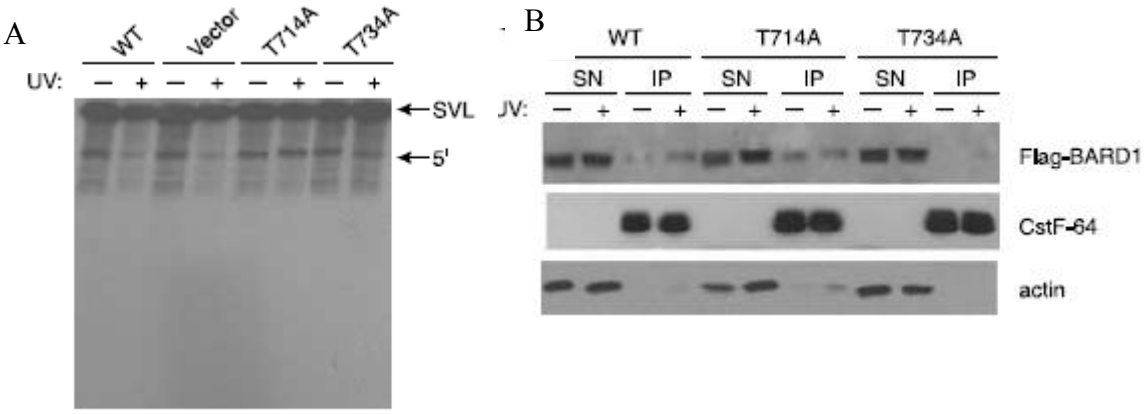

Figure 28 - BARD1 T714A and T734A mutants are defective in UV-induced 3' cleavage inhibition and in the UV-induced CstF/BARD1 complex formation. Nuclear extracts were prepared from cells stably transformed with various BARD1 mutants, either untreated or treated with UV. **(A)** *In vitro* RNA cleavage 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. **(B)** 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. Modified from Kim et al. 2006.

In addition, as discussed earlier in Chapter II, we have presented evidence that p53 associates with CstF-50 and BARD1, connecting p53 to the RNA 3' processing machinery. We also found that the tumor suppressor BARD1 can coexist in complexes with CstF and p53 in nuclear extracts of UV–treated cells, suggesting that p53 may play a role in the UV-induced inhibition of mRNA 3' processing. Consistent with this, we showed that p53 can inhibit the 3' cleavage reaction *in vitro* and that p53 siRNA-treatment has different effects on mRNA 3' processing in cells expressing different levels of p53. Taken together, those studies indicate a functional link between CstF, BARD1 and p53 following DNA damage.

We were intrigued by the fact that BARD1, which participates in the UV-induced inhibition of 3' processing, is phosphorylated under DNA damaging conditions (Kim et al. 2006). We wanted to investigate the possible effects of this UV-induced modification of BARD1 on p53 phosphorylation following UV treatment. Here I present preliminary results that indicate that phosphorylation of T734 BARD1 is critical for the UV-induced phosporylation of p53 at Ser 15, suggesting that this modification of p53 might be important for its role in 3' mRNA processing.

Results

As BARD1 plays a role in phosphorylating and stabilizing p53 following DNA damage (Feki et al.2005), we first wanted to determine the effect of UV-induced phosphorylation of BARD1 on p53 levels. To examine this, Western blot analysis was performed on nuclear extracts (NE) prepared from U2OS cells stably transfected with empty vector, WT and mutant BARD1 at residues T714A and T734A. As mentioned before, 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 p53, BRCA1, RNAP II, RNAP IIO, CstF-64 and actin.

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. Consistent with these previous studies, 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 29). 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 Thr714 and Thr734 is important for the preferential degradation of RNAP IIO mediated by the BRCA1/BARD1 complex in response to DNA damage.

Extending those studies, we analyzed the expression levels of p53 in U2OS cells transfected with mutant BARD1 at T714 and T734. As expected, cells expressing the WT and empty vector showed UV-induced increase in p53 expression (Figure 29). However, cells expressing either T714A or T734A BARD1 mutants showed lower levels of p53 and did not show the increase in p53 levels following DNA damage (Figure 29), suggesting that the UV-induced phosphorylation of BARD1 at T714 or T734 may play a role in the stability of p53 following UV treatment in U2OS cells.

