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New Insights into the Roles of Human DNA Damage Checkpoint Protein ATR in the Regulation
of Nucleotide Excision Repair and DNA Damage-Induced Cell Death

A dissertation
presented to the faculty of
the Department of Biomedical Sciences
East Tennessee State University
In partial fulfillment
of the requirements for the degree
Doctor of Philosophy in Biomedical Sciences

by

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December 2013

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ABSTRACT

New Insights into the Roles of Human DNA Damage Checkpoint Protein ATR in the Regulation of Nucleotide Excision Repair and DNA Damage-Induced Cell Death

by

Zhengke Li

Integrity of the human genome is frequently threatened by endogenous and exogenous DNA damaging reagents that may lead to genome instability and cancer. Cells have evolved multiple mechanisms to repair DNA damage or to eliminate the damaged cells beyond repair and to prevent diverse diseases. Among these are ataxia telangiectasia and Rad3-related (ATR)-mediated DNA damage checkpoint and nucleotide excision repair (NER) that are the major pathways by which cells handle ultraviolet C (UV-C)- or other exogenous genotoxin-induced bulky DNA damage. However, it is unclear how these 2 pathways may be coordinated. In this study we show that ATR physically interacts with NER factor *xeroderma pigmentosum* group A (XPA) where an ATR phosphorylation site on serine 196 is located. Phosphorylation of XPA on serine 196 is required for repair of UV-induced DNA damage. In addition, a K188A point mutation of XPA that disrupts the ATR-XPA interaction inhibits the UV-induced XPA phosphorylation and DNA repair. Moreover, we show that depletion of p53, a downstream checkpoint of ATR, and inhibition of p53 transcriptional activities reduced the UV-induced XPA import. Furthermore, we found that the ATR-directed XPA nuclear import happens primarily in the S phase of the cell cycle. In effort to determine the mechanism involved in the XPA nuclear import, we found that, in addition to the nuclear localization signal (NLS) of XPA, importin- α 4 is required for the UV-induced XPA nuclear import in an ATR-dependent manner. These data suggest that NER could be regulated by the ATR-dependent checkpoint via modulation of XPA

phosphorylation and nuclear import. In a separate study we show that, upon UV damage, cytoplasmic ATR translocates to mitochondria, blocks the recruitment of proapoptotic Bcl-2-associated X (Bax) protein to mitochondria and prevents the loss of mitochondrial membrane potential ($\Delta\Psi$) and apoptosis. Bax-depletion reduces the effect of ATR on $\Delta\Psi$. Remarkably, the cytoplasmic ATR exhibits no checkpoint kinase activity, a hallmark function of nuclear ATR. Silencing of ATR's kinase activity failed to affect Bax relocalization to mitochondria. These results reveal a novel checkpoint-independent antiapoptotic function of ATR at mitochondria in the cellular response to DNA damage.

DEDICATION

This manuscript is dedicated to my lovely wife, Julie. Thanks for all her hard work and patience. She honors me. She is not self-seeking, not easily angered, and keeps no record of wrongs. Without her support, this achievement would not be possible.

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CHAPTER 1

INTRODUCTION

Human DNA Damage and Cellular Responses

A high integrity of genomic DNA must be maintained in order to pass on correct genetic information to the next generation of cells. However, this integrity is frequently threatened by DNA damage induced by endogenous and exogenous genotoxic agents that may cause DNA structural changes leading to genomic instability and cancer (1-5). To counter these threats, cells have evolved multiple systems to signal DNA damage in order to mediate repair or to eliminate the damaged cells that are beyond repair (4, 6-8). Such responses include a wide range of cellular events and are biologically crucial because they prevent a series of diseases (5). There are 4 primary mechanisms that build such responses: DNA damage checkpoint-mediated cell cycle arrest, activation of transcriptional programs, DNA repair, and apoptosis (Figure 1-1) (4, 6, 7). DNA damage checkpoint proteins sense DNA damage, activate the checkpoint, arrest cell cycle progression to give cells sufficient time to remove the damage before the damage can be converted to mutations due to error-prone DNA synthesis, and modulate cell fate (6, 9, 10). DNA repair removes the damage and restores DNA structural integrity. DNA damage-induced apoptosis functions to eliminate cells damaged beyond repair (8, 11, 12). Changes in transcriptional programs balance the specific proteins that are needed in these 3 events (7). Precise and coordinated functions of these DNA damage responses are required to maintain genome integrity and to prevent development of diseases.

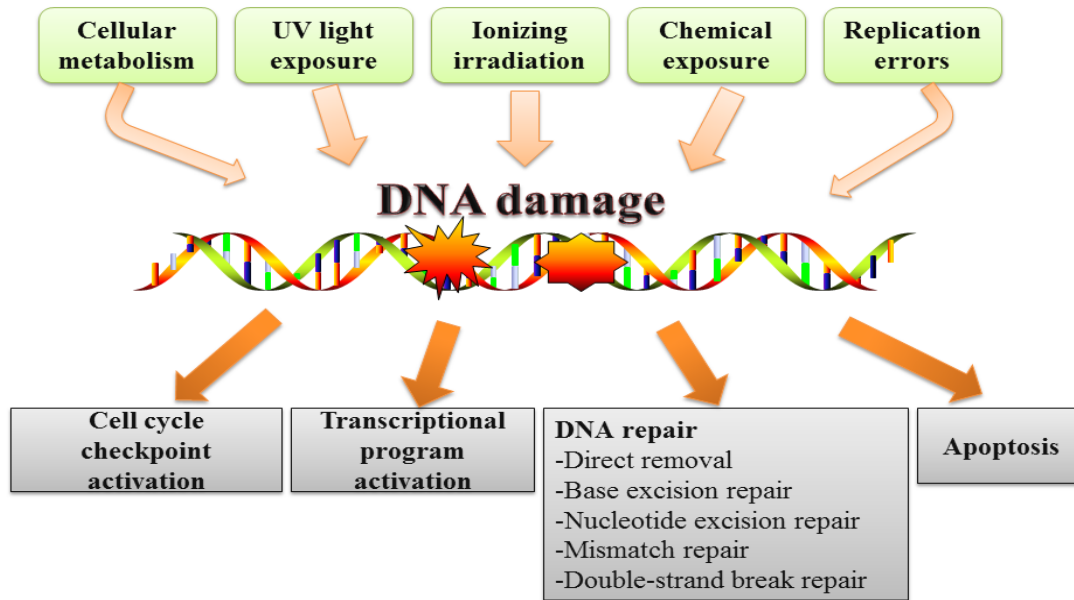


Figure 1-1. Molecular mechanisms of mammalian DNA damage responses. Modified from Sancar *et al.* 2004 (7)

Damage to DNA can be induced by endogenous or exogenous agents (Figure 1-1), generating 3 major types of structural or chemical abnormalities: nucleotide base damage, DNA backbone breakages, or DNA cross-links. The type and amount of DNA damage determines which DNA damage checkpoint, repair pathway, and apoptotic program is initiated to restore the structure of DNA or to promote irreversible cell death (7, 8, 11, 12). My dissertation research has focused on the modulation of nucleotide excision repair (NER) via regulation of *xeroderma pigmentosum* group A (XPA) by DNA damage checkpoint protein ataxia telangiectasia and Rad3-related (ATR), and the role of ATR in mitochondria-mediated apoptosis pathways. Therefore, DNA damage checkpoints, NER, and DNA damage-induced apoptosis are discussed primarily in this dissertation.

DNA Damage Checkpoints

The DNA damage checkpoints survey the structural integrity of genomic DNA and coordinate multiple cellular pathways to ensure timely and efficient removal of DNA damage (7, 10, 13-15) or to determine whether cells are damaged beyond repair and should be eliminated (8, 11, 12). These pathways are comprised of a series of DNA damage sensors, signal mediators, and transducers as well as downstream effectors. In mammalian cells 2 major DNA damage checkpoint systems are built from the DNA damage checkpoint proteins *ataxia telangiectasia mutated* (ATM) and ATR (16, 17). Both ATM and ATR are protein kinases belonging to the phosphoinositide 3-kinase-like kinase (PIKK) family. Activation of these 2 kinases leads to phosphorylation of many downstream mediators/effectors which include checkpoint kinase 1 (Chk1), checkpoint kinase 2 (Chk2), p38/MAPKAP Kinase-2 (MK2), p53, *etc.* These downstream proteins play roles in arresting cell cycle progression (major effects), regulating DNA repair and apoptosis (Figure 1-2) (7, 8, 10, 18-20). There is significant cross-talk between these two pathways; however, it is currently believed that ATM is primarily activated by DNA double-strand breaks while ATR is activated by replication protein A (RPA)-coated single-stranded DNA (ssDNA) generated from DNA damage-induced replication stresses (7, 10, 21-23). Notably, ATR is an essential gene (24, 25) while ATM is not.

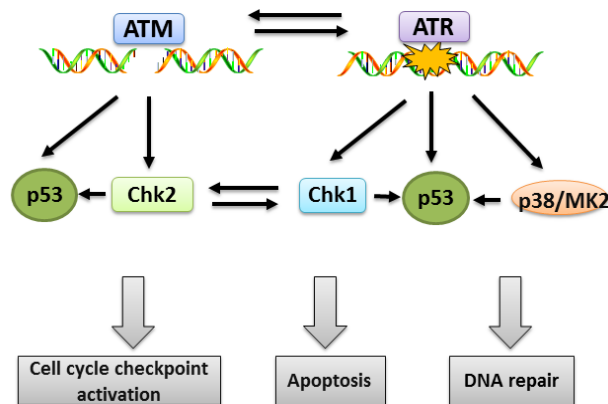


Figure 1-2. Major mammalian DNA damage checkpoints

Genetic diseases found to be associated with ATR mutations include Seckel syndrome (26, 27) and oropharyngeal cancer syndrome (28). Seckel syndrome is caused by a single base substitution that causes mis-splicing of exon 9 resulting in a lower level of ATR protein (26). The selective failure of the brain's germinal elements in these patients that results in their small brains and small heads is a result of defects in ATR-dependent DNA damage signaling leading to the premature death of proliferating cells (26, 29, 30). Interestingly, cells from oropharyngeal cancer syndrome patients have normal ATR protein level; relative ATR kinase activity towards downstream proteins is also normal. To date, the only abnormality observed in the oropharyngeal cancer syndrome cells is a lower level of the DNA damage-induced increase of p53 due to heterozygous missense mutation in ATR (28). Additionally, somatic mutations in exon 10 of the ATR gene are found in endometrial, colon, and stomach tumors (30, 31). Mutations in ATM lead to ataxia telangiectasia (A-T), an autosomal recessive neurodegenerative disease characterized by cancer proneness, telangiectasia, immunodeficiency, and extreme radiosensitivity (32, 33).

Nucleotide Excision Repair (NER)

In mammals the NER pathway is the major mechanism for the removal of helix-distorting, replication-blocking bulky DNA adducts that are induced by exogenous agents such as UV irradiation and a variety of genotoxic chemicals (7, 14). NER is the primary mechanism preventing potential mutations resulting from UV irradiation that induces DNA damage mostly in the form of cyclobutane pyrimidine dimers (CPDs) and 6, 4 photoproducts (6-4PPs) (7, 34-36). NER can be divided into 2 subpathways according to whether the damage occurs in transcriptionally active or inactive regions of the genome: transcription-coupled NER (TC-NER) or global genomic NER (GG-NER) (Figure 1-3). The 2 subpathways differ only in their method

of DNA damage recognition. In TC-NER recognition is initiated by stalled RNA polymerase II (RNAPII) in a process involving the Cockayne syndrome B (CSB) and CSA protein complex. In the GG-NER the damaged DNA is recognized by the *xeroderma pigmentosum* group C (XPC) that is facilitated by XPE (complex of UV-damaged DNA-binding proteins (DDB) 1 and 2). Following recognition, the 2 subpathways merge into a common mechanism starting with the unwinding of DNA containing the lesion, removal of the damaged DNA strand, and creation of a single-strand gap that is subsequently filled by DNA polymerase using the undamaged strand as a template.

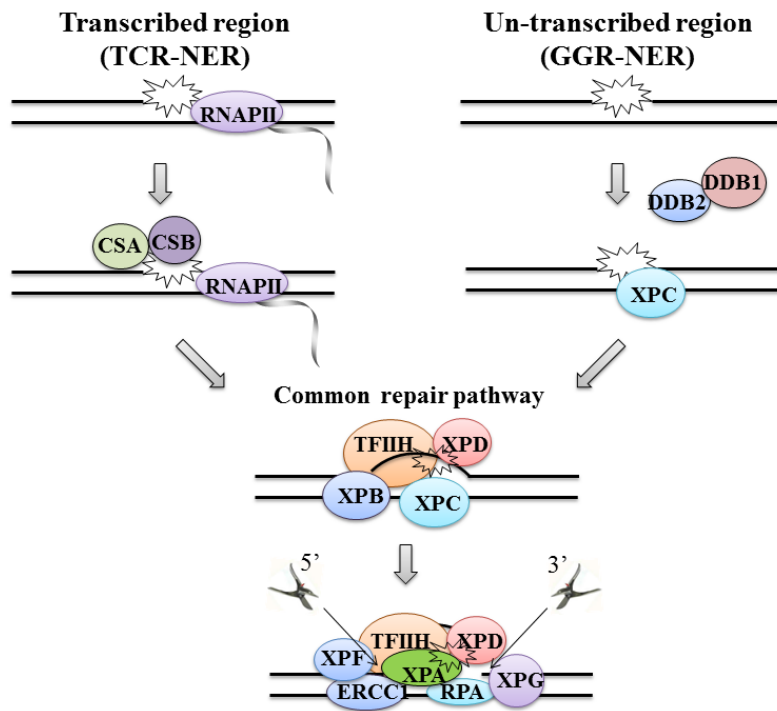


Figure 1-3. Nucleotide excision repair pathways. Modified from Cleaver *et al.* 2009 (37)

In humans deficiency in 11 of the approximately 30 total NER proteins can lead to a severe cancer-prone disease Xeroderma pigmentosum (XP) (38-40) or to premature aging-like disorders including Cockayne syndrome (CS) and trichothiodystrophy (TTD) (37, 41). Most

patients with XP, such as patients with a *xeroderma pigmentosum* complementation group A (XPA) deficiency, have defects in both GG-NER and TC-NER and are characterized by extreme photosensitivity and a 1000-fold increased risk of skin cancer (36, 38, 41). Patients with CS or TTD have defects in TC-NER (36, 41). A hallmark of all of these syndromes is hypersensitivity to UV-irradiation resulting from defective repair of UV-induced DNA damage by NER.

DNA Damage-Induced Apoptotic Cell Death

There are 2 major outcomes from DNA damage: the damage is removed / tolerated or the cells that harbor damages are eliminated by apoptosis. Given that DNA damage checkpoints and DNA repair are the primary mechanisms for removing DNA damage, the regulated apoptotic cell death is fundamental in eliminating cells harboring DNA damage (8, 11, 12). To date research indicates that there are 2 main apoptotic pathways (42-44): the extrinsic cell death receptor pathway and the intrinsic mitochondrial pathway. Each pathway activates its own initiator procaspases (cysteine-dependent aspartate-directed proteases that exist as inactive proenzymes) that activate other procaspases allowing for the initiation of an irreversible commitment towards cell death.

A central player in DNA damage-induced apoptosis is the tumor suppressor p53. DNA damage-induced intrinsic apoptosis can be divided into 2 pathways according to the involvement of p53: p53-dependent and p53-independent apoptosis (8, 11, 12). In the p53-dependent apoptotic pathways, p53 plays a direct role through relocalization to mitochondria (45, 46) and/or interacting with apoptotic proteins (47), or it works indirectly by transcriptional activation of apoptotic genes (8, 11, 12, 47). Cells also can trigger p53-independent apoptosis pathways: (i) p53 function can be replaced by the homologs such as p63 and p73 (48, 49) and (ii) apoptosis

that is initiated by transcriptional inhibition (50), transcription factor nuclear factor-kB (NF-kB) (51), B-cell lymphoma 2 (Bcl-2) degradation (52, 53), caspase-2 (54-56), or caspase-3 (52, 57, 58). With regard to diseases, inappropriate apoptosis is involved in many human diseases including neurodegenerative diseases, ischemic damage, autoimmune disorders, and cancer (42-44).

The ATM and ATR-dependent DNA damage checkpoint pathways are thought to be the key nodes in making the decision between survival and death following genotoxic stresses (8, 11, 12, 59). Many proteins are phosphorylated by these 2 kinases (60) and cell death decisions can be conducted by those involved in apoptotic pathways (8, 11, 12).

DNA Damage Checkpoint Protein Ataxia Telangiectasia and Rad3-Related (ATR) Serine/Threonine Kinase

ATR is a member of the phosphoinositide 3-kinase-like kinase (PIKK) family. Its kinase activity has a strong preference for serine or threonine residues that are followed by a glutamine (“S/TQ” sites) (61). The full activation of this kinase requires the localization of the ATR-ATRIP (ATR-interacting protein) complex to the RPA-coated ssDNA, as well as other regulators including TopBP1 (DNA topoisomerase 2-binding protein 1) (62, 63), 9-1-1 complex (Rad9-Rad1-Hus1 proteins) (64, 65) and RHINO (RAD9, HUS1, RAD1-interacting nuclear orphan protein) (66). Following activation, ATR phosphorylates hundreds of downstream proteins that arrest the cell cycle and maintain cell survival (22, 25, 60, 67-69).

Unfortunately, there is currently no high-resolution structural data available for ATR. Nonetheless, the domain architecture of ATR is similar to other PIKKs based on their sequence similarity (Figure 1-4) (22, 68-70). The kinase domain is flanked by the FAT

(73, 74). XPA is required for both TC-NER and GG-NER (76, 77) and has been proposed as a rate-limiting factor for human NER activity (78-80). XPA^{-/-} cells complemented with recombinant phosphorylation-deficient XPA protein displayed an increased sensitivity to UV-C irradiation compared with cells complemented with wild-type XPA (73); furthermore, inhibition or knockdown of ATR delayed repair of UV-induced DNA damage (74, 75). In addition to its functions in NER, ATR regulates inter-strand crosslink repair by phosphorylation of FANCD2 (Fanconi anemia group D2) (81); ATR phosphorylates several proteins needed in recombination (82-84).

Regulation of DNA Replication

ATR is an essential gene as evidenced by the fact that knockout of ATR in mice is embryonically lethal (25, 85) and cells generated from these ATR-knockout mice die of apoptosis (25, 72). There are several mechanisms proposed by which ATR can regulate DNA replication in the absence of exogenous stresses (71, 86). These mechanisms include: control of replication initiation by regulating timing of origin firing (87-89), monitoring of replication progression (90-93), prevention of premature chromatin condensation and entry into mitosis without completion of DNA replication (94-97), and/or protection from the accumulation of DNA breaks associated with replication (98, 99).

Antiapoptotic Functions of ATR in DNA Damage Responses

In contrast to ATM, ATR is essential for viability of mice and the cells from ATR-knockout mice died of apoptosis (25, 85, 100). Furthermore, inhibition of ATR enhanced apoptosis through a p53-independent mechanism (58, 101-105) regardless of ATR's kinase

activation in some cancer cells (105), and suppressed UV-induced carcinogenesis (102, 103). Also interestingly, a body of evidence from human epidemiologic and mouse model studies has demonstrated that caffeine consumption significantly decreased the risk of cancer (24, 106-116). Notably, caffeine is an inhibitor of cell cycle checkpoint pathways (111), but the inhibition may be independent of the kinase activities of ATM or ATR (117). Together the current evidence suggests that unlike ATM, ATR may contain an antiapoptotic activity and inhibition of ATR suppresses carcinogenesis. However, the molecular mechanism remains unknown. Given the potential antitumor activity of ATR inhibition, ATR has drawn attention as a promising target for anticancer chemotherapy, further underscoring the importance for understanding the mechanism. In this dissertation study, our data revealed a possibility that ATR also may have cytoplasmic functions in the mitochondria-mediated apoptosis pathways.

Regulation by Posttranslational Modifications

Posttranslational modifications of ATM are important markers for studying the ATM-mediated signaling pathways (118). Therefore, it is anticipated that posttranslational modification of ATR also may serve as an indicator for ATR activation that could be enormously useful, as previous approaches to monitor ATR signaling relies solely on assessment of its downstream substrates (22, 69). In recent studies 2 groups identified auto-phosphorylation of ATR on Thr-1989 (70, 119) which could serve as a marker for ATR kinase. The Thr-1989 phosphorylation of ATR is induced by DNA damage in an ATR kinase-dependent manner. Compared with cells complemented with wild type ATR, cells expressing the non-phosphorylatable ATR (T1989A) mutant exhibit modest ATR functional defects (70). More detailed studies show that this phosphorylation of ATR is dependent on RPA and ATRIP and is

recognized by TopBP1 to facilitate ATR substrate recognition (119). Interestingly and importantly, this phosphorylation of ATR, together with a later report of other 16 potential autophosphorylation sites of ATR (120), does not alter its migration in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). In this study we found a DNA damage-induced migratory change of cytoplasmic ATR (possible novel modifications on ATR) that has important antiapoptotic roles in DNA damage responses.