The loss of UV-induced increase in the p53 levels in cell lines expressing BARD1 mutants at residues T714 and T734 (Figure 29) might be due to a decrease in p53 stability. Since phosphorylation at Ser 15 enhances p53 stability by reducing its interactions with Mdm2 (Shieh et al. 1997), we wanted to determine the effect of the UV-induced phosphorylation of BARD1 on the phosphorylation of p53 at Ser 15. The phosphorylation of p53 at Ser 15 was analyzed using NEs from cell lines expressing WT, empty vector or T714A and T734A BARD1 mutants by Western blot with specific antibodies against the Ser 15 phosphorylated form of p53. As shown in Figure 30, cells expressing WT-BARD1, empty vector or the BARD1 T714A mutants showed the increase in Ser 15 phosphorylated forms of p53 following UV treatment. However, the increase in p53 phosphorylated in Ser 15 was not observed in cells expressing the BARD1 T734A mutant following UV treatment. While cells expressing WT BARD1 showed an increase in both Ser 15 phosphorylated forms of p53 (Figure 30) and total p53 (Figure 29) after UV treatment, cells expressing the T714A BARD1 mutant showed an increase only in Ser 15 phosphorylated forms of p53 (Figure 30) but not in total p53 (Figure 29) following UV treatment. This suggests that the observed lack of increase in p53 stability in T714A mutant cells is probably not due to a loss of Ser-15 phosphorylation on p53. It is possible that the residue

T714 is involved in the regulation of p53 stability by a mechanism other than the Ser 15 phosphorylation. All together, these results suggest that the UV-induced phosphorylation of BARD1 at Thr 734 is required for p53 phosphorylation at Ser 15, affecting the stability of p53.

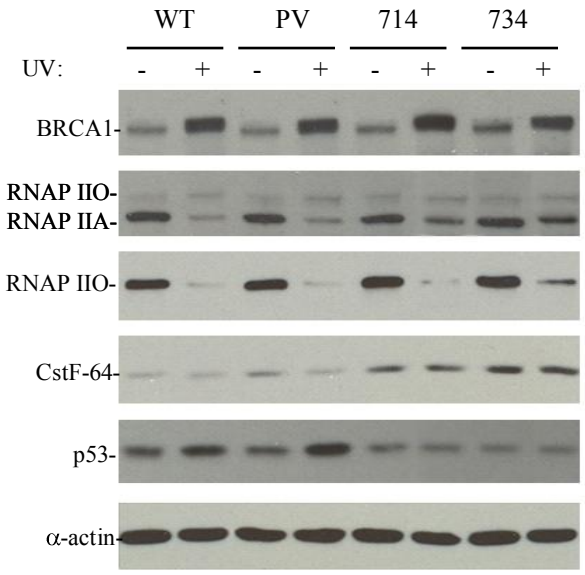

Figure 29: BARD1 T714A and T734A mutants are defective in UV-induced increase in p53 levels. U2OS cells stably transformed with WT, empty vector (PV) and T714A and T734 BARD1 mutants were untreated or treated with UV (20 J/m^2). After 2 hours, cell extracts were immunoblottted with the indicated antibodies: BRCA1 (C-20, Santa Cruz), RNAP II (8WG16 Covance), p53 (DO-1 Santa Cruz). Anti-actin (A2066, Sigma) blot shows relatively equal loading in all samples.

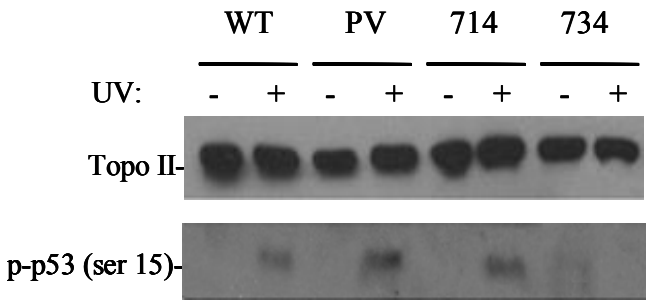

Figure 30: BARD1 T734A but not T714A mutant shows phosphorylation of p53 at Ser 15 following UV treatment. U2OS cells stably transformed with WT, empty vector (PV) and T714A and T734 BARD1 mutants were untreated or treated with UV (20 J/m^2). After 2 hours, cell extracts were immunoblottted with antibodies against Topo II (SC-25330, Santa Cruz) and phosphorylated p53 at Ser 15 (Cell signalling 9286).

Discussion

Reversible phosphorylation is a critical mechanism regulating the cellular response to DNA damage, fine tuning this response depending on the type and severity of damage. Here, I have shown preliminary data that indicate that the phosphorylation state of one tumor suppressor, BARD1, affects the phosphorylation of another, p53, in response to DNA damage. Ser-15 phosphorylation of p53, which stabilizes p53 after UV treatment (Shieh et al. 1997), is abrogated in cells expressing the BARD1 mutant T734A, which is defective in the UV-induced phosporylation of BARD1 (Kim et al. 2006). Interestingly, cells stably transformed with T734A version of BARD1 show no inhibition of mRNA 3' cleavage following DNA damage (Kim et al. 2006), suggesting that BARD1 phosphorylation at residues T734 is required for inhibition of cleavage following DNA damage. The complex formation between BARD1 and CstF is abrogated by the expression of the BARD1 mutant T734A. Considering that BARD1 and p53 interact with CstF-50 following DNA damage, the reversible phosphorylation of these factors could be an elegant mechanism to regulate gene expression controlling the cellular response to DNA damage to promote either cell survival or apoptosis. Consistent with the idea that CstF together with BARD1 and p53 play a role in the DNA damage response, both the polyadenylation factor CstF and p53 are involved in NER (Hanawalt 2002) and are found at sites of DNA damage together with BRCA1/BARD1 (Mirkin et al.2008). Taken together, these studies suggest that the BARD1/CstF/p53 complex is an ideal candidate to sense the repair of the DNA damage and control apoptosis by interconnected phosphorylation events.

Kim and colleagues (2006) show that phosphorylation in Thr 714 is also important for the UV-induced inhibition of 3' cleavage. However, complex formation between BARD1 and CstF is abrogated only in the case of expression of the BARD1 mutant T734A but not T714A.

Interestingly, UV-induced phosphorylation of p53 at Ser-15 is also abrogated only in cells expressing the BARD1 mutant T734A. This suggests that p53 phosphorylation at Ser 15 and the BARD1/CstF complex formation are functionally related to the UV-induced phosphorylation of BARD1 at Thr 734, suggesting that phosphorylation of p53 at Ser 15 might be required for BARD1/CstF complex formation. It would be interesting to extend these studies to determine the effect of p53 phosphorylation on the CstF/BARD1/p53 complex formation as well as mRNA 3' processing. Ongoing experiments from Dr. Kleiman's lab suggest that the form of p53 responsible for the inhibition of mRNA 3' cleavage is the phosphorylated form (data not shown). Now it would be important to determine which site of phosphorylation in p53 is responsible for that effect. An important question is does phosphorylation of p53 stabilize the BARD1/CstF/p53 complex to enhance inhibition of mRNA 3' end processing? Since inhibition of mRNA 3' processing is linked to the survival mode of BARD1 and phosphorylation of p53 is linked to the apoptotic mode of BARD1 (Irminger-Finger and Jefford 2006), it is now important to examine how these two pathways are interconnected. Are other BARD1-mediated phosphorylation events on p53 involved in this process? To elucidate more processes related to this mechanism of response to DNA damage, it would be relevant to study the role of BARD1-dependent p53 phosphorylation on the mRNA 3' processing pathway and on p53-dependent apoptosis.

CHAPTER V

CONCLUDING REMARKS AND FUTURE DIRECTIONS

The results presented in this dissertation provide a broader understanding of the interplay between tumor suppression and mRNA processing pathways following DNA damage. More specifically, the work carried out focuses on the functional interconnections between the p53 and BARD1 tumor suppressor pathways and RNA 3' end processing and their role in the cellular response to DNA damage. The results presented here suggest that an interplay between these pathways could control gene expression and eventually cell fate in a novel and unexpected manner.

First, evidence has been provided that CstF-50 interacts directly with the tumor suppressor p53 and that a complex comprising CstF-50, BARD1 and p53 plays a role in the inhibition of mRNA processing following UV-induced DNA damage. It has also been shown that this inhibition of 3' end processing results in reduced levels of poly(A) mRNA of housekeeping genes but not p53-regulated genes. We determined that the C-terminal domain of p53 is responsible for this inhibition and that cell lines carrying a p53 mutation in this region are defective not only in forming the p53/BARD1/CstF complex but also in the inhibition of mRNA 3' processing. These studies are particularly significant because they prove the existence of a p53-dependent mechanism to regulate posttranscriptional gene expression, providing new insights to the p53 network.

Second, we contributed to the attempts to characterize the BARD1 structural biochemistry responsible for CstF-50 binding. Studies carried out in Dr. Glover's laboratory (Edwards et al. 2009) show that the interaction of BARD1 with CstF-50 occurs on the flexible linker region between the ankyrin repeats and the BRCT domain of BARD1. The CstF-50 binding domain of BARD1 lies in a region that shows structural plasticity, which might be important to the regulated assembly of different protein complexes. It has been suggested that

this interaction between CstF-50 and the BARD1 linker could rigidify and, thereby, control placement of the BARD1 C-terminal region, facilitating interactions with other factors, such as p53, to regulate different aspects of the DNA damage response. Importantly, these structural-functional studies are consistent with the functional interconnection between BARD1, CstF and p53 in the DNA damage response described above.