DNA Damage-Induced Phosphorylation and Nuclear Import of *Xeroderma Pigmentosum* Group

A (XPA)

The XPA protein is 1 of 8 factors that were found to be deficient in XP disorders (121), and XPA-deficient cells exhibit the highest UV sensitivity among the XP cells (122). XPA is an indispensable factor for both TC-NER and GG-NER (76, 77) (Figure 1-3). Because of XPA's crucial functions in NER, the activity of NER and sensitization of cancer cells to chemotherapy can be regulated by transcriptional and posttranscriptional control of the XPA protein (73, 74, 78-80, 123). Functionally, XPA is believed to play roles in verifying DNA damage, stabilizing repair intermediates, and recruiting other NER factors to the damaged DNA (76, 124-131). To accomplish these functions XPA protein contains multiple functional domains and interacts with various other proteins, including replication protein A (RPA), ERCC1, DNA damage-binding protein 2 (DDB2), and transcription factor II H (TFIIH), as well as with chemical carcinogen-damaged or UV-damaged DNA (Figure 1-5) (131).

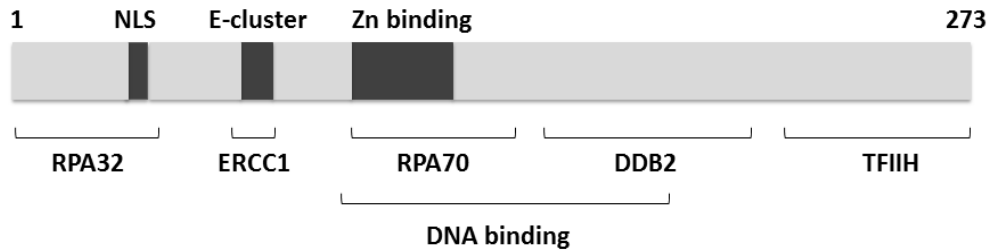


Figure 1-5. A map of XPA protein illustrating the locations of the binding sites for various DDR proteins or for binding damaged DNA. Modified from ref Bartels *et al.* 2007 (131)

Extensive studies have been dedicated to understand the transcriptional and posttranscriptional regulation of XPA protein with a hope to manipulate the activity of NER because NER is responsible for removal of DNA damage introduced by chemotherapeutic agents. Manipulation of NER has potential benefits in anticancer therapy (78-80, 123, 132). Previous studies indicate that ATR directed a time- and dose-dependent cytosol-to-nucleus redistribution of XPA in response to UV irradiation (74). This damage-induced translocation of XPA is dependent on ATR as a checkpoint because inhibition of ATR's kinase activity inhibited XPA nuclear import (74). Furthermore, inhibition or knockdown of ATR inhibited phosphorylation of XPA (73) as well as NER activity (73, 75, 133). These findings lead us to propose that ATR regulation of XPA nuclear importation and/or phosphorylation may coordinate the ATR checkpoint activity with NER. The details of the ATR-directed XPA nuclear import and the roles of ATR-mediated XPA phosphorylation, however, are still unknown and are part of the research presented in this dissertation.

Questions to be Answered in the Studies

In our previous studies we found that ATR directly binds to and phosphorylates XPA in an UV-dependent manner (73). In addition, the ATR-mediated DNA damage checkpoints direct the nuclear import of XPA in response to UV-irradiation. A clear interaction between ATR and XPA has been established, and there is growing evidence that the ATR-dependent checkpoint pathway coordinates with NER via regulation of XPA subcellular localization and/or phosphorylation to promote DNA repair. However, whether the phosphorylation of XPA by ATR is required for removal of UV-induced DNA damage (73); as well as how the ATR-XPA interaction (73) occurs and its significance have not been defined. The molecular basis of the ATR-XPA interaction remains to be elucidated as there is no structural information available for the ATR kinase domain or a model for how it binds to target proteins. Using a mass spectrometry-based protein footprinting method, a former lab member (Steven M. Shell) found that ATR interacts with a helix-turn-helix (HTH) motif in the minimal DNA binding domain of XPA where an ATR phosphorylation site (serine 196) is located. I continued this avenue of research and investigated whether this ATR interaction and/or phosphorylation of XPA is required for DNA repair. This work has been published in the Journal of Biological Chemistry (2009, 284(36): 24213-22) and is presented here as Chapter 2 (134).

After we published our initial work on the regulation of NER via posttranslational regulation of XPA by ATR, another group reported a requirement of ATR in maintaining NER activity primarily during S phase in human cells (15, 75). This leads to additional questions: (1) is the ATR-directed nuclear import of XPA upon DNA damage also cell cycle specific; (2) are any of ATR downstream targets involved in the UV-induced XPA import? To address these questions, human cells were synchronized, and the nuclear import of XPA as well as NER

activity was measured. The dependence of XPA nuclear import on the ATR's major phosphorylation targets such as p53, Chk1, and MK2 also were investigated. We demonstrated that the XPA-mediated regulation of NER by ATR is p53-dependent and occurs primarily in S phase. This work has been published in PLoS ONE (2011, 6(12): e28326) and is presented here as Chapter 3 (135).

Given the important nuclear functions of XPA in NER (7, 136) as well as the fact that targeting protein trafficking could be a strategy to improve the sensitivity of cancer cells to chemotherapeutic agents (137), it is of interest to study the DNA damage-dependent nuclear import of XPA (74, 135, 138). We have established that DNA damage-induced nuclear import of XPA from the cytoplasm, for NER during S phase, is mediated by DNA damage checkpoint proteins ATR and p53 (74, 135). However, despite the importance of XPA nuclear import in NER, how XPA is imported through the nuclear pore complex (NPC) remains unknown. In a separate study, protein factors potentially involved in the nuclear import XPA were investigated. We found that in addition to dependence on the nuclear localization signal (NLS) of XPA, importin- α 4 and importin- α 7 are required for the XPA nuclear import. This manuscript is published in PLoS ONE (2013, 8(7): e68297) and is presented here as Chapter 4.

Our recent data suggests that UV-induced XPA nuclear import is regulated by ATR through a p53 signaling pathway (135). Given that p53-deficient cancer cells rely on the p38MAPK/MK2 pathway for survival of DNA damages rather than the ATR/p53 signaling pathway (19, 139), it is of interest to determine whether the DNA damage-induced XPA nuclear import occurs in p53 deficient cancer cells and whether the import is regulated by different pathways in p53-proficient and deficient cancer cells. In addition, cisplatin is one of the 3 most commonly used chemotherapeutic drugs (140). NER-mediated repair of cisplatin-induced DNA

damage is a major factor in modulating the therapeutic efficacy of cisplatin (141, 142). Therefore, the status of NER is an important factor in the success of chemotherapy using cisplatin (142, 143), which makes it interesting to study whether cisplatin also induces a nuclear import of XPA. Interestingly, our finding indicates that although the ATR dependence of XPA nuclear import occurs in p53-proficient normal and cancer cells treated with cisplatin or UV, it does not happens in p53-deficient lung cancer cells such as H1299 cells. This work is published in the Int J Biochem Mol Biol. 2(2): 138-145 and is presented as Chapter 5 (138).

ATR has been shown to have antiapoptotic functions in replicative stress response (58, 101-105, 144); however, the mechanism by which it carries out its intrinsic apoptotic functions is not well defined. ATR-mediated regulation of checkpoints p21 and Chk1 contribute to its antiapoptotic function (102, 144). Interestingly, ATR also was suggested to have kinase-independent antiapoptotic roles in sensitization of cells to DNA damage (105). In addition to its antiapoptotic functions in cells treated with exogenous DNA damaging agents, there are lines of evidence showing possible proapoptotic function of ATR when cells fail in initiating DNA replication (57, 145). However, it was not clear to us how ATR carries out its intrinsic roles in apoptosis. We reasoned that ATR may have a role in the mitochondria-mediated apoptotic pathways. As a novel finding, our results suggest a role for ATR in regulating mitochondrial membrane potential/apoptosis via kinase-independent inhibition of Bax relocalization to the mitochondria. This manuscript is in submission and is presented here as Chapter 6.

CHAPTER 2

ATR PROMOTES NUCLEOTIDE EXCISION REPAIR OF UV-INDUCED DNA DAMAGE VIA PHYSICAL INTERACTION WITH XPA (134)

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Abstract

In response to DNA damage, eukaryotic cells activate a series of DNA damage-dependent pathways that serve to arrest cell cycle progression and remove DNA damage. Coordination of cell cycle arrest and damage repair is critical for maintenance of genomic stability. However, this process is still poorly understood. Nucleotide excision repair (NER) and the ATR-dependent cell cycle checkpoint are the major pathways responsible for repair of UV-induced DNA damage. Here we show that ATR physically interacts with the NER factor *xeroderma pigmentosum* group A (XPA). Using a mass spectrometry-based protein footprinting method, we found that ATR interacts with a helix turn-helix motif in the minimal DNA-binding domain of XPA where an ATR phosphorylation site (serine 196) is located. XPA-deficient cells complemented with XPA containing a point mutation of S196A displayed a reduced repair efficiency of cyclobutane pyrimidine dimers as compared with cells complemented with wild-type XPA, although no effect was observed for repair of (6-4) photoproducts. This suggests that the ATR-dependent phosphorylation of XPA may promote NER repair of persistent DNA

damage. In addition, a K188A point mutation of XPA that disrupts the ATR-XPA interaction inhibits the nuclear import of XPA after UV irradiation and, thus, significantly reduced DNA repair efficiency. By contrast, the S196A mutation has no effect on XPA nuclear translocation. Taken together, our results suggest that the ATR-XPA interaction mediated by the helix-turn-helix motif of XPA plays an important role in DNA-damage responses to promote cell survival and genomic stability after UV irradiation.

Introduction

NER is the primary mechanism in mammalian cells for the removal of bulky DNA lesions induced by exogenous agents such as UV radiation and a variety of genotoxic chemicals (7). In eukaryotic cells NER requires more than 25 proteins to perform the DNA damage recognition, excision, and DNA synthesis steps necessary to remove the lesion and restore the integrity of DNA (146, 147). In humans, defects in NER lead to the clinical disorder *xeroderma pigmentosum* (XP) that is characterized by increased sensitivity to UV light and a predisposition to development of skin cancer (38-40).

Xeroderma pigmentosum group A protein (XPA) is one of eight factors found to be deficient in XP disorder (121). XPA is a 32-kDa zinc metalloprotein that is believed to verify the damage site after initial recognition of the presence of a lesion, stabilize repair intermediates, and play a role in recruiting other NER factors (76, 124-130). XPA is an indispensable factor for both the transcription-coupled repair and global genome NER pathways (76, 77). Given its central role in NER, patients with XPA deficiency display the most severe XP phenotypes (121, 147). In addition, XPA also has been implicated to play a role in laminopathy-induced premature aging syndromes (148, 149).

The DNA damage checkpoint pathways serve to monitor genomic integrity and to coordinate multiple cellular pathways to ensure efficient repair of DNA damage (13). The ATM (*ataxia-telangiectasia* mutated) and ATR (ATM and RAD3-related)-mediated checkpoint pathways represent two major DNA damage-dependent checkpoints. Both ATM and ATR are protein kinases belonging to the phosphoinositide 3-kinase-like kinase family. These pathways are composed of a series of DNA damage sensors, signal mediators and transducers, and downstream effector molecules (7, 13, 74). The ATR-dependent checkpoint pathway serves to sense replication stress and responds primarily to DNA damage typically generated by UV irradiation (7, 18, 21, 150). ATR is targeted to the sites of elongated RPA-coated single-strand DNA generated when DNA replication forks stall because of DNA damage. This event is mediated by interactions between RPA and the ATR interaction protein ATRIP (21). Upon sensing DNA damage, ATR initiates a complex signaling cascade via phosphorylation of downstream protein substrates, which ultimately leads to cell cycle arrest (7, 18, 20).

Previous studies have implied a role for the ATR-mediated checkpoint pathway in regulation of the NER pathway (73-75). In particular, ATR kinase activity may participate in the regulation of global genome NER uniquely during the S phase of the cell cycle. Additionally, XPA has been defined as a direct ATR target for phosphorylation and cytoplasm-to-nucleus redistribution in response to UV-C irradiation (73, 74). XPA^{-/-} cells complemented with recombinant phosphorylation-deficient XPA protein displayed an increased sensitivity to UV-C irradiation compared with cells complemented with wild-type XPA (73). In addition, ATR directed the nuclear import of XPA in both a dose-dependent and time-dependent manner for regulation of NER activity (74).

Although there is growing evidence that the ATR-dependent checkpoint pathway coordinates with NER via an ATR-XPA interaction to promote DNA repair, how the interaction occurs and its significance have not been defined. Furthermore, the molecular basis of the ATR-XPA interaction remains to be elucidated. There is no structural information available for the ATR kinase or a model for how it binds to target proteins. In this study we investigated the molecular basis for the ATR-XPA interaction. Using our mass spectrometric protein footprinting technique, we identified an α -helix in the XPA minimum DNA-binding domain that mediates the XPA-ATR interaction. In addition, we demonstrate that regulation of XPA activity by ATR via ATR-XPA interaction is required for promoting repair of UV-C induced DNA damage by NER.

Materials and Methods

Cell Lines and Tissue Culture

XPA^{-/-} cells (GM04429) were obtained from Coriell Cell Repositories (Camden, NJ) and were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. XPA-complemented cells were generated by stably transfecting GM04429 cells with pcDNA3.1 vectors (Invitrogen) containing either wild-type or mutated XPA cDNA with the indicated point mutations as described previously (73). U2OS stably transfected with the doxycycline-inducible FLAG-tagged ATR expression construct were a generous gift from Dr. Paul Nghiem (University of Washington Medical Center) and were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, 0.2 mg/ml neomycin, and 0.2 mg/ml hygromycin. All cell lines were grown at 37 °C, 5% CO₂. UV-C irradiation was performed using a 254-nm lamp at a fluence of

1.3 J/m²/s. For time course analysis cells were incubate at 37 °C, 5% CO₂ for the indicated amounts of time.

FLAG-ATR Immunoprecipitation and ATR-XPA Complex Formation

U2OS-FLAG-ATR cells were grown overnight in 10-cm tissue culture dishes in Dulbecco's modified Eagle's medium supplemented with doxycycline (5 µg/ml). Cells were harvested by scraping and resuspended in lysis buffer (50 mM HEPES-KOH, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1× protease inhibitor mixture (Roche Applied Science)). Clarified lysates were immunoprecipitated using monoclonal mouse-anti-FLAG M2 antibody (Sigma) and captured with Protein G-coated Sepharose beads (Amersham Biosciences). Beads were rinsed with wash buffer A (50 mM HEPES-KOH, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.05% NP-20) before washing with high salt buffer (50 mM HEPES-KOH, pH 7.4, 1 M NaCl, 1 mM EDTA, 0.05% Nonidet P-40). Immunoprecipitates then were equilibrated in the XPA-ATR binding buffer (50 mM HEPES-KOH, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM ATP), and purified His₆-XPA was added and incubated at room temperature for 30 min. Unbound XPA was washed away using buffer B (50 mM HEPES-KOH, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM ATP, 0.05% Nonidet P-40). The resulting complex was modified by the addition of NHS-biotin (1 mM final concentration) for 30 min. The reactions were quenched with 10 mM lysine in its free form. The interacting proteins were separated by SDS-PAGE and visualized by Coomassie stain. The XPA bands were excised from the gel and subjected to in-gel trypsin hydrolysis.

Mass Spectrometric Analysis

The tryptic peptide fragments were analyzed with matrix-assisted laser desorption time-of-flight (MALDI-TOF) mass spectrometry using an Axima-CRF instrument (Shimadzu Scientific Instruments). Samples were ionized with an α -cyano-4-hydroxycinnamic acid matrix.

To identify XPA peptide peaks, the experimental data were compared with the theoretical values obtained with Protein Prospector Version 4.0.6. The experimental measurements indicated the mass accuracy of 0.1–0.01%. Modified lysine residues were assigned by identifying the peptide peaks formed upon the NHS-biotin treatment of the protein. The experimental mass/charge data for modified peptides were then compared with the predicted theoretical values considering that each modification adds 226 Da to the affected Lys and renders the residue resistant to tryptic hydrolysis. For accurate quantitative analysis of the modified peptide peaks, at least two unmodified proteolytic peptide peaks were used as internal controls. A protection was considered to be significant when the intensity of the given modified peptide peak derived from NHS-biotin-treated free protein was reduced at least 10-fold in the context of the protein-protein complex. A modified peptide peak was considered unprotected when the intensities of the given peptide obtained from free protein and protein-protein complexes were within $\pm 20\%$ of each other. The data were reproducibly compiled and analyzed from at least four independent experimental groups.

Co-Immunoprecipitation

Coimmunoprecipitation experiments were conducted by lysing cells with lysis buffer A and adding dilution buffer to reduce the final NaCl concentration to 150 mM. One mg of total protein was immunoprecipitated using 2 μ g of monoclonal mouse-anti-XPA (Clone 12FA, Kamiya Biochemical) or anti-ATR antibody (A300-137A, Bethyl). Samples were resolved on a 4–12% gradient SDS-PAGE for western blot analysis.

Immunofluorescent DNA Repair Assay

For immunofluorescence microscopy, cells were grown on coverslips and UV-C-irradiated through 3- or 5- μ m polycarbonate isopore filters (Millipore) and allowed to recover for

the indicated amounts of time. Cells were fixed with 100% methanol and treated with 1 M HCl to denature the DNA. Cyclobutane pyrimidine dimers (CPD) or (6-4) photoproducts ((6-4)PPs) were detected with monoclonal mouse-anti-CPD (TDM-2, MBL International Corporation) or anti-(6-4)PPs (D195-1, MBL) and donkey-anti-mouse Alexa Fluor 568 antibodies (Molecular Probes). Coverslips were mounted in ProLong Antifade with DAPI (Molecular Probes) and visualized using 100× magnification. Data were recorded under single-blind conditions in which the individual performing the microscopy did not know the identity of the samples. Repair of CPD or (6-4)PPs damage was quantified as a percentage of DAPI-stained nuclei containing at least one well defined CPD or (6-4)PPs focus by overlaying the anti-CPD or anti-(6-4)PPs and DAPI images. At least 50 nuclei per time point were counted for damage repair quantification. Samples then were normalized, with time point 0 h representing 100% nuclei containing CPD or (6-4)PPs foci. Images were analyzed using Photoshop CS.

Slot-blot DNA Damage Repair Assay

Cells were seeded at 1×10^6 cells per 10-cm tissue culture dish and allowed to grow for 48 h before UV-C irradiation. Cells were allowed to recover for the indicated amounts of time, and genomic DNA was purified using the PureLink Genomic DNA kit (Invitrogen). Purified DNA was quantified by measuring the $A_{260 \text{ nm}}$, and samples were diluted to 0.2 $\mu\text{g/ml}$ in a final volume of 200 μl of TE buffer containing 10 mM Tris-HCl, pH 7.4, and 1 mM EDTA. Samples were denatured by incubating at 90 °C for 10 min then rapidly chilled on ice for 10 min before adding an equal volume of 2 M ammonium acetate. Samples were filter-immobilized on a nylon membrane and probed using monoclonal mouse-anti-CPD.

Subcellular Fractionation

The subcellular protein fractionation was performed using the Proteo JET™ cytoplasmic and nuclear protein extraction kit (Fermentas) and by following the procedures as suggested by the manufacturer. Briefly, 10 volumes of cell lysis buffer (with protease inhibitors) were added to 1 volume of packed cells. After vortexing for 10 s and incubation on ice for 10 min, cytoplasmic proteins were separated from nuclei by centrifugation at $500 \times g$ for 7 min. Isolated nuclei were washed once with 500 μ l of the nuclei washing buffer and then collected by centrifugation. The collected nuclear pellets were resuspended in ice-cold nuclear storage buffer, and 1/10 volume of the nuclear lysis reagents were added to the mixtures to lysis the nuclei by shaking for 15 min at 4 °C. Then nuclear lysate was collected after rinsing by centrifugation at $20,000 \times g$ for 12 min.