Third, preliminary results presented here show that cells deficient in UV-induced phosphorylation of BARD1 show no p53 phosphorylation at Ser 15 and the consequent increase in p53 levels following UV treatment, both of which are important events in the activation of the p53 pathway. Consistent with the idea of a functional interconnection between the p53 network and 3' end processing during the DNA damage response, it has been shown by the Kleiman's lab that the UV-induced phosphorylation of BARD1 also plays an important role in the UV-induced BARD1/CstF complex formation; the UV-induced degradation of RNAP II and the UV-induced inhibition of 3' end processing (Kim et al. 2006).

Based on these findings, a working model has been proposed where the CstF/BARD1/p53 interaction not only regulates RNA 3' end processing, and thereby controls gene expression, but also may perform a sensory/regulatory role in detecting the severity of DNA damage to promote either cell survival or apoptosis. This model is based on our studies as well as on the following findings by others:

1- CstF, BARD1 BRCA1 and p53 are all involved in NER pathways and found at sites at DNA damage (Hanawalt 2002, Mirkin et al. 2008). It has been shown that cells with reduced levels of CstF display decreased viability following UV treatment, reduced ability to ubiquitinate RNAP II, and are defective in the repair of the DNA damage by the

TCR pathway (Mirkin et al. 2008). These data support the idea that the CstF/BARD1/BRCA1/p53 complex plays a critical role in the DNA damage response.

2- We have shown that the CstF/BARD1/p53 complex inhibits mRNA 3' end processing following DNA damage. The mRNA poly(A) tail is important for mRNA stability and transport across the nuclear membrane (Zhao et al. 1999). It is possible that the UV-induced inhibition of 3' processing results in reduced mRNA nuclear export, which might induce nuclear accumulation of p53. It has been shown that the nuclear accumulation of p53 plays a role in the TCR response (Ljungman 2007). This suggests that the p53 function in regulating mRNA processing could also affect the DNA repair functions of p53.3- p53 plays a role in the recovery of cellular mRNA levels following exposure to UV light in a TCR-dependent manner (McKay and Ljungman 1999) and the recovery of mRNA levels protects the cell against apoptosis (McKay et al. 2001). We have shown that p53 plays a role in the mRNA 3' processing pathway following DNA damage, participating in the UV-induced decrease of cellular mRNA levels. All together, these studies suggest that p53 could control DNA repair/cell survival and apoptosis by its role in controlling RNA 3' processing machinery.

4- Our results also indicate that the CstF/p53 interaction occurs through the C-terminal domain of p53 (Figure 8). It has been determined that the C-terminal domain of p53 could act as positive or negative regulator for DNA binding, affecting p53 transcriptional activity (Mckinney et al. 2004, Sauer and Steiwe 2008). Other proteins that bind to the C-terminal domain of p53, such as Y-box-binding protein YB1 and members of the E2F family, have been shown to induce either apoptosis or arrest of cell proliferation (Braithwaite et al. 2006). In view of this, it is possible that the binding of CstF and/or

BARD1 to the p53 C-terminal domain causes the retention of p53 in the nucleus and contributes not only to the p53 functions in DNA repair as discussed earlier but also controls the apoptotic response by preventing nuclear export of p53 and induction of apoptosis through the transcription independent apoptotic program.

5- BARD1 plays a role as mediator of apoptosis by binding, stabilization, and phosphorylation of p53 by binding to the Ku-70 subunit of DNA-PK (Feki et al. 2005). .In addition, a recent study has shown that the Ku-70 is also part of the cleavage/polyadenylation complex (Shi at al. 2009). It has been suggested that BARD1 has a dual mode of action, a cell survival pathway mediated by the BRCA1/BARD1 heterodimer, and a cell death pathway, which is independent of BRCA1. Consistent with this, BARD1 together with BRCA1 are involved in mRNA 3' processing and BARD1 independently of BRCA1 is involved in p53 phophorylation, leading to a DNA repair/survival pathway or apoptotic pathway, respectively.

6- The pro-apoptotic activity of BARD1 is stimulated by nuclear export and involves binding to p53 (Rodriguez et al. 2004). Recently, it has been shown that BARD1 apoptotic activity is linked to its translocation to mitochondria and oligomerization of the pro-apoptotic factors in the mitochondrial membrane (Tembe and Henderson 2007). This is reminiscent of the mechanism by which p53 induces apoptosis in a transcription-independent manner, linking these two proteins in this apoptotic pathway. As will be explained later, it is possible that BARD1 and p53 apoptotic effects might be regulated by the binding of these two factors to CstF in the nucleus.

Based on these observations we propose a model whereby CstF, p53 and BARD1 interaction acts as a molecular switch to control cell survival or apoptosis (Figure 31). Following DNA damage and BARD1 phosphorylation, mRNA 3' end processing is inhibited by the interaction between BARD1/CstF/p53. Ongoing studies in Dr. Kleiman's lab show that indeed the form of p53 involved in inhibition of 3' end processing is phosphorylated at Ser 15 (data not shown). If the DNA lesions are repaired rapidly, p53 may be de-phosphorylated and mRNA processing might be restored, protecting the cell against apoptosis and promoting cell survival. However, if the DNA damage remains unrepaired, mRNA processing might stay inhibited for a long time not allowing the mRNA levels to recover. In that scenario, it is probable that CstF facilitates the interaction between p53 and BARD1 after DNA damage, allowing the phosphorylation and stabilization of p53 that leads to the inhibition of mRNA processing, keeping the total mRNA levels low and inducing apoptosis. The inhibition of 3' end processing also results in reduced nuclear export of mRNA, which could cause nuclear retention of p53 and induction of TCR. Moreover, the retention of p53 in the nucleus and induction of TCR might also occur by masking of the nuclear export signal of p53 that lies in the C-terminal domain with BARD1 and CstF. Extending this model, when DNA damage persists, RNAP II, which is another interactor of CstF-50, could be degraded via the ubiquitin ligase activity of BARD1/BARD1. Since the C-terminal domain of RNAP II acts as a scaffold for RNA processing factors, ubiquitination and degradation of RNAP II could cause dissociation of the RNA processing machinery, affecting the recovery of mRNA processing and the cellular levels of mRNA and inducing apoptosis.

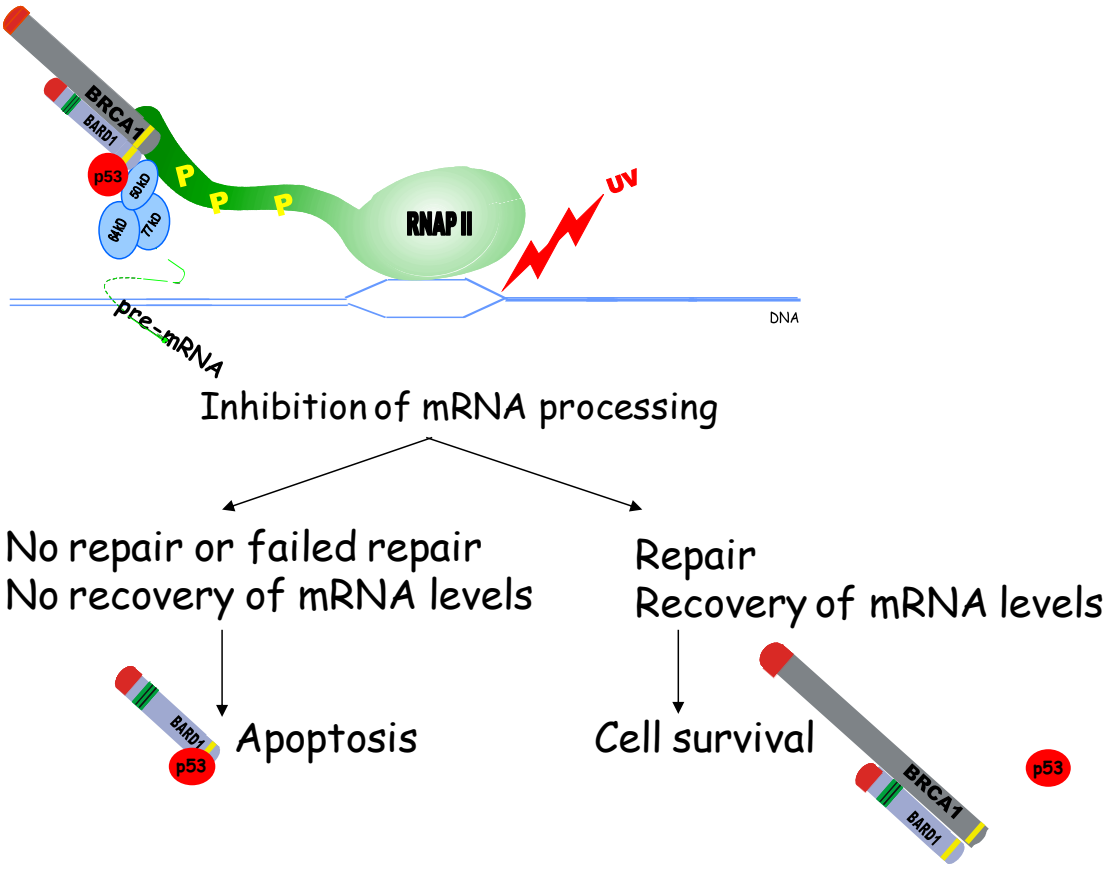

Figure 31 - Model showing role of mRNA processing in the regulation of cell survival and apoptosis. Following DNA damage, 3' end processing is inhibited due to the complex formation between BARD1, CstF-50 and p53. If the damage is repaired rapidly, BARD1 and p53 dissociate from the 3' processing machinery and cellular mRNA levels are restored, promoting cell survival. If the DNA damage remains unrepaired, BARD1 and p53 continue to be bound to CstF, mRNA levels are not recovered, and BARD1 and p53 are translocated to the mitochondria, promoting apoptosis.