Results

Mass Spectrometric Footprinting of the XPA-ATR Complex

To form the protein-protein complex, purified His₆-XPA was added in excess to FLAG-ATR immobilized on Protein-G beads and incubated in the binding buffer (see “Materials and Methods”). For control experiments, XPA was also incubated with the Protein-G beads that were preincubated with anti-FLAG-treated cell lysates from un-induced U2OS cells under the same experimental conditions. Unbound XPA was then washed away, and the resulting samples were treated with NHS-biotin. In parallel experiments, free XPA was also subjected to the NHS-biotin treatment. The modified proteins were then resolved by SDS-PAGE. The XPA bands were excised from the gel and subjected to in-gel trypsin proteolysis to generate small peptide fragments amenable for MALDI-TOF analysis. To identify the XPA surface lysine(s) interacting

with ATR, we compared the modification profiles of free XPA and the XPA-ATR complex. Figure 2-1 depicts representative MALDI-TOF fragment profiles used for comparison. Figure 2-1A demonstrates that Lys-188 was readily susceptible to modification in free XPA but was inaccessible to NHS-biotin in the XPA-ATR complex. These results indicate that Lys-188 is surface-exposed in free XPA and becomes shielded upon ATR binding. In contrast, Figure 2-1B illustrates an m/z peak corresponding to biotinylated XPA fragment 31-32 (K31+ biotin) present in the spectra for both the free XPA and the XPA-ATR complex. These data suggest that surface topology of Lys-31 is not affected by the bound ATR. Peaks C1, C2, and C3 are unmodified peptide peaks of XPA and provide internal reference. The lysine foot-printing results for the XPA-ATR complex are summarized in Table 2-1: 16 lysine residues were biotinylated in XPA, of which one residue, Lys-188, was protected from modification in the presence of ATR.

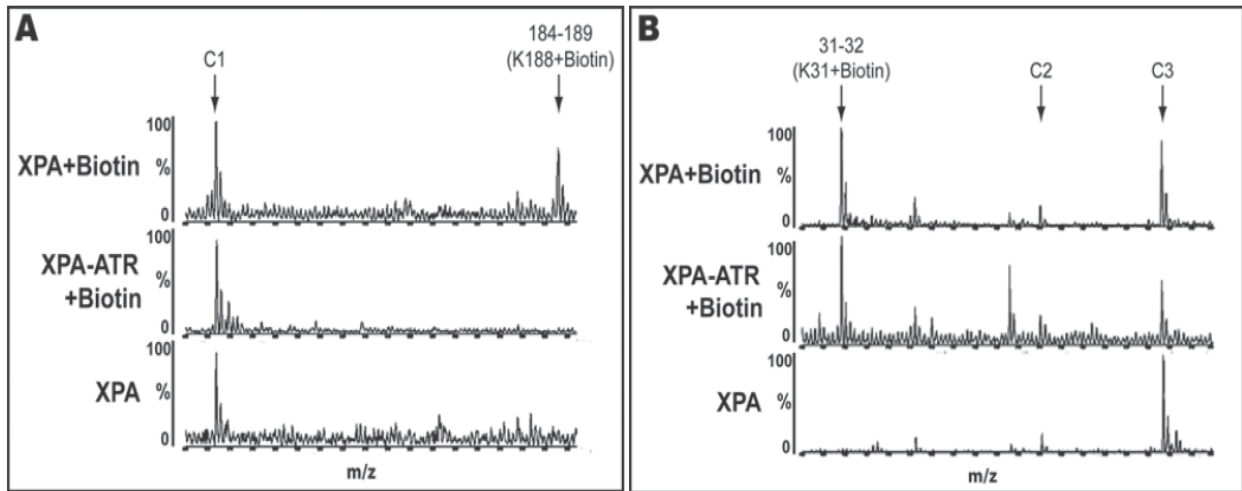


Figure 2-1. MALDI-TOF analysis of lysine protection in the XPA-ATR complex

A, top and middle spectra show free XPA and the XPA-ATR complex treated with NHS-biotin. The bottom spectrum shows untreated free XPA. The peak corresponding to XPA tryptic peptide fragment amino acids 184–189 containing a single modified Lys at position 188 is indicated. This peak is detected in free XPA samples and is significantly diminished in the XPA-ATR complex. B, unlike Lys-188, lysine residue 31 is modified in both free XPA and the XPA-ATR

complex. Peaks C1, C2, and C3 are unmodified peptide fragments of XPA and serve as internal controls. Data in this Figure were generated by Steven M. Shell.

Table 2-1. Summary of MALDI-TOF analysis of biotinylated XPA peptides Presented are tryptic digest fragments containing biotinylated lysine residues. Lysine residues protected from modification in the presence of ATR are indicated by a -, whereas residues not protected by ATR are indicated by a +. The asterisk indicates that the biotinylated fragment is located in the His tag. The intensity of the biotinylated peptide peak 184–189 (Lys-188 + biotin) was at least 10-fold higher for free XPA treated with NHS-biotin than for XPA-ATR complex modified under identical conditions. Intensities of other biotinylated XPA peptide fragments in the absence and presence of ATR varied within $\pm 20\%$. This Table was created by Steven M. Shell.

Fragment	Modified Lys	Protection
(-18)-(-5)*	-12*	-
31-32	31	-
87-110	89	-
136-141	137	-
152-158	157	-
164-168	167	-
184-189	188	+
190-207	204	-
218-221	218	-
216-221	217, 218	-
219-224	221, 222	-
222-227	222, 224	-
223-227	224	-
232-237	236	-
259-273	259	-
260-273	272	-

Lys-188 and the XPA-ATR Interaction

Figure 2-2A is a view of the minimum DNA-binding domain structure of XPA as determined by NMR spectroscopy (PDB code 1D4U) (134). ATR phosphorylates XPA at serine

196 (shown in green), which is located in the turn of a helix-turn-helix motif, a part of the proposed DNA-binding cleft. Protected lysine residue Lys-188 (shown in blue) is also located in the helix-turn-helix motif and is oriented in nearly the same plane as Ser-196. This is consistent with the fact that phosphorylation requires binding of ATR to XPA.

Given that Lys-188 is shielded from the solvent in the presence of ATR (Table 2-1), site-directed mutagenesis of Lys-188 was performed to investigate its possible role in XPA-ATR complex formation. Thus, pcDNA3.1 expression constructs were generated in which Lys-188 was changed to either alanine (K188A) or glutamic acid (K188E), and the vectors were stably expressed in XPA^{-/-} cells. Coimmunoprecipitation assays were performed to examine the effects of the Lys-188 mutations on ATR binding to XPA. As shown in Figure 2-2B top, anti-XPA antibody efficiently co-immunoprecipitated ATR from whole cell lysates generated from XPA^{-/-} cells complemented with wild-type XPA protein. Consistent with previous observations, UV-C irradiation increased the affinity of ATR for XPA but was unnecessary for the interaction (73). Interestingly, XPA-K188E protein co-immunoprecipitated with ATR in a similar pattern to that observed for the wild-type protein, although its affinity seemed slightly reduced (this work was done by Steven M. Shell). The K188A mutation, however, completely abolished the XPA-ATR interaction in both the irradiated and un-irradiated cells. These results were confirmed by the reversed coimmunoprecipitation in which XPA was coimmunoprecipitated by anti-ATR antibody (Figure 2-2B bottom).

Effect of K188A Mutation on Nuclear Import of XPA upon UV Irradiation and DNA Damage Repair

We previously reported that after DNA damage, XPA translocates from the cytoplasm to the nucleus in an ATR-dependent manner (74). Thus, an interesting question is whether the

ATR-XPA interaction is required for XPA nuclear import. To address this question, a cellular fractionation assay was performed using XPA^{-/-} cells expressing recombinant wild-type XPA (XPA-WT) or XPA-K188A mutant. After UV-C irradiation (20 J/m²), wild-type XPA is re-distributed from the cytoplasm to the nucleus. However, UV-C irradiation does not affect the cellular distribution of the XPA-K188A protein (Figure 2-2C top). These results suggest that the K188A mutation negatively affects the ability of ATR to induce nuclear accumulation of XPA in response to DNA damage. The results were confirmed by an immunofluorescence microscopy assay in which the subcellular localization of XPA was directly visualized (Figure 2-2C bottom). Interestingly, the phosphorylation of XPA at Ser-196 appears to have no effect on XPA nuclear import. Collectively, these suggest that physical interaction between XPA and ATR, but not Ser-196 phosphorylation, may play a role in DNA damage-induced XPA nuclear import. It should be noted that although the total levels of XPA appeared to be different in cells expressing XPA-WT and mutant, the effect of total XPA level on the UV-induced subcellular translocation patterns of XPA should be very minimal. To determine the dependence of NER on the Lys-188-mediated ATR-XPA interaction, XPA-WT or XPA-K188A mutant cells were cultured on glass coverslips and irradiated through isopore filters to generate localized DNA damage. Damage foci were visualized by immunofluorescence microscopy and measured as a function of repair time. As shown in Figure 2-2D, foci removal is impaired in XPA^{-/-} and XPA-K188A cells compared with XPA-WT cells, suggesting that the mutation largely abrogated the repair.

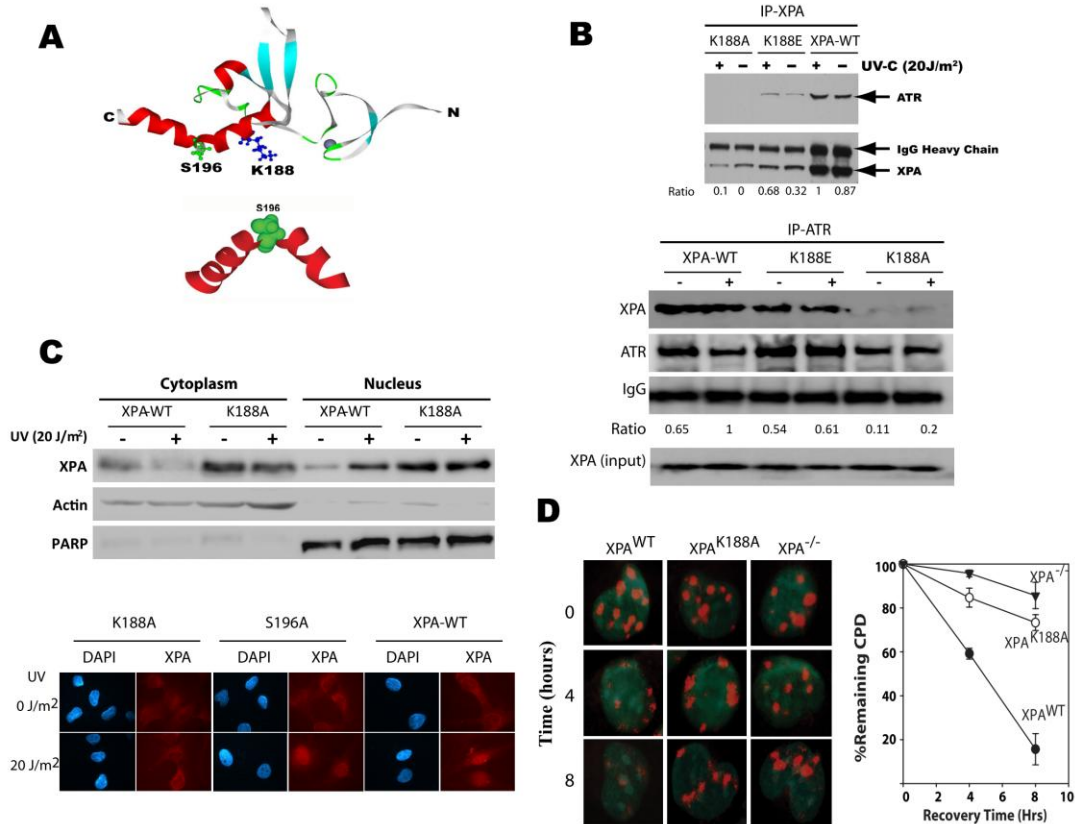


Figure 2-2. Residue Lys-188 is required for XPA-ATR complex formation, XPA nuclear import upon UV damage, and NER

A, ribbon diagram of XPA minimum DNA-binding domain (PDB code 1D4U), indicating that the identified lysine residue Lys-188 (shown in *blue*) is located in the helix-turn-helix motif-containing ATR phosphorylation site serine 196. B, point mutations were generated in the pcDNA-XPA expression construct generating alanine (K188A) and glutamic acid (K188E) substitutions. The mutated constructs as well as wild-type XPA were stably expressed in XPA^{-/-} cells, and their effects on the XPA-ATR interaction were investigated by coimmunoprecipitation (IP). The K188A mutant protein was unable to coimmunoprecipitate ATR or vice versa from lysates generated from UV-irradiated or un-irradiated cells. The K188E mutant maintained the interaction between XPA and ATR and exhibited a similar UV-induced pattern as seen for XPA-WT. The relative amounts of the co-immunoprecipitated ATR were estimated by its ratio to those of the immunoprecipitated protein that were normalized to the loading control IgG. Data in this panel was generated by Steven M. Shell. C, XPA cells complemented with wild-type XPA and XPA-K188A were subjected to subcellular fractionation and immunofluorescence

microscopy analysis. The specificity of the fractionation assay is demonstrated by the presence and absence of cytoplasm-specific and nucleus-specific proteins β -actin and PARP (poly(ADP-ribose) polymerase), respectively, in the cytosol and nucleus. D, cells were irradiated with UV of 50 J/m^2 through isopore filters to induce localized DNA damage and then fixed at the indicated times for immunofluorescence analysis with anti-CPD antibody. Nuclei containing at least one defined CPD focus were counted as a percentage of total DAPI-stained nuclei and plotted *versus* time post-irradiation. At least 50 DAPI-stained nuclei were randomly chosen for the quantification at each time point.

XPA Phosphorylation and DNA Damage Repair

Another possible role of ATR binding to XPA in cells is to phosphorylate the NER protein. The ATR-dependent phosphorylation of XPA previously has been shown to play a role in cell survival after UV-C irradiation (73). Given the unique role of XPA in NER, we hypothesized that XPA phosphorylation may play a role in promoting removal of UV photoproducts. Previous experiments in which ATR kinase activity was inhibited by small interfering RNA knockdown reduced the repair rate of (6-4) photoproducts ((6-4)PPs) (74); however, most of the lesions were removed within several hours post-irradiation, which appears to be earlier than XPA phosphorylation (73). This suggests that the phosphorylation may not be involved in (6-4)PP repair. We, therefore, reason that XPA phosphorylation might play a role in promoting the removal of persistent lesions, such as CPDs.

To test the notion, repair of UV-C-induced photoproducts was monitored by immunofluorescence microscopy in XPA^{-/-} cells complemented with either wild-type XPA or XPA in which serine 196 was replaced by alanine (S196A). As shown in Figure 2-3A, CPD and (6-4)PP foci (stained in red), formed in the nuclei of cells complemented with XPA-WT protein, decrease in size and frequency as the recovery time increases up to 24 h. However, CPD foci are

more persistent in nuclei from cells complemented with XPA-S916A. Nuclei from cells transfected with empty vector alone show little change in CPD focus size or frequency across time. These results suggest that CPD lesions are repaired at a slower rate in cells complemented with XPA-S196A compared with XPA-WT protein (Figure 2-3, A and B). In agreement with previous results, no substantial difference in repair rate was observed for (6-4)PPs between cells complemented with wild-type and phosphorylation-deficient XPA (Figure 2-3B). In a parallel DNA repair assay (Figure 2-3C), XPA-complemented cells were irradiated with UV-C (20 J/m²) followed by extraction of the genomic DNA. Equal amounts of purified DNA were then immobilized on nylon membrane and probed with antibody specific for CPDs. Consistently, CPD lesions were removed more efficiently in cells expressing XPA-WT compared with cells expressing phosphorylation-deficient XPA-S196A protein, whereas little repair occurred in XPA cells transfected with empty vector. These results suggest that XPA phosphorylation promotes the repair of persistent UV-induced photolesions. Also importantly, from the data generated by Moises A. Serrano, the phosphorylation is dependent on the ATR-XPA interaction as demonstrated in Figure 2-3D in which the XPA phosphorylation was abolished in cells expressing XPA-K188A mutant. Taken together, these results support the observation on the effects of XPA-K188A mutation on CPD repair in Figure 2-3D.

To determine whether the reduced repair of CPD lesions by XPA-S196A was because of reduced nuclear translocation, we performed a cellular fractionation assay to determine the subcellular distribution of XPA-S196A in response to UV-C irradiation. As demonstrated in Figure 2-3E, phosphorylation-deficient XPA-S196A is imported into the nucleus with the same efficiency as wild-type XPA, suggesting that XPA translocation is not affected by the phosphorylation. The result is consistent with that determined by the immunofluorescence

analysis (Figure 2-3C). Taken together, XPA phosphorylation is likely involved in a mechanism independent of subcellular protein redistribution to promote repair of persistent DNA lesions.

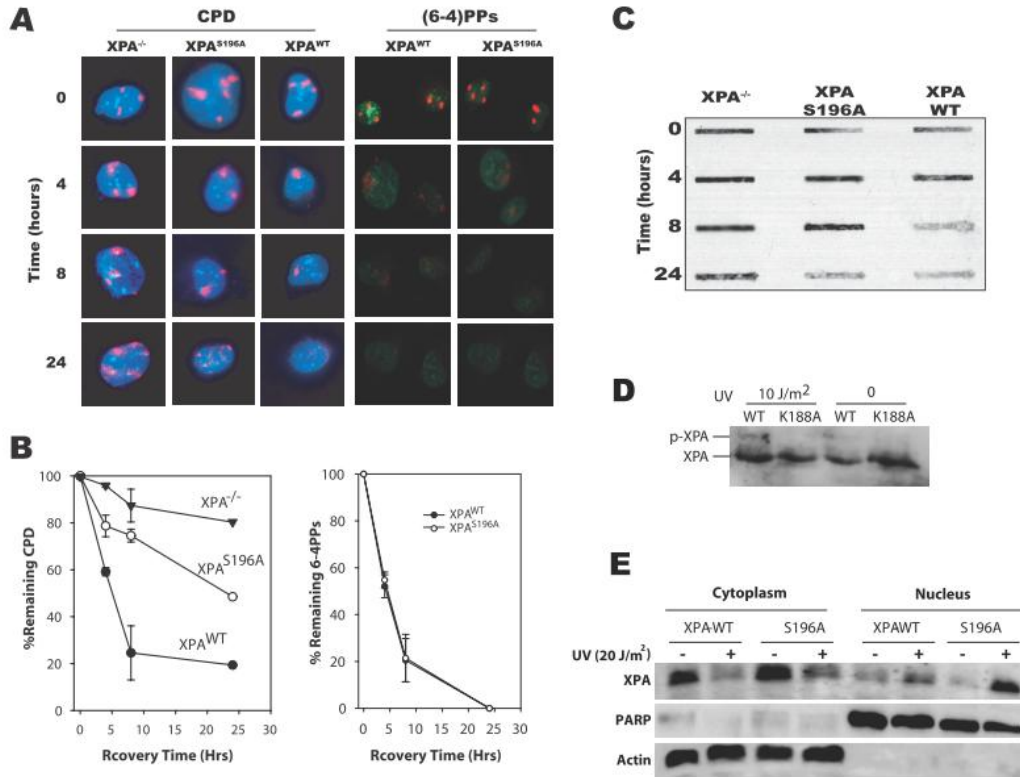


Figure 2-3. Effects of XPA phosphorylation on repair of cyclobutane pyrimidine dimers. Recombinant pcDNA constructs containing wild-type XPA or XPA-S196A cDNA were stably expressed in XPA^{-/-} cells. A, cells were grown on coverslips and UV-irradiated at 20 J/m² through isopore filters to induce localized DNA damage followed by immunofluorescence staining with anti-(6-4)PPs and anti-CPD antibodies at the indicated time points. B, nuclei containing at least one DNA damage focus were counted as a percentage of total nuclei and plotted *versus* time post-irradiation. At least 50 DAPI-stained nuclei were randomly counted for each time point. C, genomic DNA was isolated from cells complemented with wild-type or phosphorylation-deficient XPA after UV-C irradiation. The DNA was immobilized on nylon membranes, and total CPDs were detected using mouse-anti-CPD. D, cells expressing recombinants XPA-WT and XPA-K188A, respectively, were UV-irradiated and then subjected to western blot analysis of phosphorylated and intact XPA. This data was generated by Moises A. Serrano. E, subcellular distribution of XPA-WT and XPA-S196A after UV-C irradiation was

determined by cellular fractionation. β -Actin and PARP (poly(ADP-ribose) polymerase), cytoplasm- and nucleus-specific proteins, respectively, demonstrate the specificity of the assay and serve as loading controls.

Discussion

Our previous work demonstrated that XPA is phosphorylated by the DNA damage checkpoint kinase ATR in response to UV irradiation at serine residue 196 located in the minimum DNA-binding domain of XPA (73). In addition, ATR directed the UV-induced subcellular redistribution of XPA from the cytosol to the nucleus in both a dose-dependent and time-dependent manner (74). Given the central role of XPA in nucleotide excision repair, an interaction between XPA and ATR may represent a novel regulatory mechanism for NER to be modulated by DNA damage checkpoints. The dependence of NER on ATR is further supported by a recent report by Auclair *et al.* (75).

Our lab's previous results (134) strongly imply a key role for the N-terminal α -helix of the XPA helix-turn-helix motif in the interaction with ATR and suggest that Lys-188 mediates the effects on this interaction indirectly by modulating the stability of the helix rather than through direct interaction with ATR. The significance of the ATR-XPA interaction has been shown by its requirement for XPA nuclear import in response to UV irradiation of the cells (Figure 2-3C). Because XPA is an indispensable factor for NER and the nuclear availability of XPA is critical for NER, it is reasonable to expect that NER may depend on ATR-XPA interaction. Indeed our result indicates that the nucleotide excision repair of UV-induced photolesions requires the ATR-XPA interaction in cells. There are at least two possible scenarios in which XPA nuclear translocation and, thus, NER could depend on ATR-XPA interaction. First, the interaction could occur in the nucleus, which reduces the nuclear concentration of free

XPA. The depletion of free XPA in the nucleus disrupts the concentration balance across the nuclear membrane and subsequently allows more XPA to be imported into the nucleus driven by favorable free energy. In the second scenario, XPA could form a complex with ATR in the cytoplasm and then be imported into the nucleus across the nuclear membrane in the complex form.

It is obvious that ATR-XPA complex formation also is required for the phosphorylation of XPA. Phosphorylation of XPA at serine 196 has been previously shown to moderately promote cell survival after UV-C irradiation (73). Given that the only known function for XPA is in NER, we assayed UV-C-induced photoproducts removal to investigate the role of XPA phosphorylation in NER. We found that XPA^{-/-} cells complemented with XPA-S196A displayed a slower repair rate for CPD photoproducts when compared with wild-type XPA. By contrast, no effect has been observed for repair of (6-4) photoproducts. It has been well established that CPD is much more persistent DNA damage than (6-4) photoproducts (151). Taken together, these results suggest that XPA phosphorylation may play a role in stimulating NER activity for removal of persistent DNA damage through an as yet undetermined mechanism. This also appears consistent with our previous observation that phosphorylated XPA represents only a small portion of the cellular pool of XPA (73). We hypothesized that phosphorylation of XPA may modulate NER activity by altering the affinity of XPA for the damage site. Indeed, phosphorylated XPA was shown to associate more tightly with UV-damaged chromatin in cells than the wild-type XPA (73).

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CHAPTER 3

XPA-MEDIATED REGULATION OF GLOBAL NUCLEOTIDE EXCISION REPAIR BY ATR IS P53-DEPENDENT AND OCCURS PRIMARILY IN S PHASE (135)

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Abstract

Cell cycle checkpoints play an important role in regulation of DNA repair pathways. However, how the regulation occurs throughout the cell cycle remains largely unknown. Here we demonstrate that nucleotide excision repair (NER) is regulated by the ATR/p53 checkpoint via modulation of XPA nuclear import and that this regulation occurs in a cell cycle-dependent manner. We show that depletion of p53 abrogated the UV-induced nuclear translocation of XPA, while silencing of Chk1 or MAPKAP Kinase-2 (MK2) had no effect. Inhibition of p53 transcriptional activities and silencing of p53-Ser15 phosphorylation also reduced the damage-induced XPA nuclear import. Furthermore, in G1-phase cells the majority of XPA remained in the cytoplasm even after UV treatment. By contrast, while most of the XPA in S-phase cells was initially located in the cytoplasm before DNA damage, UV irradiation stimulated bulk import of XPA into the nucleus. Interestingly, the majority of XPA molecules were always located in the nucleus in G2-phase cells no matter whether the DNA was damaged or not. Consistently, the UV-induced Ser15 phosphorylation of p53 occurred mainly in S-phase cells, and the cyclobutane pyrimidine dimers (CPDs) were removed much more efficiently in S-phase cells than in G1-phase cells. Our results suggest that upon DNA damage in S phase, NER could be regulated by

the ATR/p53-dependent checkpoint via modulation of the XPA nuclear import process. In contrast, the nuclear import of XPA in G₁ or G₂ phase appears to be largely independent of DNA damage and p53.

Introduction

The human genome is under constant threat of damage from exogenous genotoxic pollutants and carcinogens. Removal of DNA damage requires the dual action and coordination of cell cycle checkpoints and DNA repair machineries in each phase of the cell cycle (14). The nucleotide excision repair (NER) pathway is the primary mechanism in cells for the removal of helix-distorting, replication-blocking DNA adducts induced by exogenous agents such as UV radiation and a variety of genotoxic chemicals (7). In humans, defects of NER lead to the clinical disorder *Xeroderma pigmentosum* (XP) which is characterized by an increased sensitivity to UV radiation and a predisposition to the development of skin cancers (38, 40). It remains elusive how NER is regulated by DNA damage checkpoints throughout the cell cycle.

The *xeroderma pigmentosum* group A protein (XPA) is one of eight factors that were found to be deficient in XP disorders (121), and the XPA-deficient cells exhibit the highest UV sensitivity among the XP cells (122). XPA is an indispensable factor for both the transcription-coupled NER (TC-NER) and global genome NER (GG-NER) (76, 77). NER can be regulated by transcriptional and posttranscriptional control of the XPA protein (73, 74, 78-80, 123). Functionally, XPA is believed to play roles in verifying DNA damage, stabilizing repair intermediates, and recruiting other NER factors to the damage site (76, 124-130).

The DNA damage checkpoints survey the structural integrity of genomic DNA and coordinate multiple cellular pathways to ensure timely and efficient removal of DNA damage.

The ATM (ataxia telangiectasia mutated)- and ATR (ATM- and RAD3-related)-mediated checkpoint pathways are two major genome surveillance systems in human cells. Both ATM and ATR are protein kinases belonging to the phosphoinositide 3-kinase-like kinase (PIKK) family. These pathways are comprised of a series of DNA damage sensors, signal mediators and transducers, and downstream effectors (7, 13, 14, 103). Checkpoint kinase-1 (Chk1), p53, and MAPKAP Kinase-2 (MK2) are the three main downstream checkpoint proteins that can be directly or indirectly activated by ATR following UV irradiation (19, 152, 153).

ATR can be activated by genotoxic agents that cause replication stress associated with accumulated RPA (Replication Protein A)-coated ssDNA (154). In our previous studies, ATR and its kinase activity were found to be required for modulating XPA translocation into the nucleus from the cytosol upon UV-DNA damage (74). Consistently, ATR was reported to be required for maintaining NER activity primarily during S phase in human cells (75). When the XPA translocation is inhibited by disruption of the ATR-XPA interaction in the nucleus, DNA repair efficiency is significantly reduced (134). Regulation of nuclear import is necessary for timely localization of the repair proteins that participate in DNA repair (136). These findings lead us to propose that ATR regulation of the XPA nuclear import may directly coordinate the ATR checkpoint activity with NER. However, the question as to whether the ATR-regulated nuclear import of XPA upon DNA damage is cell cycle specific remains to be addressed.

In the current work, we demonstrate that UV-induced XPA nuclear import is cell cycle dependent and happens primarily in the S-phase, which may contribute to the ATR-regulated NER process. We also identified p53 as the ATR-regulated downstream protein required for the UV-induced XPA nuclear import and the removal of UV-DNA damage.

Materials and Methods

Tissue Culture, Drugs, and Antibodies

The A549/LXSN (p53+) and A549/E6 (p53-) cells were gifts from Dr. Jeffrey L. Schwartz (155). Cells were maintained in D-MEM supplemented with 10% FBS and 1% penicillin-streptomycin. All cell lines were grown at 37°C, 5% CO₂. UV-C irradiation was performed using a 254 nm lamp at a fluence of 0.83 J/m²/sec. For time course analysis, cells were incubated at 37°C, 5% CO₂ for the indicated amounts of time. For inhibition of p53 transcriptional activities, pifithrin- α (Sigma Chemical Co.) was added into the culture medium at 30 μ M and the cells incubated for 20 hours. For western blotting and immunofluorescence staining of XPA, primary mouse polyclonal antibody against XPA was purchased from Kamiya Biomedical Company. For western blotting, mouse monoclonal antibody against PARP, rabbit polyclonal antibody against p53, mouse monoclonal antibody against Chk1, and goat anti-MK2 antibody were purchased from Santa Cruz Biotechnology Co. A FITC conjugated primary mouse anti-actin antibody was obtained from Sigma Chemical Co. The anti-actin and anti-PARP antibodies were used in western blots to confirm successful subcellular fractionations and protein loadings.

RNAi and Transfections

p53 and XPA siRNA duplexes were purchased from Santa Cruz. MK2 siRNA and Chk1 siRNA duplexes were synthesized by Genepharma using the following sequences: MK2 siRNA, sense strand 5'-UGACCAUCACCGAGUUUAUdTdT-3' and antisense strand 5'-AUA AACUCGGUGAUGGUCAdTdT-3'; Chk1 siRNA, sense strand 5'-ACAGUAUUUCGGUAUAAUATT-3' and antisense strand 5'-UAUUAUACCGAAAUACUGUTG-3'. The siRNA transfection reagent was purchased from

Polyplus-transfection and the transfections were done by following their instructions. Briefly, cells were grown to 30-40% confluence and washed with FBS- and antibiotic-free medium. siRNA duplexes were added to a small volume of FBS- and antibiotic-free medium and incubated with transfection reagent for 10 min. This siRNA/reagent mixture then was added to cells in FBS- and antibiotic-free medium at a final siRNA concentration of 5-10 nM. After 5-7 hours incubation, concentrated FBS and antibiotic medium were added into the transfection medium for further incubation. The cells were UV-irradiated at either 48 or 72 hrs of post-transfection. For time course experiments, siRNA-containing medium was taken off the cells for UV irradiation and added back for further cell growth. The p53 3'-UTR siRNA duplexes were purchased from QIAGEN Incorporation. Plasmids of human wild-type p53 and the Ser15Ala p53 mutant were gifts from Dr. Karen Vousden at the Beatson Institute for Cancer Research, United Kingdom. The co-transfection of p53 3'-UTR siRNA with p53 plasmids were done using Lipofectamine™ 2000 (Invitrogen) by following the company's instructions.

Immunoblotting

Cells were harvested by scraping or trypsin digestion, and re-suspended in lysis buffer (50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1x protease inhibitor cocktail [Roche]). 2X SDS loading buffer was added to the lysates and the mixtures were heated at 90°C for 10 min to denature proteins. After running the samples in SDS-PAGE, proteins in the gel were transferred onto PVDF membrane. The membranes then were blocked with 5% nonfat milk in TBST and probed with specific primary and secondary antibodies. Chemiluminescence signal was captured with a FUJI Film camera, and western blot images were processed with Multi-Gauge 3.0 software.

Cell Synchronization, Flow Cytometry, and BrdU Incorporation Assay

Cells were synchronized by mitotic “shake off” as described previously (156). A549 cells were synchronized by seeding cells into four 300 cm² flasks and grown to ~70 % confluence. Mitotic cells were collected by physically shaking the flasks to dislodge the loosely attached cells with monitoring by phase contrast microscopy at 100X magnification. For synchronizing HeLa cells, the cells were cultured in four 300 cm² flasks and treated with nocodazole at 100 ng/ml for 8 hours (to enrich the mitotic cells) before shaking off. Collected mitotic cells then were seeded into 12-well or 6-well plates at 30-40% confluence and left to grow in standard culture condition. The synchronization was confirmed using BrdU labeling at each 4-hr time point after “shake off” and by flow cytometric cell cycle analysis after propidium iodide staining of the nuclear DNA.

For propidium iodide staining, cells were fixed in cold 70 % ethanol for 1- to 16-hrs at 4°C. Fixed cells were centrifuged at 10,000xg for 10 sec to pellet the cells, and propidium iodide solution (PBS with 20 ug/mL propidium iodide and 100 ug/mL RNase (Invitrogen)) was added to re-suspend the cell pellet. The resuspended cells were incubated for another 30 min at 37°C. Stained cells then were analyzed using an Accuri C6 flow cytometer or a BD Biosciences flow cytometer to assess the DNA content. The cell synchronization results were processed by FCS software.

BrdU incorporation was performed following the company’s instructions (Cellomics) with a few modifications. Briefly, cells were grown on coverslips and labeled with BrdU by adding the nucleoside to a final concentration of 160 μM; the cells were grown for an additional 15 min to label those in S phase. Cells then were fixed with 4% paraformaldehyde solution before treatment with permeabilization buffer. After blocking with 15% BSA for 1hr at room

temperature, primary mouse anti-BrdU antibody and fluorescence-conjugated secondary antibodies (Invitrogen) were used to detect BrdU incorporation. Stained cells were visualized using 20X magnification with fluorescence microscopy.

Subcellular Fractionation

Subcellular fractionation was performed using the Proteo JET™ cytoplasmic and nuclear protein extraction kit (Fermentas) by following the procedures suggested by the manufacturer. Briefly, 10 volumes of cell lysis buffer (with 1x protease inhibitors) were added to 1 volume of packed cells. After a short vortexing and incubation on ice for 10 min, cytoplasm was separated from nuclei by centrifugation at 500xg for 7 min at 4°C. Isolated nuclei were washed once or twice with 500 µL of the nuclei washing buffer and then collected by centrifugation. The collected nuclear pellets were re-suspended in ice-cold nuclear storage buffer, and 1/10 volume of the nuclear lysis reagents was added to the mixtures to lyse the nuclei with rotation for 15 min at 4°C. Nuclear lysate was collected by centrifugation at 20,000xg for 15 min at 4°C. In all of the fractionation experiments, western blotting of β-actin and PARP were assessed to check cytoplasmic and nuclear protein loading, respectively.

Immunofluorescence Microscopy

For immunofluorescence microscopy of proteins, cells were grown on coverslips before the initiation of experimental treatments. After UV-C irradiation and specified recovery times, the cells were fixed with 100% cold methanol and blocked with 15% BSA for 1hr at room temperature. Proteins were detected with primary antibodies and fluorescence-conjugated secondary antibodies (Invitrogen). Cells on coverslips were coated with prolong gold antifade reagent containing DAPI (Invitrogen) before microscopic examination using 100X magnification.

Slot-blot DNA Repair Assay

Cells were seeded at 1×10^6 cells per 10 cm tissue culture dish and allowed to grow indicated times prior to UV-C irradiation. After irradiation, cells were allowed to recover for the indicated periods, followed by genomic DNA purification using the QIAGEN DNA Mini Kit. The purified DNA was quantified by measuring the light absorbance at 260 nm and diluted to 0.2 $\mu\text{g}/\text{mL}$ in a final volume of 200 μL TE buffer (10 mM Tris-Cl, pH 7.5. 1 mM EDTA). The DNA was denatured by incubating at 90°C for 10 minutes, followed by rapid chilling on ice water before adding an equal volume of 2 M ammonium acetate. Samples were immobilized on a nylon membrane and probed using monoclonal mouse anti-CPD or anti-(6-4)PP antibodies (Kamiya Biomedical Co.).

Statistical Analysis

The statistical analysis of samples was performed with a two-tailed student's t-Test, and a p-value of less than 0.05 was considered as significant.

Results

UV-induced XPA Nuclear Import Depends on p53

We previously demonstrated that UV-induced XPA nuclear translocation is dependent on ATR (74). Since p53 is a major downstream substrate of ATR, it is of interest to determine if p53 is required for XPA nuclear import. Thus, cells were transfected with p53 siRNAs. As shown in Figure 3-1A, the p53 silencing inhibited the UV-induced nuclear import of XPA. In the control siRNA-transfected cells, most of XPA molecules were imported into the nucleus after DNA damage (compare the nuclear XPA-to-cytoplasmic XPA (nXPA/cXPA) ratio in lanes 6 and 2 with the ratio in lanes 5 and 1; also see the adjacent plot). By contrast, the nuclear

XPA/cytoplasmic XPA ratio was significantly reduced in the cells with p53 depletion (Figure 3-1A). β -actin and PARP are cytoplasmic and nuclear proteins, respectively, and were probed as controls to indicate the quality of the cytoplasmic/nuclear protein fractionation. An immunofluorescence microscopy assay also was performed and the same effect of p53 on the XPA nuclear import in the cells was observed (Figure 3-1B). In the absence of DNA damage, most XPA molecules were located in the cytosol of the cells transfected with control siRNA, but were translocated into the nucleus following UV irradiation. However, only a small portion of cytosolic XPA was translocated into the nucleus in the p53-silenced cells even after UV irradiation. To further confirm these results, A549/E6 (p53-) and A549/LXSN (p53+) cells were employed. In A549/E6 (p53-) cells, p53 is abrogated due to overexpression of human papillomavirus type 16 E6 protein (155), while in A549/LXSN (p53+) cells wild-type p53 is expressed. As shown in Figure 3-1C, the UV-induced XPA nuclear translocation was disrupted in the p53-deficient cells as compared to the p53-proficient cells.

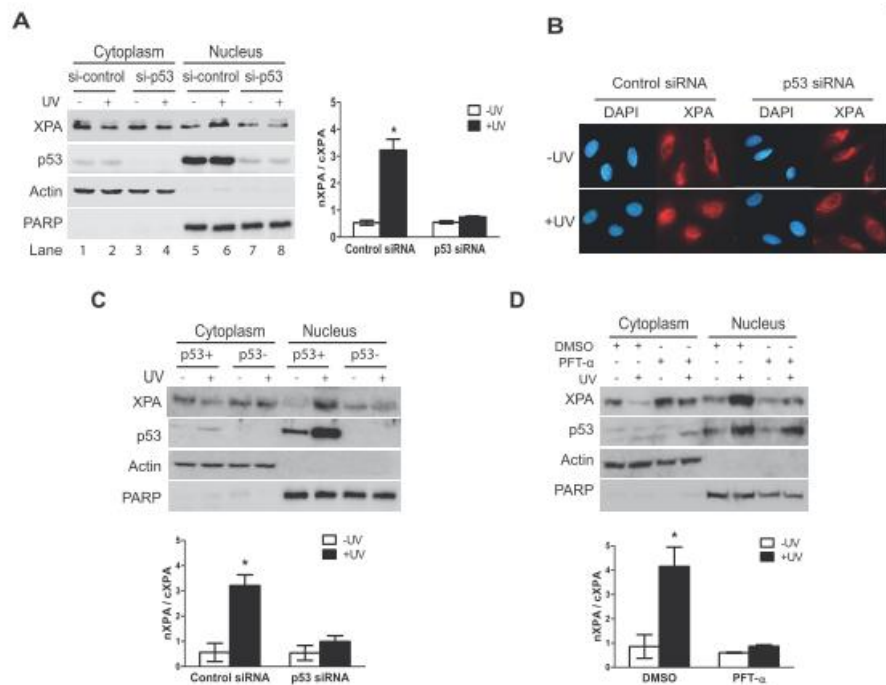


Figure 3-1. p53 is required for the XPA nuclear import upon UV irradiation

A, p53 was transiently knockdown with siRNA duplexes in HeLa cells. After treatment with or without 20 J/m² UV followed by a 2-hr recovery, subcellular fractionation and western blotting were performed to assess the re-distribution of XPA. β -actin and PARP were probed as cytoplasmic and nuclear protein controls, respectively. The quantitative data were obtained from at least three independent experiments. nXPA/cXPA represents the ratio of nuclear XPA to cytoplasmic XPA. B, Immunofluorescence microscopy analysis of cells transfected with control or p53 siRNA and with or without UV irradiation. C, A549/LXSN (p53+) and A549/E6(p53-) cells were mock- or UV-irradiated. Cytosol and nuclear fractions were collected and analyzed by western blotting. D, A459 cells were treated with pifithrin- α (30 μ M), an inhibitor of p53 transcriptional activity, for 20 hrs. After irradiation with UV followed by a 2-hr recovery, the cells were analyzed for subcellular localization of XPA. The * in the quantification plots indicates a statistically significant ($p < 0.05$) difference between the groups being compared.

We next examined the effect of transcriptional function of p53 on XPA nuclear import. A549 cells were pre-incubated with pifithrin- α , a p53 transcriptional activation inhibitor, before the UV treatment. As shown in Figure 3-1D, the presence of pifithrin- α significantly reduced the UV-induced XPA nuclear import (the nXPA/cXPA ratio) as compared to the DMSO-treated cells. These data suggest that p53 may regulate the UV-induced nuclear import of XPA through its transcriptional activity.