In the future, it would be interesting to test this model by studying the correlation between inhibition of 3' processing and levels of apoptosis following DNA damage. Furthermore, it would be relevant to identify if other p53-regulated genes are also regulated in a posttranscriptional manner via control of mRNA 3' processing. The following proposed studies could provide more information that would help to understand some aspects proposed in this dissertation.

Correlation of the levels of mRNA 3' processing and the induction of p53-dependent apoptosis

Ljungman et al. (2007) suggested that reduced nuclear export of p53 contributes to the TCR response and that the recovery of mRNA levels protects the cell against apoptosis. To determine if indeed a correlation exists between changes in levels of 3' processing and p53-dependent apoptosis, levels of mRNA 3' cleavage and apoptosis will be assessed following UV treatment in various cell lines expressing different levels of p53. These studies will be extended to analyze the effect of siRNA-mediated knockdown of p53. Alternatively, cell lines carrying mutant p53 transfected with inducible WT-p53 will be used. NEs from those cells will be used to monitor 3' cleavage of the radiolabeled L3 pre-mRNA substrate as described. Nuclear mRNA from those cells will be also used to monitor the expression levels of p53-dependent and -independent genes by RT-PCR. The levels of apoptosis of these cells will be assessed using flow cytometric analysis. Based on previous results and the proposed model, it is expected that mRNA 3' processing would be inhibited transiently following UV treatment, and recovered in a p53-dependent manner and this would correlate with protection against apoptosis. However, if

inhibition of mRNA 3' processing is prolonged and no recovery of mRNA levels is observed**,** this would correlate with the induction of apoptosis.

Identify p53 target genes regulated by changes in mRNA 3' processing.

Although the p53 pathway has been intensively studied, unexpected functions and regulatory mechanisms of p53 and its targets are still being discovered. For instance, many reports suggest that p53 regulates the expression of certain genes but the mechanisms behind these regulatory functions have not been described yet (Spurgers et al.2006). The discovery of the p53-dependent expression of microRNAs offer one possible mechanism by which p53 could regulate expression of target genes posttranscriptionally (He et al. 2007). In this dissertation, it has been identified that p53 plays a role in the inhibition of mRNA 3' processing following DNA damage. This raises the possibility that control of 3' end processing by p53 could be another novel mechanism by which p53 could control the expression of certain target genes. This could also be mechanism to selectively control either cell cycle arrest or apoptotic target genes in order to favor one pathway over the other, depending on the type and severity of the damage.

To identify a wider set of p53 target genes controlled by changes in mRNA 3' processing, Total RNA from UV-treated cells expressing different levels of p53 and having different levels of 3' cleavage will be analyzed by RT-PCR. This analysis will be carried out for known p53-dependent genes, such as genes responsible in the induction of cell survival or apoptosis, as well as suspected p53 targets. Then the levels of poly(A)/total RNA ratios will be determined as described (Gomes et al. 2006). If p53 indeed controls certain target genes via inhibition of mRNA processing, we would observe a decrease in the poly(A)/total RNA ratio of these targets along with a relative decrease in mRNA levels. For example, it has been shown in this

dissertation that the poly(A)/total RNA ratio of p21 mRNA is not affected following UV damage (Figure 23). It will be interesting to extend this study to analyze poly(A)/ total RNA ratios of other p53-target genes. To determine the possible correlation between the regulation of expression of different types of p53 targets and the severity of the damage, it will be also important to determine the poly(A)/total RNA ratios for p21 as well as other p53 targets following various doses of DNA damage. These studies would provide insights if indeed control of RNA 3' processing, like recently described for the miRNA pathway, is an alternative mechanism to control p53-regulated pathways, and if this constitutes a new mechanism to control cell fate decisions.

Characterization of the effects of various p53 phosphorylation events on mRNA 3' processing.