Neither Chk1 nor MK2 is Required for UV-Induced XPA Nuclear Import

Chk1 is a close downstream kinase substrate of ATR and may play an important role in transducing damage signal in the ATR pathway by phosphorylating p53. MAP kinase-activated protein kinase 2 (MAPKAPK2 or MK2) is another downstream kinase of ATR recently was identified as an alternative checkpoint in p53-deficient cancer cells (19). It is of interest to investigate whether Chk1 and/or MK2 are involved in the UV-induced nuclear translocation of XPA. To this end, A549 cells were transfected with Chk1 or MK2 siRNA followed by

subcellular fractionation. The results in Figures 3-2A and 3-2B indicate that neither Chk1 nor MK2 is required for the UV-induced XPA nuclear import as no difference of the import was observed between the control siRNA- and the Chk1 or MK2 siRNA-transfected cells. Similar results also were obtained for cells depleted of both Chk1 and MK2 (Figure 3-2C). By contrast, siRNA knockdown of ATR in the A549 cells did significantly reduce the UV-induced nuclear import of XPA (Figure 3-2C), confirming the involvement of ATR/p53 checkpoint pathway in the regulation of XPA nuclear import.

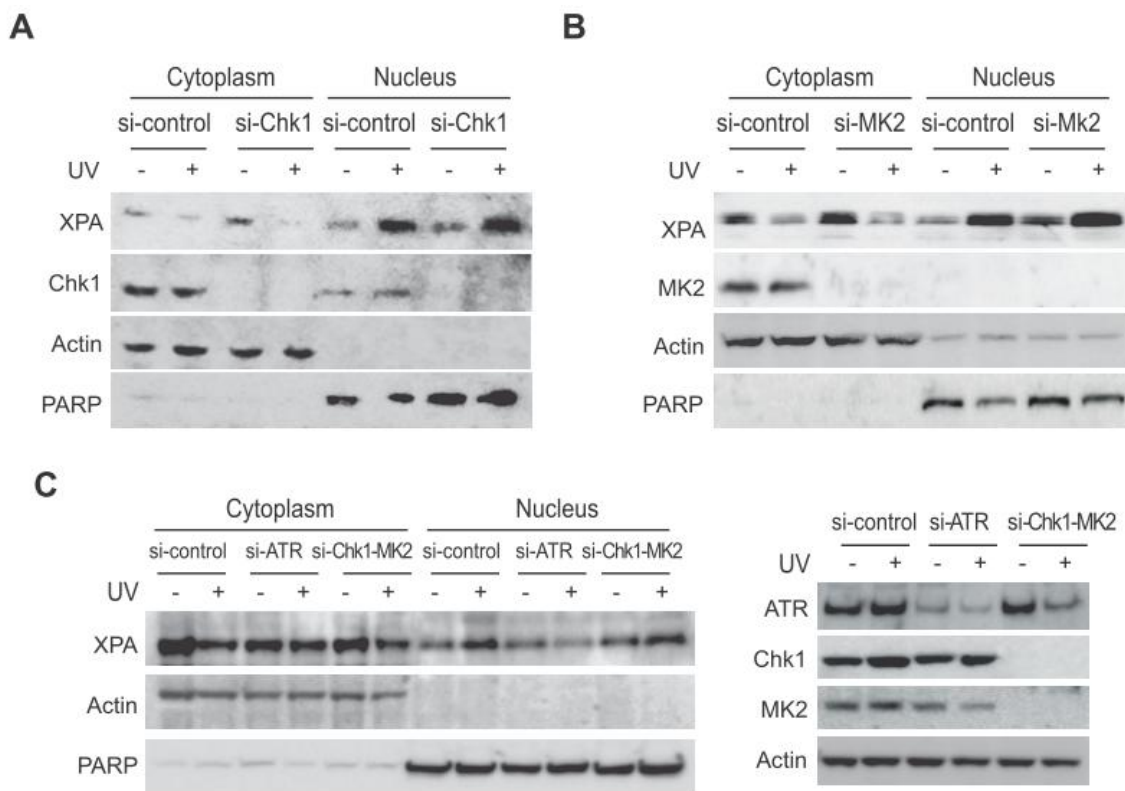


Figure 3-2. Cell cycle checkpoint proteins Chk1 and MK2 are not required in the UV-induced nuclear import of XPA

A, siRNA duplexes targeting Chk1 were transiently transfected into A549 cells followed by mock or 20 J/m² UV irradiation and a 2-hr recovery. The localization of XPA was assessed using subcellular fractionation followed by western blot analysis. PARP and β -actin proteins were probed as nuclear and cytoplasmic protein controls, respectively. B, A549 cells were treated with MK2 or control siRNA, followed by UV irradiation. After a 2-hr recovery period, irradiated cells

were fractionated and analyzed by western blotting. C, Chk1 and MK2 were either simultaneously knocked down by Chk1 and MK2 siRNAs, or knocked down by ATR siRNA. A549 cells were transfected by siRNAs either for simultaneous knockdown of Chk1 and MK2, or for ATR. Then the UV-induced XPA nuclear import in these cells was assessed by fractionation and western blotting.

UV-damage Induced XPA Nuclear Import Occurs Primarily in S phase

Next we addressed the question of whether the ATR-dependent XPA nuclear import following DNA damage is cell cycle specific as is the ATR checkpoint (157). To avoid any physiological perturbation associated with drug treatments, A549 cells were synchronized by mitotic “shake off” as described previously (156). The collected mitotic cells were seeded into cell culture dishes and maintained at standard culture condition to generate a synchronized cell population for subsequent analysis. The synchronization efficiency was assessed by propidium iodide (PI) staining followed by flow cytometric analysis and by assessing the cells positively labeled with BrdU under fluorescence microscopy (Figure 3-3A). Based on the results (data generated by Zhiping Dong), the 4-hour post-“shake off” time point was selected as the G1 cell population (most cells with a 2C DNA content and the lowest level of BrdU labeling); the 14-hour time point was selected as the S-phase cell population (DNA content between 2C and 4C and highest BrdU labeling); the 18-hour post- “shake off” time point was selected as the G2 cell population (4C DNA content and low BrdU labeling). The location of XPA molecules in the synchronized cell populations was assessed using immunofluorescence. Figure 3-3B shows that in the G1 cells most of the XPA molecules were located in the cytosol, while there was only a slight accumulation of XPA in the nucleus after UV irradiation. By contrast, although most of the XPA molecules were located in the cytosol in S phase before UV irradiation, they were imported into the nucleus after UV treatment. Interestingly, in the G2 cell population most cells showed significant levels of XPA in the nucleus either with or without UV irradiation.

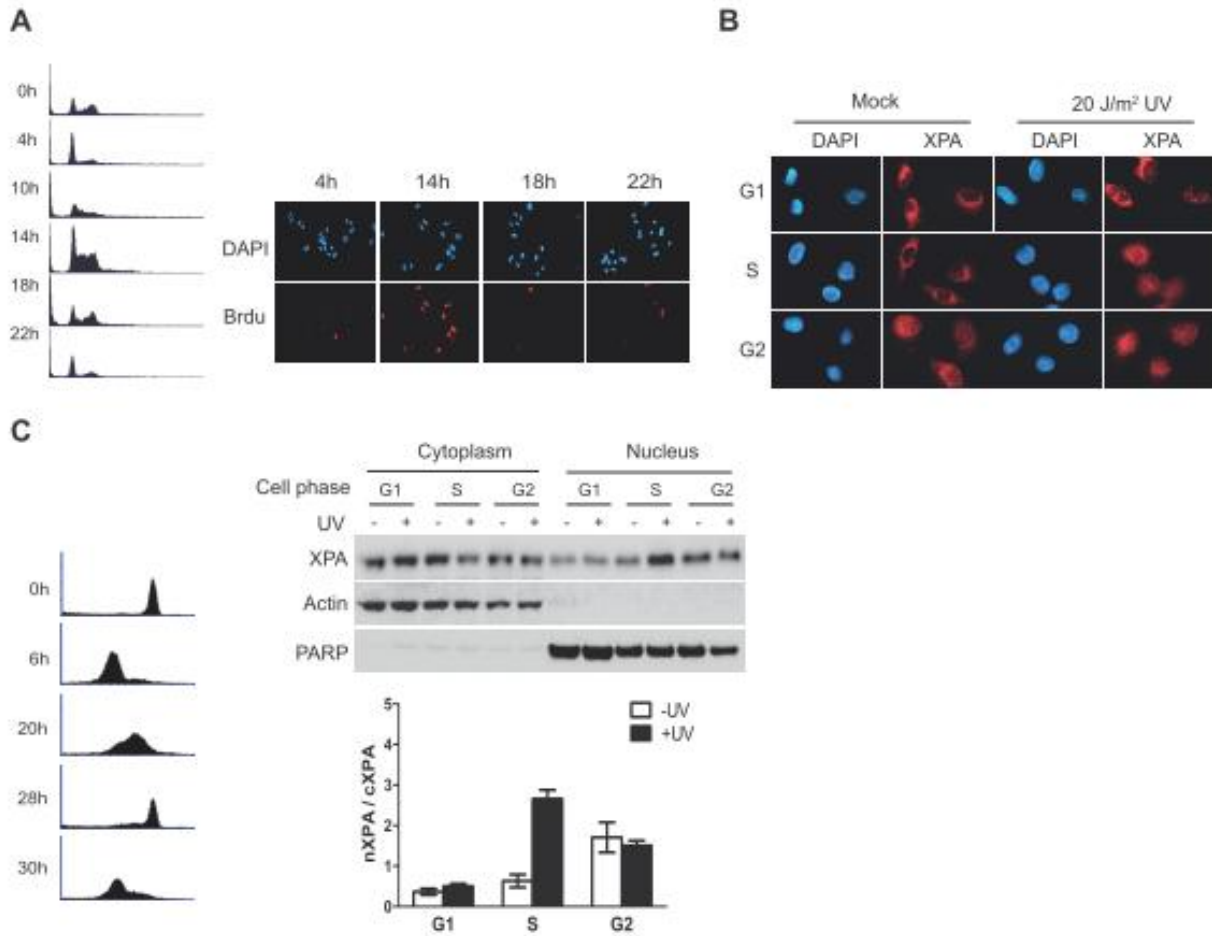


Figure 3-3. DNA damage-induced XPA nuclear accumulation occurs primarily in S phase

A, Mitotically-synchronized A549 cells grown for the indicated time periods were stained with propidium iodide for analysis of the cell cycle distribution (Panel A, left) or labeled with BrdU to identify synchronized S-phase cells (panel A, right). B, Immunofluorescence microscopic analysis of the subcellular localization of XPA in the synchronized cells. Synchronized A549 cells were mock- or UV-treated (20 J/m²) and left to recover for 2 hrs. Cells were fixed and stained with primary and fluorescence--conjugated secondary antibody to determine the localization of XPA. At least 100 cells were examined, and the representative data is shown. C, Left: Mitotically-synchronized HeLa cells were stained with propidium iodide followed by flow cytometry analysis. Right: Subcellular fractionation followed by western blotting was performed to analyze the subcellular localization of XPA in each phase of the cell cycle after UV irradiation of the synchronized HeLa cells. PARP and β -actin were probed as nuclear and cytoplasmic

protein loading controls, respectively. At least three independent experiments were performed and representative data is presented.

To determine whether this cell phase-specific translocation of XPA is a general response, synchronized HeLa cells (Figure 3-3C, left) were treated with or without UV and then subjected to subcellular fractionation for analysis of subcellular localization of XPA in each phase of cell cycle. Consistent with the observation in the A549 cells, we found that in HeLa cells, the UV-induced XPA nuclear translocation also occurs primarily in the S phase (20 hrs post- “shake off”) (Figure 3-3C, right). Similarly, in the G1 phase cells (6 hrs post- “shake off”), most XPA remained in the cytoplasm even after UV treatment, while in the G2 phase cells (28 hrs post- “shake off” time point), a large amount of XPA was found in the nucleus even without UV irradiation.

Repair of CPDs is Significantly Slower in G1 than in S phase

As described above, the UV-induced XPA import occurs primarily in S-phase cells, particularly in comparison with that in G1 phase (Most of the XPA is located in the nucleus in G2 phase even in the absence of DNA damage). Thus, it was expected that repair of the UV-induced DNA damage could be more efficient in S phase cells than in G1 phase cells. To confirm this, HeLa cells were mitotically synchronized and the cells in either G1 or S phase were UV-irradiated at a dose of 10 J/m^2 , followed by indicated periods of recovery (Figure 3-4A). As shown in Figure 3-4B, more CPDs were generated in S phase than G1 phase cells following UV irradiation, likely due to the more open chromatin structures in S phase than in G1 phase (thus less protection of DNA from UV damage). As expected, the repair rate of CPDs was much higher in S phase than in G1 phase cells. In contrast, no difference in repair efficiency for 6-4PPs was observed between G1 and S phase cells (Figure 3-4B). It is well known that 6-4PPs

can be removed in cells within a few hours while CPDs are the much persistent DNA damage that is responsible for the UV-induced cell death in NER-proficient cells (158). It also is known that 6-4PPs are the minor lesions induced by UV in cells as compared with CPDs (159, 160). Since the repair of 6-4PPs is generally so efficient, it is possible that the relatively low level of XPA in the nuclei of G₁ phase cells is adequate for efficient removal of the relatively small quantity of 6-4PPs.

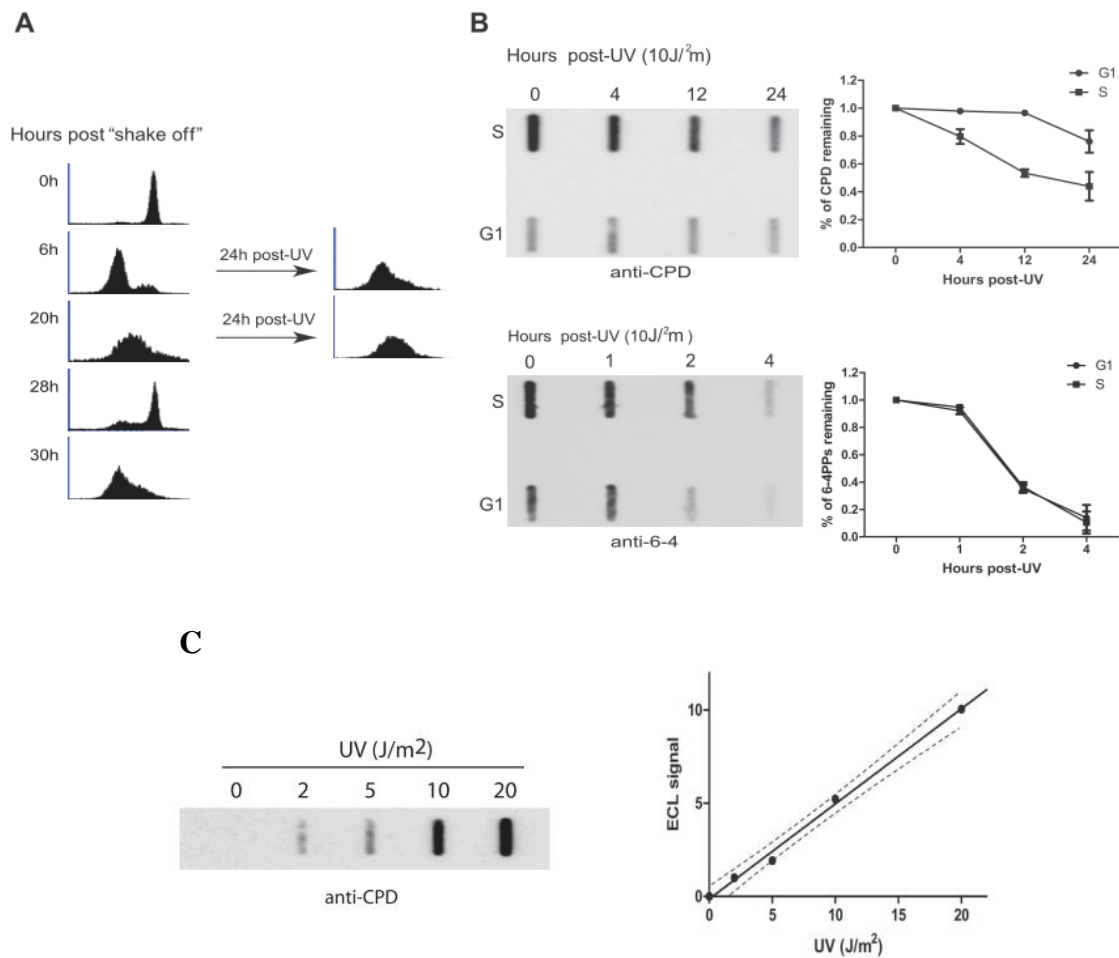


Figure 3-4. Removal of UV-induced DNA damage in G₁- and S-phase cells

A, Mitotically-synchronized HeLa cells were fixed and stained with propidium iodide at indicated time points following the mitotic "shake off". The cell cycle distribution then was analyzed by flow cytometry. Cells at G₁ (at the 6 hours post-"shake off") or S phases (20 hours post-"shake off"), were UV irradiated at 10 J/m², followed by a recovery of 24 hours. B, Cells at

G₁ or S phases were UV irradiated at 10 J/m², followed by the indicated periods of repair. Removal of CPDs and 6-4PPs was measured by slot-blot assay. The amounts of CPDs or 6-4PPs were normalized to the values at zero hour and quantified based on three independent measurements. C, An evaluation of the slot-blot DNA repair assay. HeLa cells were treated with different doses of UV-C and harvested immediately. The amount of CPDs on the blot were detected (left) and subjected to linear regression analysis (right). It shows that the detection of CPDs in our assay (panel B) is in a linear range within a 95% confidence.

Phosphorylation of p53 on Serine15 is Involved in the UV-induced XPA Nuclear Import

Although our results showed that p53 is required for the XPA nuclear import (Figure 3-1), it is unclear whether the requirement involves the checkpoint activity of p53. Therefore, we examined the effect of the phosphorylation of p53 at Ser15 on XPA nuclear import. The p53 phosphorylation at Ser15 plays an important role in ATR-dependent checkpoint signaling (7, 152). Consistent with the observed cell phase-specific nuclear import of XPA induced by UV, the UV-induced phosphorylation of p53 on Ser15 was found to occur predominantly in the S-phase cell population (Figure 3-5A). We next assessed the requirement of p53 serine15 for the UV-induced XPA nuclear import. For this purpose, the endogenous p53 in A549 cells was depleted using 3'UTR siRNA while the cells transiently expressed recombinant siRNA-resistant wild-type p53 or S15A-mutant p53. The deficiency of the Ser15 phosphorylation in the p53-S15A construct-transfected cells was confirmed by western blotting (Figure 3-5B). Also shown in Figure 3-5B, the UV-induced XPA nuclear import in the cells expressing p53-S15A was significantly lower than in the cells expressing wild-type p53.

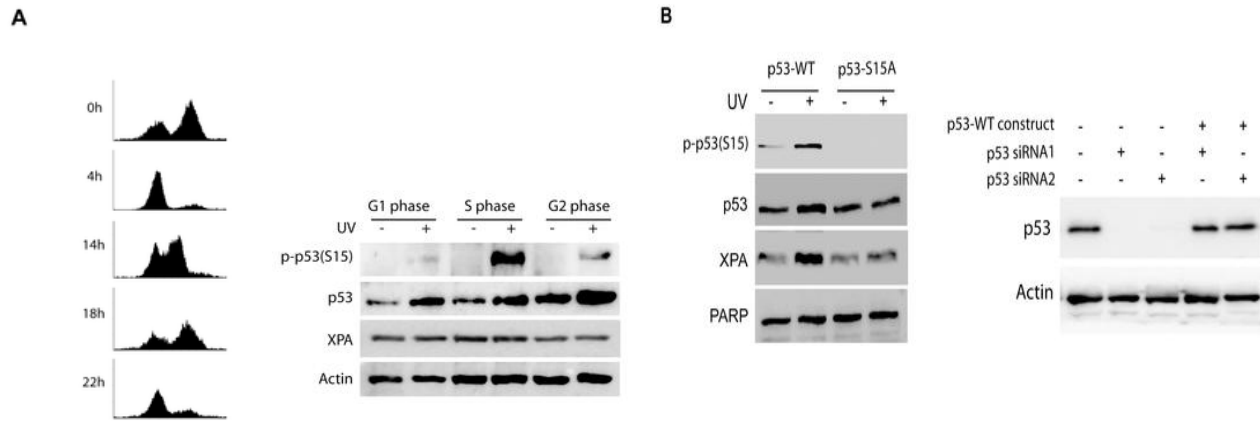


Figure 3-5. Phosphorylation of p53 is involved in the UV-induced XPA nuclear import
 A, Mitotically synchronized A549 cells were mock-treated or irradiated with 20 J/m² of UV-C, and allowed a 2-hr recovery before accessing the phosphorylation of p53 at Ser15 by western blotting. B, Constructs for expressing human wild-type p53 or the S15A mutant of p53 were co-transfected with p53 3'-UTR siRNA into A549 cells. 72 hours after transfection, the A549 cells were mock- or UV (20 J/m²)-treated and allowed a 2-hr recovery. The UV-induced phosphorylation of p53 and the XPA in nuclear fractions were then analyzed by western blotting. The right panel shows the efficiency of siRNA knockdown of endogenous p53 and the level of recombinant p53 in the cells co-transfected with p53 3'-UTR siRNA and wild-type p53 construct.

Discussion

A precise regulation of DNA repair is essential for cells to function normally in response to DNA damage. Given the key role of XPA in NER, results from our previous (74, 134) and current studies suggest that the ATR-dependent regulation of the damage-induced XPA nuclear import may represent a novel mechanism by which NER activity can be regulated by DNA damage checkpoints. Here we found that this regulation occurs primarily in S-phase, which well reflects the fact that DNA is most vulnerable to insult in S phase in terms of maintaining genome

integrity. This is also consistent with the recent report that ATR kinase is required for GG-NER exclusively during S phase (75).

We also examined whether any of the major downstream checkpoint substrates of ATR such as p53, Chk1 or MK2, is involved in the regulation of the UV-induced XPA nuclear import. Our results indicate that the XPA nuclear import is dependent on p53 in cells responding to UV damage (Figure 3-1), but neither Chk1 nor MK2 are required for this XPA nuclear translocation (Figure 3-2). The results suggest a regulatory role of p53 in NER, which is in agreement with previous studies (161-166). We found that not only is the p53 protein itself necessary (as shown in the siRNA knockdown experiments), but also the transcriptional function of p53 and the damage signaling via p53-ser15 phosphorylation are required for the UV-induced XPA nuclear import (Figures 3-1 and 3-5). In fact, the Ser15 phosphorylation of p53 has been shown to stimulate the transcriptional functions of p53 through its increased association with p300 co-activator (167-169) and stabilization via disruption of binding to MDM2 (170). Thus, the effect of p53-ser15 phosphorylation appears to converge with that of the p53 transcription activation inhibitor pifithrin- α . The Ser15 of p53 can be phosphorylated either directly by ATR or by ATR-activated Chk1 in response to UV irradiation (4, 7, 152, 153). Since Chk1 was not required for UV-induced XPA nuclear import (Figure 3-2), our results suggest that ATR kinase may directly phosphorylate p53 for transcriptional activation of the XPA nuclear translocation. Given that p53 activates transcription of multiple genes involved in numerous cellular processes (47), it is possible that p53 may enhance the transcription of the genes involved in XPA trafficking from cytoplasm to nucleus (171-173). More recently, studies have showed that the transcription factor E2F1 plays a role in facilitating the recruitment of XPA and other NER factors to the UV-induced DNA damage sites and this appears to be mediated by ATM- and ATR-dependent

phosphorylation of E2F1 at Ser31 (174, 175). Evidently, future investigation is needed to identify the p53-regulated transcriptional targets that are involved in protein nuclear import process.

In contrast to the case in S phase, most of the XPA molecules remained in the cytoplasm of G1-phase cells after UV irradiation, while XPA normally accumulated in the nucleus in G2-phase cells even without DNA damage. In addition, the level of UV-induced Ser15-phosphorylation of p53 was much lower in G1- and G2-phase cells than in S-phase cells (Figure 3-5A). These results suggest that the UV-induced XPA nuclear import happens predominately in S phase, while the XPA nuclear import in G1 and G2 phases is largely, if not fully, independent of UV irradiation and p53. Consistently, DNA repair of UV-induced CPDs was much more efficient in S phase than in G1 phase (Figure 3-4). Also interestingly, it was previously demonstrated that p53 deficiency had a negative impact on GG-NER but not on TC-NER (165, 166). Given the indispensable role of XPA in both GG-NER and TC-NER, the observation of a p53 requirement for the UV-induced XPA nuclear import in S-phase cells implies that the TC-NER may predominately occur in other cell cycle phases in a p53-independent manner. Consistently, the rate of transcription is generally low during S phase except the transcription for histone production.

Compared to the UV-induced XPA nuclear translocation in S phase, the subcellular distributions of XPA in the absence and presence of UV are very different in G1 and G2 phases. For G1 phase in typical human cells, it lasts much longer than S phase and the G1-phase cells do not experience a replication pressure requiring a fast repair of DNA damage. Thus, it is possible that the relatively lower rate of NER could be sufficient for timely removal of DNA damage by the end of G1 phase. In addition, the G1/S DNA damage checkpoint could also prevent the cells

from entering S phase before the damage has been removed. In the case of G2 phase, since XPA nuclear accumulation occurs even in the absence of DNA damage, the accumulation is not a residual effect of an S phase accumulation. This may imply a quick removal of DNA damage in G2. It may also implicate a possible role of XPA in G2 maintenance or G2/M checkpoint regulation in normal cell cycle. Indeed, depletion of XPA protein in cells changed the population distribution of cells in the cell cycles (data not shown). Although the potential role of XPA in normal cell cycle is of interest, it is out of scope of this study and deserves further investigations.

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CHAPTER 4

UV-INDUCED NUCLEAR IMPORT OF XPA IS MEDIATED BY IMPORTIN-A4 IN AN ATR-DEPENDENT MANNER (139)

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Abstract

Xeroderma pigmentosum Group A (XPA) is a crucial factor in mammalian nucleotide excision repair (NER) and nuclear import of XPA from the cytoplasm for NER is regulated in cellular DNA damage responses. In this study, experiments were carried out to determine the transport mechanisms that are responsible for the UV-induced nuclear import of XPA. Using subcellular fractionation, RNAi and immunofluorescence microscopy assays, we found that, in addition to the nuclear localization signal (NLS) of XPA, importin- α 4 and/or importin- α 7 are required for the XPA nuclear import. Further investigation indicated that importin- α 4 and importin- α 7 directly interacted with XPA in cells. Interestingly, the binding of importin- α 4 to XPA was dependent on UV-irradiation, while the binding of importin- α 7 was not, suggesting a role for importin- α 7 in nuclear translocation of XPA in the absence of DNA damage, perhaps with specificity to certain cell-cycle phase. Consistent with the previous report of a dependence of UV-induced XPA nuclear import on ataxia telangiectasia and Rad3-related protein (ATR) knockdown of ATR reduced the amount of XPA interacting with importin- α 4. In contrast, the GTPase XPA binding protein 1 (XAB1), previously proposed to be required for XPA nuclear import, showed no effect on the nuclear import of XPA in our siRNA knockdown analysis. In

conclusion, our results suggest that upon DNA damage transport adaptor importin- α 4 imports XPA into the nucleus in an ATR-dependent manner, while XAB1 has no role in this process. In addition, these findings reveal a potential new therapeutic target for the sensitization of cancer cells to chemotherapy.

Introduction

Human genomic DNA is constantly exposed to endogenous and exogenous damaging reagents, which may lead to genome instability. Removal of these structural and chemical abnormalities in the DNA requires timely and coordinate recruitment of DNA repair factors to the damaged DNA (7, 14). The NER pathway is the primary mechanism in cells for the removal of helix-distorting, replication-blocking bulky DNA adducts that are induced by exogenous agents such as UV radiation and a variety of genotoxic chemicals (7, 14). In humans, defects in nucleotide excision repair (NER) lead to the clinical disorder *xeroderma pigmentosum* (XP) which is characterized by an increased sensitivity to UV radiation and a predisposition to the development of skin cancers (40, 177). The XPA protein is one of eight factors that were found to be deficient in XP disorders (121), and the XPA-deficient cells exhibit the highest UV sensitivity among the XP cells (122). XPA is an indispensable factor both for transcription-coupled NER and global genome NER (76, 77). Functionally, XPA is believed to play roles in verifying DNA damage, stabilizing repair intermediates, and recruiting other NER factors to the damaged DNA (7, 51, 124, 127, 128, 130, 146, 178-182). Because of XPA's crucial functions in NER, the activity of NER and sensitization of cancer cells to chemotherapy can be regulated by transcriptional and posttranscriptional control of the XPA protein (73, 74, 78-80, 123). In addition, recent studies demonstrated that besides its anticipated functions in NER, XPA also

may participate in other cellular events in the absence of genotoxic insults, such as facilitating chromatin modification for transcription (183), inhibiting DNA double-strand break repair in progeria (149), and potential involvement in DNA replication (173).

Given the important nuclear functions of XPA in NER (7, 136), it is of interest to study the DNA damage-dependent nuclear import of XPA (74, 135, 138), and also targeting protein trafficking could be a strategy to improve the sensitivity of cancer cells to chemotherapy agents (137). It was previously shown that the DNA damage-induced nuclear import of cytoplasmic XPA for NER in S phase is a DNA damage checkpoint-dependent process mediated by ataxia telangiectasia and Rad3-related (ATR) and p53 tumor suppressor protein (74, 134, 135). Both ATR and free p53 localize in the nucleus with or without DNA damage. Despite the importance of XPA nuclear import for NER, it remains unresolved how XPA is imported into the nucleus from the cytoplasm.

In this study, protein factors potentially involved in the transport of XPA through the nuclear pore complex (NPC) were investigated. We found that in the presence of the nuclear localization signal (NLS) of XPA, importin- α 4 and importin- α 7 served as the transport adaptors for the UV-induced nuclear import of XPA from the cytoplasm. Consistent with the requirement of ATR for the UV-induced XPA nuclear import, the role of importin- α 4 in the import also was dependent on ATR. Surprisingly, however, XAB1 protein, suggested to be the GTPase involved in XPA nuclear import, showed no effect on the XPA nuclear import. Given the indispensable role of XPA in human NER, our findings demonstrate a cytoplasmic regulatory mechanism important for NER. The therapeutic disruption of this transport process may provide a means of sensitizing cancer cells to chemotherapeutic drugs.

Materials and Methods

Cell Culture, UV-Irradiation and Antibodies

Cells were maintained in D-MEM supplemented with 10% FBS and 1% penicillin-streptomycin. XPA-complemented cells were generated by stably transfecting H1299 cells with pcDNA3.1 vectors (Invitrogen) containing either wild type or NLS-mutated XPA cDNA with indicated mutations. All cell lines were grown at 37°C, 5% CO₂. UV-C irradiation was performed using a 254 nm lamp at a fluence of 0.83 J/m²/sec. For blotting primary rabbit polyclonal antibody against XPA and mouse monoclonal antibody against poly (ADP-ribose) polymerase (PARP) were purchased from Santa Cruz Biotechnology Co. Antibodies against importins- α 1, - α 3, - α 4, - α 5, or - α 7 were from Genetex Biotechnology Co. A FITC-conjugated primary mouse anti-actin antibody was obtained from Sigma Chemical Co. The anti-actin and anti-PARP antibodies were used in western blots to confirm successful subcellular fractionations and protein loadings. The rabbit monoclonal antibody against XAB1 is from Sigma Chemical Co.

Immunoblotting

Cells were harvested by scraping or trypsin-EDTA release, and re-suspended in lysis buffer [50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1x protease inhibitor cocktail (Roche)]. 2X SDS loading buffer was added to the lysates and the mixtures were heated at 100°C for 10 min to denature proteins. After running the samples in SDS-PAGE, proteins in the gel were transferred onto a PVDF membrane. The membrane then was blocked with 5% nonfat milk in TBST buffer and probed with specific primary and secondary antibodies. Chemiluminescence signal was captured using a Fuji Film camera, and the blot images were processed with Multi-Gauge 3.0 software.

RNAi

XAB1 siRNA duplexes were purchased from Santa Cruz. siRNA duplexes targeting individual importin- α proteins in lung cells were successfully developed by Gabriel *et al.* These sequences were synthesized by Genepharma using the published sequences. The siRNA transfection reagent was purchased from Polyplus Transfection and the transfections were done by following their instructions as we described previously (135, 138).

Subcellular Fractionation

Subcellular fractionation was performed using the Proteo JETTM cytoplasmic and nuclear protein extraction kit (Fermentas) by following the procedures suggested by the manufacturer. Briefly, 10 volumes of cell lysis buffer (with 1x protease inhibitors) were added to 1 volume of packed cells. After a short vortexing and incubation on ice for 10 min, cytoplasm was separated from nuclei by centrifugation at 500xg for 7 min at 4°C. Isolated nuclei were washed once or twice with 500 μ L of the nuclear washing buffer and then collected by centrifugation. The collected nuclear pellets were re-suspended in ice-cold nuclear storage buffer, and 1/10 volume of the nuclear lysis reagents was added to lyse the nuclei with rotation for 15 min at 4°C. A clarified nuclear lysate was obtained by centrifugation at 20,000xg for 15 min at 4°C. In all of the fractionation experiments, western blotting of β -actin and PARP were assessed to check successful fractionation and cytoplasmic and nuclear protein loading, respectively.

Immunoprecipitations

Cells were lysed with NETN lysis buffer (20 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40) containing protease and phosphatase inhibitors (Thermo Scientific). For each 2 mg of protein in the whole cell lysates 2 μ g of primary antibody was added and the mixture incubated at 4°C with agitation overnight. Then, 15 μ L of high-capacity

protein G-agarose (Thermo Scientific) was added and incubated for 2 hr at 4°C to capture the antibodies. After washing three times with NETN buffer, proteins was released from beads in SDS sample loading buffer and heat denaturation, resolved on 10% SDS-PAGE, and subjected to western blotting. Immunoprecipitation of XPA-binding proteins was conducted by mixing 1.0 µg of purified recombinant XPA protein with 2 mg of cell lysates from mock or UV-irradiated GM04429 (XPA^{-/-}) cells; a goat monoclonal antibody to the C-terminal of XPA (Santa Cruz) then was added to the lysates and incubated at 4°C with agitation overnight. Protein G-agarose beads were added to capture the antibodies, washed 3 times with NETN buffer, and bound proteins eluted with SDS sample buffer, resolved on 10% SDS-PAGE, and subjected to western blotting.

In Vitro Protein-Protein Interaction

The immunoprecipitated importin proteins (prepared as above) were washed with 0.6 M NaCl (high salt wash) buffer (25 mM Tris-HCl [pH 8.0], 0.6 M NaCl, 0.2 % NP-40, 1 mM EDTA) containing protease inhibitors (Thermo Scientific) for 5 minutes at 4°C. After collection by centrifugation the beads and bound proteins were re-suspended in 200 µl of NETN lysis buffer. Then, 0.5 ug of purified XPA protein was added to each sample and incubated overnight at 4°C with agitation. The beads with bound proteins were collected by centrifugation and washed three times with NETN buffer, released in SDS sample buffer, resolved on 10% SDS-PAGE and subjected to western blotting.

Immunofluorescence Microscopy

Transfected cells were grown on coverslips before the initiation of experimental treatments. After UV-C irradiation and specified recovering times, the cells were fixed with 100% cold methanol and blocked with 15% BSA for 1 hr at room temperature. Proteins were

detected with primary antibodies and fluorescence-conjugated secondary antibodies (Invitrogen). Cells on coverslips were coated with prolong gold anti-fade reagent containing DAPI (Invitrogen) before microscopic examination using 100X magnification.

Results

The N-terminal NLS of XPA is Required for UV-Induced Nuclear Import

To maintain a functional NER, XPA protein contains multiple functional domains for interaction with various other proteins, including RPA (replication protein A), excision repair cross-complementation group 1 (ERCC1), DNA damage-binding protein 2 (DDB2), and transcription factor II H (TFIIH), as well as chemical carcinogen-damaged or UV-damaged DNA (132) (Figure 4-1A). Among these domains, a NLS-like motif is located in the N-terminus (184) (Figure 4-1A). To determine whether the DNA damage-induced nuclear import of XPA depends on this NLS-like motif or whether XPA is co-imported with proteins containing a NLS, or imported by the so-called “alternative import mechanisms” (more than one mechanism) (136), two amino acids of the XPA NLS (184) were replaced with alanine (XPA-ΔNLS) (Figure 4-1A). As shown in Figure 4-1B and Figures 4-S1A-C, no recombinant XPA protein (slower migration) was detected in the nuclear fractions of these mutated transfectants even after UV-irradiation. In comparison, after UV-irradiation the endogenous XPA increased in the nucleus as it decreased in the cytoplasm, and the recombinant wild-type XPA also accumulated in the nucleus (Figure 4-1B and Figures 4-S1A-C); indicating an UV-induced nuclear import of wild type XPA. Similarly, the immunofluorescence microscopy analysis showed that XPA-ΔNLS remained in the cytoplasm even after the cells were irradiated with UV, while the wild-type XPA accumulated

normally in the nucleus (Figure 4-1C). These results suggested the dependence of the DNA damage-induced XPA nuclear import on the NLS motif.

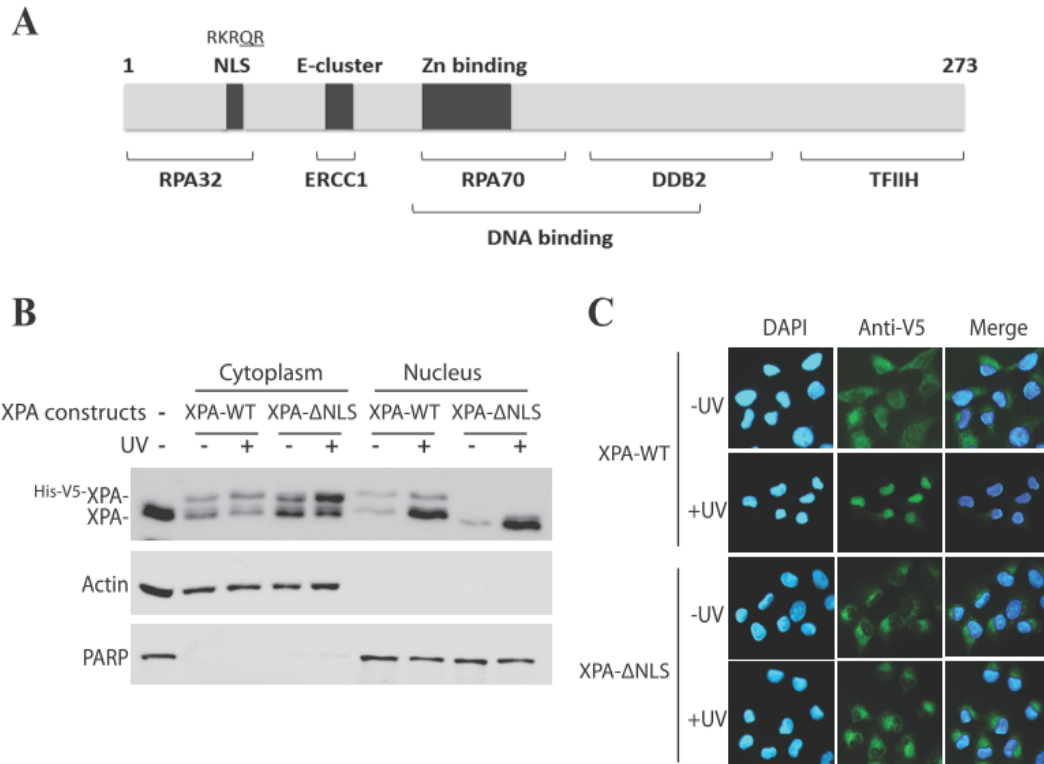


Figure 4-1. Nuclear localization of recombinant XPA requires an N-terminal NLS sequence

A, A map of XPA protein illustrating the locations of the binding sites for various DDR proteins or for binding damaged DNA. The numbers refer to the first and last amino acid in the XPA protein. The XPA- Δ NLS protein construct was made by changing the underlined amino acids within the NLS to alanine by PCR mutagenesis. B, Fractionation and western blotting demonstrates UV-induced XPA redistribution. Stably transfected H1299 cells were mock or UV-irradiated (20 J/m^2) followed by a 2-hr recovery. The recombinant His⁶-V5-tagged XPA protein migrates slower than endogenous XPA allowing us to detect each XPA protein using XPA antibody. C, Immunofluorescence microscopy of recombinant XPA using antibody against the V5-tag portion of the recombinant XPA protein. H1299 cells were treated as in A. The

localization of XPA was assessed by immunofluorescence microscopy. The nuclei were stained with DAPI.