The p53 pathway is tightly regulated by various posttrancriptional modifications, phosphorylation being the most significant. It has been shown that various phosphorylation events, either each by itself or cooperatively, can regulate various functions of p53 (Brooks and Gu 2003). The preliminary data presented here indirectly suggest p53 phosphorylation at Ser 15 might be required for BARD1/CstF/p53 complex formation and inhibition of mRNA 3' cleavage. Consistent with this, U2OS cells carrying the T734A BARD1 mutant affect not only UV-induced phosphorylation of BARD1 and p53 at Ser 15 but also CstF/BARD1 complex formation and UV-induced inhibition of mRNA 3' processing (Kim et al. 2006). Based on the proposed model, the addition of phosphorylated p53 could restore UV-induced inhibition of 3' cleavage in extracts of these cell lines. To test this, recombinant dephosphorylated and phosphorylated p53 will be added to NEs of U2OS cells expressing different BARD1 variants,

and then 3' cleavage of the radiolabeled L3 pre-mRNA substrate will be monitored as described (Kleiman and Manley 1999). Similar experiments could be performed using HeLa cells, which express low levels of p53.

To assess the effect of p53 phosphorylation at Ser 15 on 3' end processing, in vitro, p53Ser15A non phospho mimic and p53Ser15E phospho mimic mutant proteins could be generated via site directed mutagenesis. These p53 variants will be added to NEs of the above mentioned U2OS cells or HeLa cells, and then mRNA 3' cleavage will be monitored. In addition, cells could be treated with caffeine, which effectively inhibits phosphorylation of p53 at Ser15 (Kim et al.2006), and NEs from those cells could be prepared and monitored in 3' cleavage assays.

Furthermore, the effect of p53 phosphorylation on the CstF/BARD1/p53 complex formation has not been characterized yet. Therefore, CstF/BARD1/p53 complex formation will be analyzed by coimmunoprecipitation assays in NEs of U2OS cells expressing different BARD1 variants. Additionally, pull-down assays will be performed using recombinant BARD1, CstF-50 and phosphorylated and dephosphorylated p53. The p53Ser15A non phospho mimic and p53Ser15E phospho mimic mutant proteins will also be tested.

Extending these studies, it would be interesting to characterize the effect of BARD1 phosphorylation on other p53 phosphorylation sites and the consequent p53-dependent apoptosis. As mentioned before various p53 functions are regulated by different phopshorylation events. For instance, p53 phosphorylation at Ser 46 is considered to be the point of no return in which an irreversible process towards apoptosis begins (Feng et al. 2006). Studying the effect of BARD1 phosphorylation on p53 phosphorylation at Ser 46 and p53-dependent apoptosis will contribute

to our understanding of the interconnections between BARD1 and p53 pathways as well as the molecular switch signaling either cell survival or apoptosis.

To determine if BARD1 phosphorylation has an effect on p53 phosphorylation on other phosphorylation sites, first Western blot analysis with specific antibodies against phosphorylated forms of p53 will be performed using extracts from U2OS cells expressing different variants of BARD1. As mentioned before, non phospho mimic and phospho mimic mutants will be generated via site directed mutagenesis at those sites of phosphorylation in p53. The effect of those mutants on the BARD1/CstF/p53 complex formation and on 3' end processing will be assessed as described before.

Clinical implications

The determination of a correlation between inhibition of mRNA 3' processing and induction of apoptosis described above could allow the finding of meaningful therapies to induce apoptosis. Indeed, a correlation between recovery of mRNA levels and apoptosis has already been established. If prolonged inhibition of mRNA 3' cleavage causes apoptosis, this could be exploited clinically as a tool to induce apoptosis in cancer cells. Current therapies mostly use DNA damaging agents to induce the p53 response. However, if our model is correct, small molecule inhibitors of the mRNA 3' cleavage reaction could be identified and analyzed (Ryan et al. 2009) and this could be used in combination with traditional therapies in order to maximize the effect of the treatment of cancerous cells, reducing probably the toxicity to surrounding cells.

The work carried out in this dissertation provides an understanding of the interconnected functions of various tumor suppressor pathways, the inhibition of mRNA processing and regulation of the DNA damage response. The relevance of the studies presented in this dissertation is that they link for first time the mRNA 3' processing machinery to the p53 network. As p53 is mutated in most of the human cancers, the deeper understanding of the p53 pathway would allow the identification and development of new drugs to control different types of cancer.

Chapter VI

Experimental Procedures

Materials and Methods

Tissue culture methods and DNA damaging agents- HeLa and MCF7 cells were cultured in Dulbecco's modified Eagles medium (DMEM)-10% fetal bovine serum (FBS). 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.

The isogenic colon cancer cell lines DLD-1 and D-A2 were generously provided by Dr. Vogelstein (Johns Hopkins School of Medicine, Yu et al. 1999). DLD-1 cells were grown in McCoy's 5A media containing 10% FBS (v/v) and 2500 units of pen–strep. D-A2 cells required an additional 0.4 mg/ml G418, 20 ng/ml Dox, and 0.25 mg/ml hygromycin B. D-A2 cells were grown in the presence or absence of Dox as indicated. Ninety percent confluent cultures were exposed to UV and harvested after 2 hrs. UV doses (20 Jm^{-2}) were delivered in two pulses using a Stratalinker (Stratagene). Prior to pulsing, medium was removed and replaced immediately after treatment.