Importin- α 4 and Importin- α 7 are Involved in the UV-Induced XPA Nuclear Import

XPA has an N-terminal NLS (Figure 4-1), but its localization to the nucleus where it functions also depends on DNA damage and cell cycle stage (74, 135, 138). These observations indicate that there may be two stages of XPA nuclear import: one occurs naturally in the absence of DNA damage, and another, the massive transport, is initiated by specific DNA damage signaling (74, 135, 138). Among the factors involved in the DNA damage-induced XPA import, those involved in the XPA transport through the NPC are unknown. Importin- α is a constituent of the classical nuclear import pathway. It acts as an adaptor protein that recognizes the NLS of cargo proteins and is transported as a ternary complex with importin- β into the nucleus (171, 185) (Figure 4-2A). In humans, six importin- α isoforms are known. To identify the importin- α responsible for the UV-induced nuclear import of XPA, importin- α proteins in human lung cells were silenced by siRNA knockdown (Figure 4-2B). Subsequent subcellular fractionation analysis indicated that knockdown of importin- α 4 or/and importin- α 7 inhibited the XPA nuclear import (Figure 4-2C and 4-2D): XPA was present primarily in the cytoplasm without UV-irradiation in control cells, and was transported into the nucleus after UV-irradiation in the control, importins- α 1, - α 3 or - α 5 siRNA-knocked down cells (as indicated by the ratio of cXPA / nXPA). However, in cells with importin- α 4 and/or importin- α 7 silenced with siRNAs, significant amounts of XPA remained in the cytoplasm.

The similar experiments also were carried out in the cells without UV treatment. As shown in Figure 4-S1D, without UV the siRNA-knockdown of importin α 4 had no effect on XPA subcellular localization, indicating that the knockdown effect of importin α 4 is UV-

dependent. In contrast, knockdown of importin $\alpha 7$ showed some effect on XPA subcellular localization in the absence of UV, indicating that the knockdown effect of importin $\alpha 7$ is UV-independent.

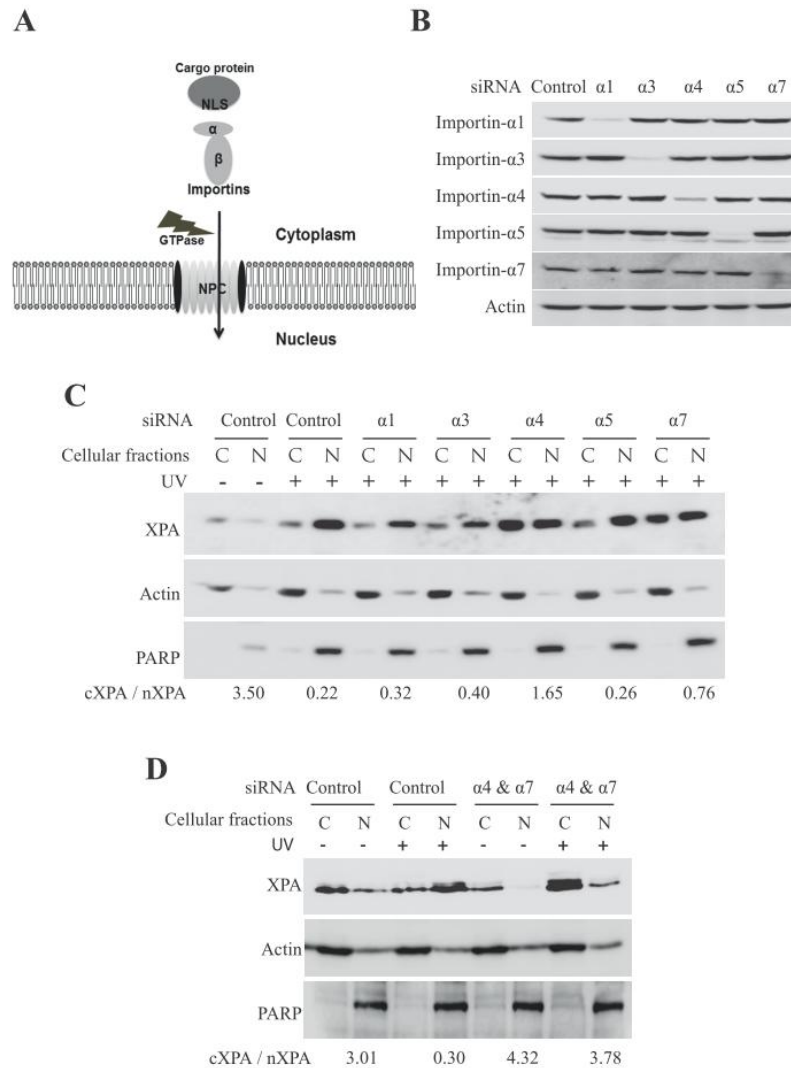


Figure 4-2. Importin- $\alpha 4$ and - $\alpha 7$ are involved in the nuclear import of XPA
 A, A model for the movement of proteins into the nucleus. B, western blotting confirms the efficiency of siRNA knockdown of five importin- α proteins. H1299 cells were transiently transfected with siRNAs targeting individual importin- α proteins. Whole cell lysates were prepared 48 hours post-transfection and assessed by western blotting. C, Fractionation and western blotting assess the localization of XPA following UV-irradiation. H1299 cells were transfected with the indicated siRNA duplexes and, at 48 hours post-transfection, cells were

mock or UV irradiated (20 J/m^2) and allowed a 2-hr recovery. Cytoplasmic and nuclear lysates were collected and analyzed by western blotting of XPA. The cXPA / nXPA numbers represent the amount of cytoplasmic XPA divided by the amount of nuclear XPA (cXPA / nXPA) and were calculated from repeated experiments. D, Similar experiments as in panel C, except that importin- $\alpha 4$ and importin- $\alpha 7$ were silenced simultaneously.

Interaction of Importin- $\alpha 4$ and Importin- $\alpha 7$ with XPA

Cargo protein with a classic NLS requires a direct interaction with importin- α to be transported through the nuclear pore complex (136, 171, 185-188) (Figure 4-2A). Further experiments were carried out to investigate if XPA forms complexes with importin- $\alpha 4$ or importin- $\alpha 7$. As shown in Figure 4-3A, XPA was co-immunoprecipitated with importin- $\alpha 4$ or importin- $\alpha 7$ antibodies. As a comparison, incubation of the cell lysates with protein G-agarose did not pull down any detectable XPA; suggesting that the presence of XPA in the immunoprecipitates is due to the interaction of importins- $\alpha 4$ or - $\alpha 7$ with XPA. Interestingly, while little XPA was found to bind to importin- $\alpha 4$ in cells prior to UV treatment, XPA was co-precipitated with importin- $\alpha 4$ after $\frac{1}{2}$ hr of recovering from 20 J/m^2 of UV-irradiation (Figure 4-3A, left panel), indicating the involvement of importin- $\alpha 4$ in the UV-induced XPA nuclear import. In contrast, however, XPA was co-precipitated with importin- $\alpha 7$ even in the absence of UV treatment (Figure 4-3A, right panel). These results were well consistent with those shown in Figures 2C and S1D.

In a reciprocal experiment, cell lysates of XPA-deficient cells were supplied with recombinant XPA, incubated, and then the XPA was immunoprecipitated using anti-XPA antibody. As shown in Figure 4-3B, importin- $\alpha 4$ and importin- $\alpha 7$ were co-immunoprecipitated with XPA. In contrast, probing the same blot with importin- $\alpha 1$ and importin- $\alpha 5$ antibodies showed no bands in the western blotting (Figure 4-3B), indicating the preferred interactions of

XPA with importin- α 4 and importin- α 7. Interestingly, the *in vitro* binding of recombinant XPA to importin- α 4 in cell lysates did not increase with UV irradiation (Figure 4-3B and Figure 4-4A-c), a significant difference from the results in Figure 4-3A where endogenous proteins were co-immunoprecipitated directly from cell lysates. This difference suggests that the binding affinity of XPA to importin- α 4 itself is not affected by UV-irradiation of cells, but the binding is regulated in a UV-dependent manner, probably through the association with other cytoplasmic factors that may mask the XPA NLS (187, 189). Indeed, it was previously reported that the UV-induced nuclear import of XPA was ATR-dependent (74). Consistently, here we showed that knockdown of ATR in cells reduced the amount of XPA interacting with importin- α 4 (Figure 4-3C).

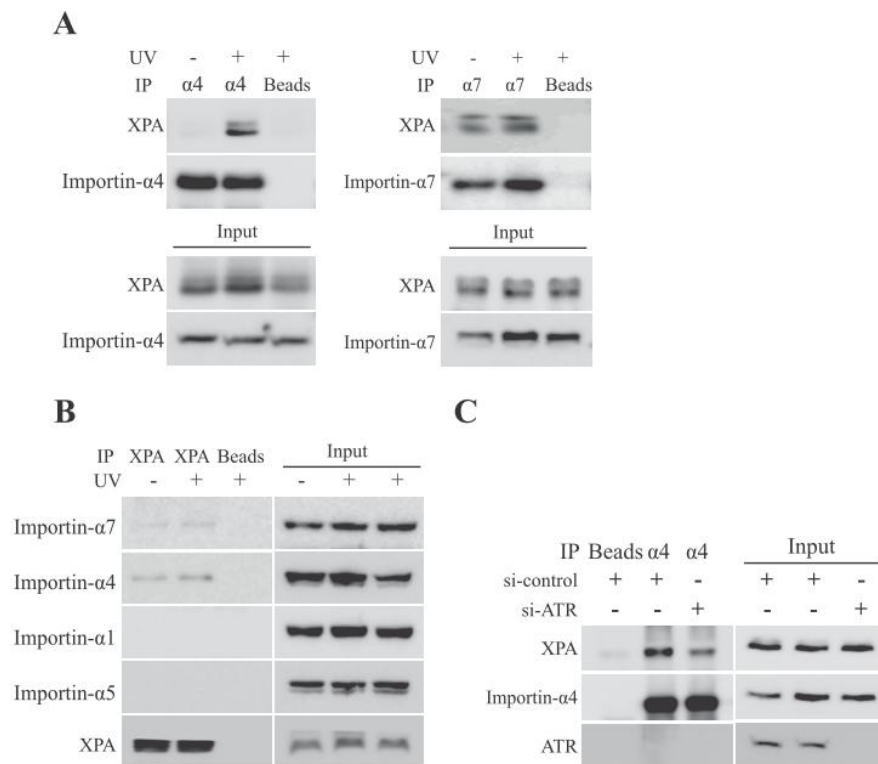


Figure 4-3. Importin- α 4 and importin- α 7 form complexes with XPA in cells

A, XPA was detected in the protein complexes of importin- α 4 or importin- α 7 by western blotting. Immunoprecipitation of importin- α 4 or importin- α 7 also pulled down XPA in lysates

prepared as described in methods from H1299 cells which were UV-C irradiated (20 J/m^2) and allowed a $\frac{1}{2}$ h recovery. XPA was not pulled down with importin- $\alpha 4$ in lysates from cells that were mock treated. B, A reciprocal experiment in which XPA was immunoprecipitated using a C-terminal XPA antibody. GM04429 (XPA^{-/-}) cells were mock or UV-C irradiated (20 J/m^2) followed by a $\frac{1}{2}$ h recovery. Cell lysates were prepared as description in the material and methods and supplied with recombinant XPA protein. Immunoprecipitation of recombinant XPA was employed to assess whether the added XPA formed complexes with importin- $\alpha 4$ or importin- $\alpha 7$ proteins. C, Knockdown of ATR inhibited cellular interaction of XPA and importin- $\alpha 4$. A549 cells were transfected with control (scrambled) or ATR siRNA. 72 hours post-transfection cells were mock-treated or exposed to UV-C radiation (20 J/m^2), followed by a 1/2-hr recovery. The amount of XPA associated with importin- $\alpha 4$ was assessed by western probing of XPA that was co-immunoprecipitated with importin- $\alpha 4$.

To further determine whether importin- $\alpha 4$ and importin- $\alpha 7$ directly or indirectly interacted with XPA, *in vitro* protein-protein interaction experiments were performed. As illustrated in Figures 4-4A and 4-4B, left panels, importin- $\alpha 4$ or importin- $\alpha 7$ was immunoprecipitated from cell lysates, and rinsed with the buffer containing 0.6 M NaCl to wash away interacting proteins as described previously (134, 190). Then purified recombinant XPA protein was added to the washed precipitates of importin- $\alpha 4$ or importin- $\alpha 7$, followed by further wash with regular binding buffer. As shown in the right panels of Figures 4-4A and 4-4B, the interaction of importin- $\alpha 4$ or importin- $\alpha 7$ with XPA was indeed direct.

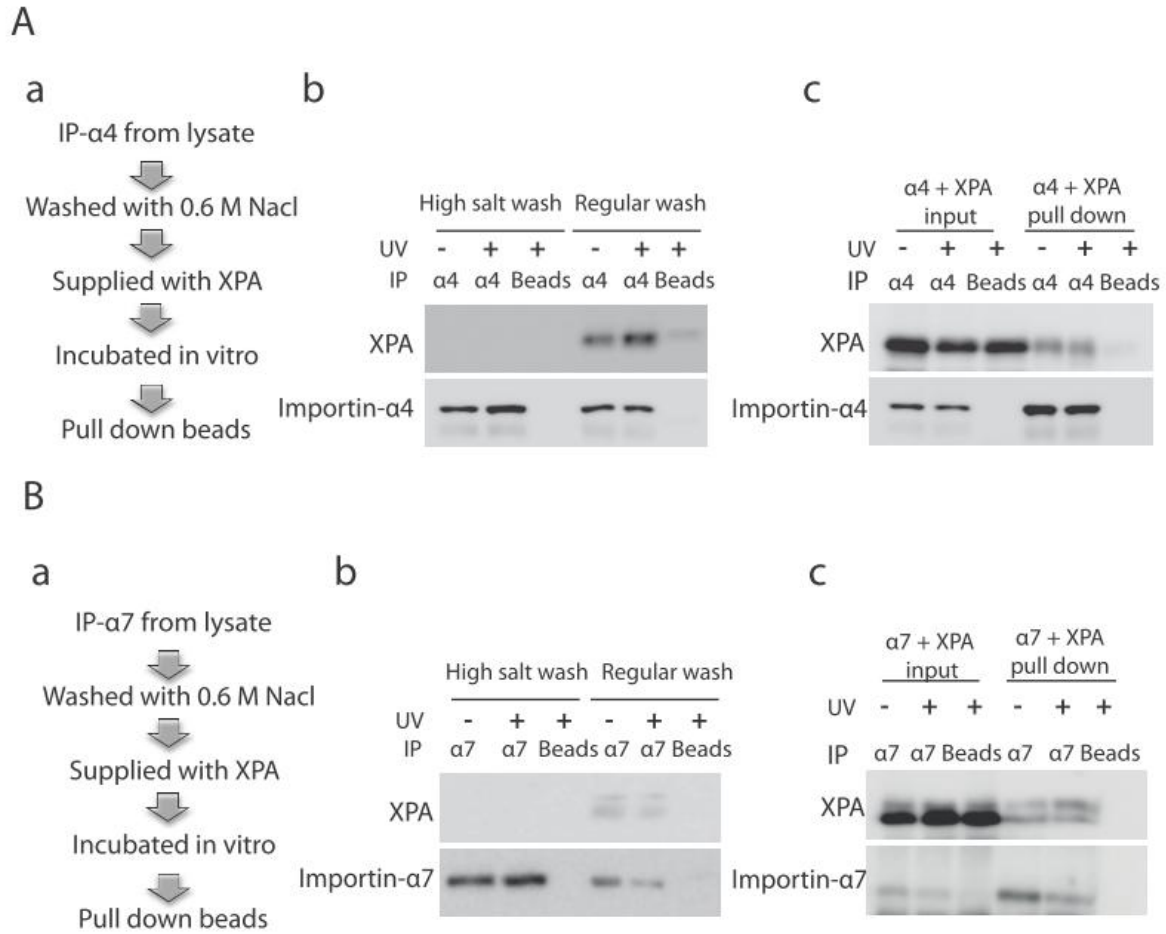


Figure 4-4. Direct interaction of importin- $\alpha 4$ or importin- $\alpha 7$ with XPA *in vitro*

A, *In vitro* interaction of importin- $\alpha 4$ and XPA was performed as description in the methods. A diagrammatic illustration of experimental procedures (a). H1299 cells were mock or UV-C irradiated (20 J/m^2) followed by a $\frac{1}{2}$ h recovery. Importin- $\alpha 4$ was immunoprecipitated as in Figure 4-3 A, and the precipitate rinsed with high salt (0.6 M NaCl) to wash away proteins interacting with importin- $\alpha 4$ without denaturation (b). Western blotting confirmed the efficiency of washing. Recombinant XPA was added and the interaction of importin- $\alpha 4$ and XPA was determined by western blotting following the pull downs (c). B, Similar to A, except that importin- $\alpha 7$ was immunoprecipitated.

Knockdown of XAB1, the Proposed GTPase, Did not Affect UV-Induced XPA Import

Transportation of cargo protein in importin- α / β protein complexes through the NPC requires energy dependent on the activities of GTPases (171, 188)(Figure 4-2A). In a yeast two-hybrid system XAB1 was observed to bind XPA and it has been proposed to be the GTPase involved in XPA nuclear import (172). To determine whether XAB1 protein is involved in human XPA nuclear transport, XAB1 was silenced by siRNA knockdown (Figure 4-5A), followed by subcellular fractionation of the cells and analysis by western blotting (Figure 4-5B) or by immunofluorescence detection of XPA (Figure 4-5C). Surprisingly, XAB1 knockdown had no effect on XPA nuclear import as compared with the control siRNA, suggesting that XAB1 alone was dispensable for the UV-induced nuclear import of cytoplasmic XPA in human cells.

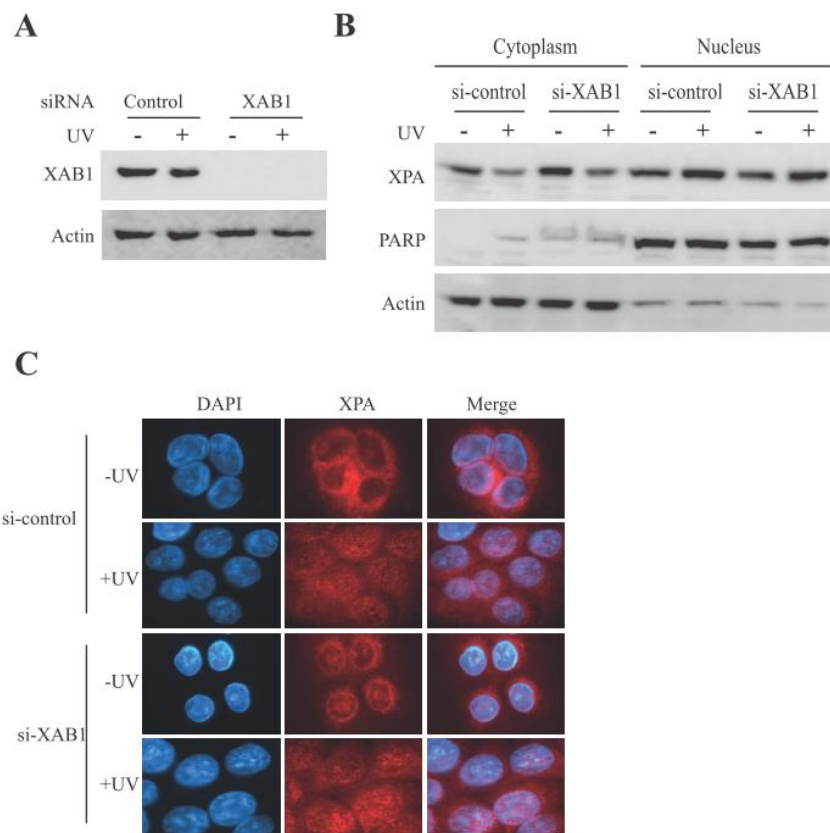


Figure 4-5. Knockdown of XAB1 did not affect UV-induced XPA nuclear import

A, Western blotting confirmed siRNA knockdown efficiency of XAB1. A549 cells were transiently transfected with siRNA targeting XAB1. 72 hours post-transfection cells were mock treated or exposed to UV-C radiation (20 J/m²) followed by a 2-hr recovery. Whole cell lysates were prepared for western blotting analysis of XAB1. B and C, Demonstration that XAB1 was not needed for the nuclear localization of XPA in A549 cells. B, 72 hours post-transfection of control siRNA or siRNA targeting XAB1, cells were mock or 20 J/m² of UV-C irradiated and allowed to recover for 2 hrs. Cytoplasmic and nuclear lysates were separated and loaded onto SDS-PAGE for analysis of XPA by western blotting. C, Immunofluorescence detection of XPA in the cells treated by UV-irradiation as in B.