Nuclear extracts preparation and immunoblotting analysis– After UV treatment, NEs were prepared from harvested cells as described (Kleiman and Manley 2001). Sixty μg of each NE was analyzed by immunoblotting with antibodies against BARD1 (H-300, Santa Cruz), p53 (SC-126, Santa Cruz), CstF-64 (generously provided by Dr. Manley, Columbia University), CstF-50 (10064-2-AP, Protein Tech Group), and Topo II (SC-25330, Santa Cruz), p-p53 (Cell Signalling, 9286)

siRNA knockdown of p53 expression in HeLa and MCF7 cells- The siRNA specific for human p53 was synthesized by Qiagen and the control RNA duplex used as nonsilencing siRNA

was obtained from Dharmacon RNA Technologies. HeLa and MCF7 cells were grown in a 10-cm plate in complete DMEM. At 60-70% confluence, the cells were transfected with 20 nM of the p53 siRNA and 50 nM of the control siRNA and Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. After culturing the cells for additional 24 hrs, the transfection procedure was repeated, and the cells were harvested for analysis 48 hrs after the initial transfection. A fraction of the cells was exposed to UV and harvested after 2 hrs. NEs were prepared and analyzed by Western blot and used in 3' cleavage reactions.

Immunoprecipitation analysis- One hundred μg total protein from each NE was immunoprecipitated with the anti-CstF-64 (generously provided by Dr. Manley, Columbia University) or p53 (SC-126, Santa Cruz) mAbs bound to protein A-Sepharose beads. The beads were recovered by centrifugation and treated at 4 °C with 50 μg of RNase A/ml for 10 min. Immunoprecipitations were carried out as described (Kleiman and Manley 2001), except that washing was with 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). Aliquots of pellets and supernatants were analyzed by SDS-PAGE and immunoblotting as described above.

Recombinant proteins– GST-BARD1 and GST-CstF-50 plasmids were constructed as described (Kleiman and Manley 2001). The plasmid encoding His-p53 was generously provided by Dr. Prives (Columbia University). His-p53 derivatives were obtained by PCR amplification with primers containing BamH1 and Nde1 restriction sites followed by inserting the PCR fragment into pET14b vector (Novagen). The Ser241Phe mutation on p53 was introduced by

site-directed mutagenesis using Quickchange kit (Stratagene) according to the manufacturer's protocol. Plasmids encoding BARD1, CstF-50, p53 and p53 derivatives were expressed in *E. coli*., and purified by binding to and elution from either glutathione-agarose or Ni-agarose columns as described (Kleiman et al. 2005).

Human BARD1 (423−777), BARD1 (423−553), and BARD1 (554−777) were expressed as GST fusion proteins in *Escherichia coli* strain BL21(DE3) and purified by glutathione affinity chromatography.

Human CstF-50 (92−431) was expressed as a GST fusion protein in *E. coli* strain BL21(DE3) and purified by glutathione affinity chromatography. Residues from 1 to 91 were excluded from the construct to limit possible self-association. The protein was then purified by anion-exchange followed by gel filtration chromatography.

Protein–Protein Interaction Assays- The GST fusion protein interaction assays with His-p53 and His-p53 derivatives were performed as described (Kleiman and Manley 2001). Alternatively, 30 μl of HeLa cell NEs were incubated with 1 μg of the indicated GST fusion proteins and increasing amounts of His-p53; the binding and washing conditions were as before. Protein samples were treated at 4 °C with 50 μg of RNase A/ml for 10 min during the binding assays. Equivalent amounts of pellets and supernatants were analyzed by immunoblotting. The formation of the CstF-50/p53/BARD1 complex in solution was analyzed by gel filtration using an analytical Superose-12 column (GE Healthcare Biosciences). A mix of each recombinant protein, at a 100 μM concentration for each, was injected in the column and fractions were analyzed by immunoblotting as described above. The buffer used for this analysis contained 20 mM Tris (pH 8.0), 100 mM NaCl, and 1 mM βME.

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) before use in processing reactions. Cleavage assays with equivalent amounts of total protein were carried out as described (Kleiman and Manley 1999).

Analysis of endogenous mRNAs by qRT-PCR– Total nuclear RNA was purified from MCF7 cells treated with control/p53 siRNA, DLD-1 and D-A2 cells using the RNeasy (Qiagen) and the Oligotex resin (Qiagen) following manufacturer's instructions. Equivalent amounts of purified RNA were used as a template to synthesize cDNA using random hexamer primers and MMLV reverse transcriptase (Promega) according to the manufacturer's protocol. 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).

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