Discussion

XPA has crucial functions in NER. It is essential to understand the mechanism by which XPA is imported into nucleus in response to DNA damage as NER depends on XPA functioning within the nucleus. In this study we identified the proteins involved in the nuclear import of XPA. We found that the DNA damage-induced nuclear import of XPA depended on an intact N-terminal NLS within the protein. In addition, importin- α 4 and importin- α 7 proteins were involved in the nuclear import of XPA. We demonstrated that importin- α 4 and importin- α 7 directly interacted with XPA, but in cells, the interaction of XPA with importin- α 4 was UV-induced and dependent on ATR. In contrast, the proposed GTPase XAB1 was not required for the DNA damage-induced nuclear import of XPA. We conclude that importin- α 4 is the transport adaptor for the UV-induced XPA nuclear import (Figure 4-6).

The observation that the nuclear import of XPA depended on its NLS in cells excludes the possibility that XPA was co-imported with other proteins containing a NLS, or imported by “alternative import mechanisms” (more than one mechanism) (136). This also suggests that the same NLS was utilized by different adaptors, importin- α 4 and/or importin- α 7, for transporting of XPA through the NPC. Interestingly, the binding of importin- α 4 was largely induced by UV-

irradiation ½ hour after exposure (Figure 4-3A, left panel). This suggests that the efficient nuclear import of XPA may occur as early as ½ hour post-UV-irradiation, consistent with the previous report (74). In contrast, substantial XPA-importin- α 7 interaction was observed in the absence of DNA damage and, thus, the DNA damage appeared to have no effect on the interaction (Figure 4-3A, right panel). These results indicate that although both importin- α 4 and/or importin- α 7 were required for XPA nuclear import, the requirement of importin- α 4 was DNA damage-dependent while that of importin- α 7 was not. Given that a small portion of XPA was present in the nucleus even without DNA damage, a possible scenario is that importin- α 7 could be involved in the nuclear import of XPA independent of DNA damage (Figure 4-S1D), while importin- α 4 participated in the damage-dependent import of XPA.

In addition, it has been shown previously that the DNA damage-induced nuclear import of XPA was cell cycle dependent, primarily occurring in S phase (135). In G2-phase cells, XPA was localized to the nucleus regardless of DNA damage (135). Thus, it is possible that importin- α 7 is mainly responsible for nuclear import of XPA in G2 phase, while importin- α 4 for S phase. This also may provide a possible explanation for the observation that knockdown of importin- α 7 had less effect on the XPA nuclear import because the percentage of G2 cells in an unsynchronized cell population is only about 15-20%.

It previously was reported that the DNA damage checkpoint protein kinase ATR was involved in the regulation of the UV-induced XPA nuclear import (74). Interestingly, here we showed that the UV-induced binding of importin- α 4 to XPA was also ATR dependent (Figure 4-3C). Although ATR phosphorylates XPA at Ser196 in cells in responses to UV damage (73), we found that abolishing the phosphorylation of XPA had no effect on XPA-importin- α 4 binding

(data not shown). This suggests that the dependence of the XPA-importin- α 4 binding on ATR may occur via ATR regulation of other cytoplasmic factors involved in binding (74, 135).

The NLS of XPA could not be efficiently recognized by importin- α 4 in the absence of UV-irradiation (Figure 4-3A), likely due to the masking of the XPA NLS by the binding of other cytoplasmic factors. Elucidation of the details, though, beyond the scope of this study, merits future investigation (187, 189). Finally, the protein XAB1 is a GTP-binding protein that was identified as an interacting partner with XPA in a yeast two-hybrid system, and was proposed to be the GTPase involved in XPA nuclear import (172). However, our result showed that siRNA knockdown of XAB1 had no effect on the UV-induced nuclear import of XPA (Figure 4-5). This inconsistency could be due to the different environments within human cells and the yeast model system. Our data support that knockdown of XAB1 alone does not affect nuclear import of XPA in human cells.

Also, it is worth noting the reports of immunofluorescence microscopic analyses that showed that XPA was fully localized to the nucleus even in the absence of DNA damage (184, 191, 192). Given that these reports exclusively focused on images of individual cells transfected with XPA-expression constructs with or without GFP tags, the observations may not be inconsistent with the results of this study since XPA subcellular localization in unstressed cells is cell cycle dependent (135). Indeed we previously reported that XPA was predominantly localized to the nucleus in unstressed G₂-phase cells (135). Though immunofluorescence images may accurately depict XPA distribution within an individual cell they do not reflect the subcellular localization of XPA within a population of cells as do our biochemical fractionation studies which represent the average XPA distribution within millions of cells (74, 135, 138). Also, in general G₂ phase cells present the sharpest images of XPA location since the protein

appears more concentrated within the smaller, defined nuclear structure. In addition, we recently found that the same monoclonal anti-XPA antibody yields distinctly different XPA distribution patterns depending on the method of cell fixation. XPA is predominately located in the cytoplasm of undamaged cells fixed with methanol; in contrast, formaldehyde fixation resulted in a predominately nuclear localization in undamaged cells (data not shown). Apparently, the fixation method affects the antigen display with methanol revealing the same antigens as seen in the western blots of SDS-denatured proteins. Formaldehyde rather than methanol was employed in the reports of a nucleus-only location for XPA (184, 191, 192). Even the basal level of nuclear XPA in the fractionation assays may vary somewhat between experiments, likely due to culture variations affecting the proportion of cells in the G₂ phase (135, 138). However, we observed a 2.8-fold average increase in the XPA and importin- α 4 interaction after UV irradiation in at least three independent experiments. Finally it is unknown if the GFP tag could have any effects on XPA in cytoplasm.

Due to its indispensable role in human NER, including both global genome and transcription-coupled NER subpathways, XPA may serve as a potential target for sensitization to cancer chemotherapy (e.g. cisplatin) via manipulation of available nuclear XPA, either at the transcriptional or posttranscriptional level (73, 78-80, 123). Indeed, we previously showed that cisplatin, like UV-irradiation, induced XPA nuclear import (74, 138). Furthermore, recent studies indicate that targeting protein-trafficking pathways altered the sensitivity of melanoma to chemotherapy (137). The results from this study suggest that targeted disruption of the XPA-importin- α 4 complex could be a potential strategy to reduce the nuclear level of XPA given that NER occurs exclusively in the nucleus. Since the DNA damage-induced nuclear import of XPA

occurs primarily in S phase (135), the inhibition is expected to be specific to replicative cells, typically cancer cells, without interruption of XPA functions in cells in other cell cycle phases.

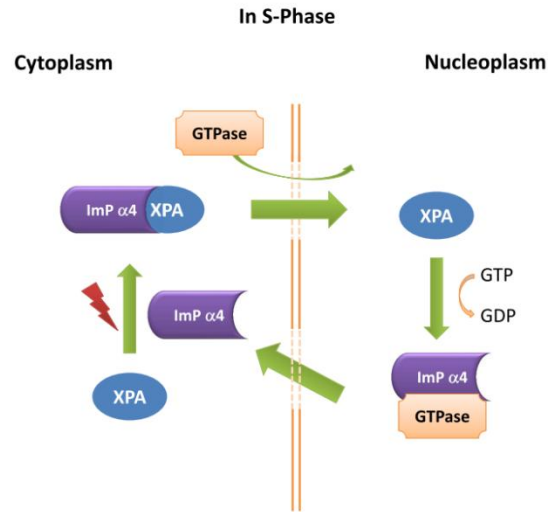


Figure 4-6. Proposed mechanisms for the UV-induced XPA nuclear import. Imp α 4 stands for importin- α 4.

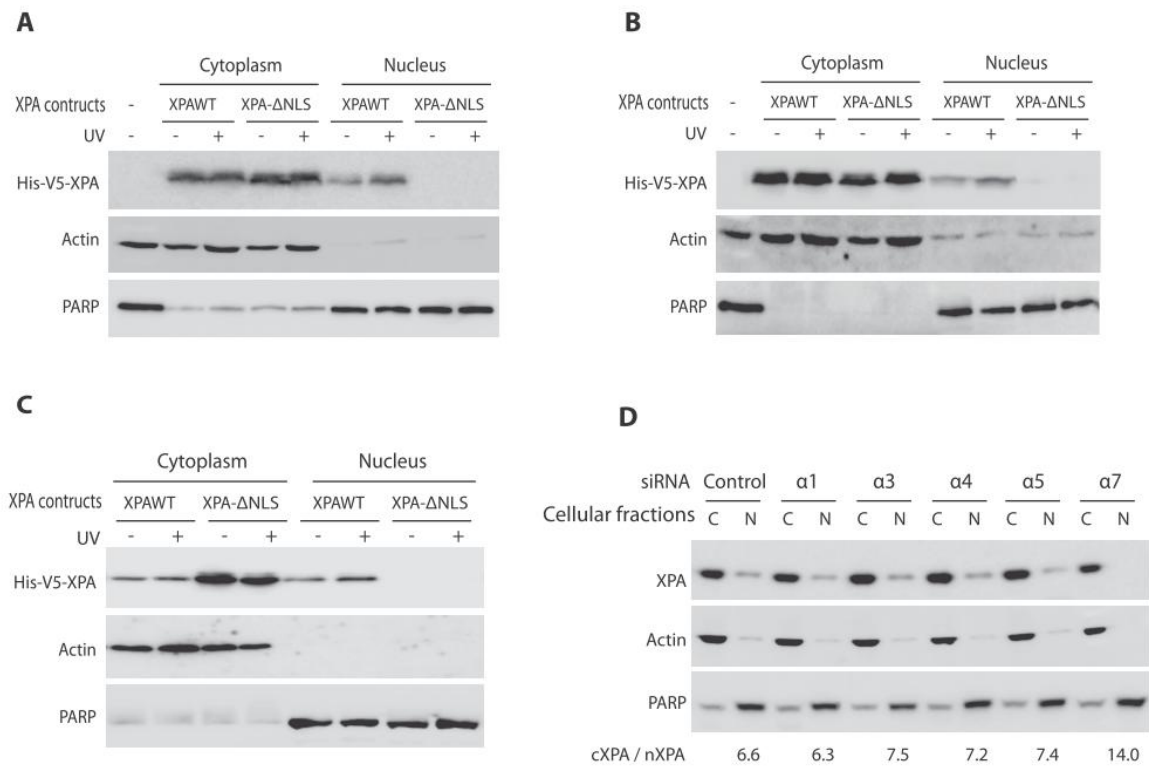


Figure 4-S1. Requirement of NLS for XPA nuclear import in different types of cells

A-C, The same XPA constructs as in Figure 4-1B were transiently transfected into human HEK 293T and GM02249 cells, as well as human primary fibroblasts BJ cells. Cells were mock or 20 J/m² of UV-C irradiated and allowed to recover for 2 hrs. Fractionation and western blotting assessed the subcellular localizations of XPA molecules. D, Effects of siRNA knockdown of importin- α proteins on XPA nuclear import in the absence of UV: H1299 cells were transfected with indicated siRNAs. At 48-hours post transfection, cellular fractions (C for cytoplasm, N for nucleus) were collected and western blotting was employed to assess the subcellular localizations of XPA.

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CHAPTER 5

DIFFERENTIAL DNA DAMAGE RESPONSES IN P53 PROFICIENT AND DEFICIENT CELLS: CISPLATIN-INDUCED NUCLEAR IMPORT OF XPA IS INDEPENDENT ON ATR CHECKPOINT IN P53-DEFICIENT LUNG CANCER CELLS (138)

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Abstract

Nucleotide excision repair (NER) and ataxia telangiectasia mutated (ATM)/ATR (ATM- and RAD3-related) DNA damage checkpoints are among the major pathways that affect the chemotherapeutic efficiency of anticancer drug cisplatin. *Xeroderma pigmentosum* group A (XPA) plays a crucial role in NER including both global genome repair (GG-NER) and transcription-coupled repair (TC-NER) subpathways, and has been a potential target for improving cisplatin therapeutic effects. We report here that XPA translocates from the cytosol into nucleus after DNA damage induced by UV irradiation and cisplatin, a mimetic of UV damage, in human cells with or without p53 deficiency. However, the damage response of XPA nuclear import was significantly slower in p53 deficient cells than in p53 proficient cells. The cells examined include the normal fibroblasts BJ, the human lung adenocarcinoma cells A549, and the p53-deficient non-small cell lung carcinoma cells H1299. We also found that while XPA is imported into nucleus upon cisplatin or UV damage in an ATR-dependent manner in p53-proficient BJ and A549 cells, the ATR checkpoint pathway has no effect on the XPA nuclear import in p53-deficient cancer cells H1299. Similarly, the XPA nuclear translocation is not

regulated by ATM checkpoint or p38MAPK/MK2 either. Our findings suggest that NER is independent on the major DNA damage checkpoint pathways in H1299 (p53^{-/-}) cells and that DNA damage responses are mechanistically different between p53-proficient and p53-deficient cells. Our results also highlight the possibility of selectively targeting XPA nuclear import as a way to sensitize cisplatin anticancer activity, but targeting ATR/ATM-dependent checkpoints may not be helpful in killing p53-deficient cancer cells.

Introduction

Chemotherapy is a critical clinical intervention for cancer patients. Cisplatin is one of the three most commonly used chemotherapeutic drugs (140). Cisplatin induces DNA intra- and inter-strand diadducts and DNA-protein crosslinks (142), which are the main cause of its cytotoxicity and hence its anti-cancer therapeutic effects. DNA repair of cisplatin-induced DNA damage is a major factor in modulating the therapeutic efficacy of cisplatin (141, 142). In humans, bulky DNA lesions produced by ultraviolet (UV) irradiation or by UV-mimetic agents such as cisplatin can be removed by nucleotide excision repair (NER) (7, 79). Therefore, the status of NER is an important factor in the success of chemotherapy using cisplatin (142, 143).

The DNA repair protein *xeroderma pigmentosum* group A (XPA) is an indispensable factor for NER including both subpathways: transcription-coupled NER (TC-NER) and global genome NER (GG-NER). XPA is believed to verify the damage sites following initial recognition of a lesion, stabilize repair intermediates and recruit other NER factors, and be involved in recruiting other NER factors (124, 127-130, 193). To our knowledge, XPA has been consistently reported to be the major factor that limits the repair of cisplatin-induced DNA

damage (79, 194, 195). Regulating the XPA at transcriptional or posttranscriptional level would affect the NER activity and repair of UV- or cisplatin-induced DNA lesions (78, 79, 194, 195).

The DNA damage checkpoints survey the structural integrity of the genome and coordinate multiple cellular pathways to ensure efficient removal of DNA damage. The ATM- and ATR-dependent checkpoint pathways are two major central components of the DNA damage response machineries in human cells. These pathways are comprised of a series of DNA damage sensors, signal mediators and transducers, and downstream effector molecules (7, 13, 14).

Among the downstream effectors of the ATR checkpoint, are three major checkpoint proteins of Chk1, p53 and p38 MARK/MAPK-activated protein kinase-2 (MK2) that can be directly or indirectly activated by ATR following UV irradiation (19, 152, 153) although p38MARK/MK2 can be also activated independent of ATR (19).

It is believed that the checkpoint pathways play an important role in regulation of NER processes (14, 15, 73-75). However, the underlying mechanisms remain elusive. Our previous studies indicated that the ATR checkpoint is required for regulation of DNA damage-induced XPA nuclear import and phosphorylation (73, 74). When the XPA nuclear translocation process is inhibited by disrupting the ATR-XPA interaction, DNA repair efficiency is significantly reduced (134). Our recent data (submitted elsewhere) also suggests that UV-induced XPA nuclear import is regulated by ATR through a p53 signaling pathway. Given that UV irradiation- and cisplatin-generating DNA lesions are repaired by NER in humans, and p53-deficient cancer cells rely on the p38MAPK/MK2 pathway for survival of DNA damages rather than the ATR/p53 signaling pathway (19, 139), it is of interest to determine whether the DNA damage-induced XPA nuclear import occurs in p53 deficient cancer cells and whether the import is regulated by different checkpoint pathways in p53-proficient and deficient cancer cells.

Materials and Methods

Cell Culture, Drugs, and Antibodies

Cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. All cell lines were grown at 37°C in 5% CO₂. UV-C irradiation was performed using a 254 nm lamp at a fluence of 0.83 J/m²/sec. For time course analysis cells were further incubated at in culture conditions for the indicated amounts of time. Cisplatin were purchased from Sigma Chemical Co. and dissolved in 0.9% NaCl to make a 3 mM stock solution just before use. A final concentration of 30 uM was obtained by making a 1/100 dilution of the stock solution into culture medium. UCN-01 and SB203580 were purchased from Sigma Chemical Co. and Calbiochem Chemical Co., respectively. Both UCN-01 and SB203580 were dissolved in DMSO to make stock solutions of 1 mM and 10 mM which were diluted into cell culture medium at 250 nM and 10 μM, respectively. For western blotting, primary rabbit polyclonal antibody against XPA, mouse monoclonal antibody against PARP, rabbit polyclonal antibody against p53, mouse monoclonal antibody against Chk1, and goat anti-MK2 polyclonal antibodies were purchased from Santa Cruz Biotechnology Co. A FITC-conjugated primary mouse anti-actin antibody was obtained from Sigma Chemical Co.

RNAi

siRNA duplexes were synthesized by GenePharma Co. using the following sequences: MK2 siRNA: sense strand 5'-UGACCAUCACCGAGUUUAUdTdT-3' and antisense strand 5'-AUA AACUCGGUGAUGGUCAdTdT-3'; Chk1 siRNA: sense strand 5'-ACAGUAUUUCGGUAUAAUAdTdT-3' and antisense strand 5'-UAUUAUACCGAAAUACUGUTGdTdT-3'; ATR siRNA: sense strand 5'-

CCUCCGUGAUGUUGCUUGAdTdT-3', and antisense strand 5'-UCAAGCAACAUCACGGAGGdTdT-3'; ATM siRNA: sense strand 5'-CAUACUACUCAAGACAUdTdT-3', and antisense strand 5'-AAUGUCUUUGAGUAGUAUGdTdT-3'. The siRNA transfection reagent was purchased from Polyplus-transfection and the transfections were carried out by following manufacturer's instructions. Briefly, cells were grown to 30-40% confluency and washed with PBS and antibiotic-free medium. siRNA duplexes were added to a small volume of FBS and antibiotic-free medium and incubated with transfection reagent for 10 min. This mixture then was added to cells with FBS and antibiotic-free medium at a final siRNA concentration between 5-10 nM. After a 5-7 hour incubation, FBS and antibiotic were added into the transfection medium and incubation continued. Experiments accessing the levels of protein expression or the effects of UV irradiation were initiated 48 or 72 hrs after transfection. For time course experiments after UV irradiation, siRNA-containing medium was temporarily removed during the UV irradiation.

Subcellular Fractionation and Western Blotting

Subcellular fractionation was performed using the Proteo JET™ cytoplasmic and nuclear protein extraction kit (Fermentas) by following the procedures suggested by the manufacturer. Briefly, 10 volumes of cell lysis buffer with protease inhibitors were added to 1 volume of packed cells. After a brief vortexing and incubation on ice for 10 min, cytoplasm was separated from nuclei by centrifugation at 500 x g for 7 min at 4 °C. Isolated nuclei were washed once or twice with 500 µL of the nuclei washing buffer and then collected by centrifugation. The collected nuclear pellets were re-suspended in ice-cold nuclear storage buffer, and 1/10 volume of the nuclear lysis reagents was added. The nuclei were lysed with shaking for 15 min at 4°C. Nuclear lysates were collected by centrifugation at 20,000 x g for 12 min at 4 °C. The lysates

were then mixed with 2x SDS loading buffer, boiled for 10 minutes and loaded into SDS-PAGE for Western blot analysis. In all of the fractionation experiments, protein levels of β -actin and PARP were assessed as cytoplasmic and nuclear protein loading controls, respectively.

Results

XPA Translocates into the Nucleus From the Cytosol Upon Cisplatin- and UV-DNA Damage

Consistent with previous observations (74, 134), the current data demonstrated that UV irradiation induced nuclear import of XPA in the normal foreskin primary cell line BJ, the human lung adenocarcinoma cell line A549, and a p53-deficient non-small cell lung carcinoma cell line H1299 (Figure 5-1A). Cisplatin is considered a mimetic of UV in inducing DNA damage that is exclusively repaired by NER. XPA is an indispensable factor of NER. To determine whether the nuclear import of XPA is part of the cellular response to cisplatin-induced DNA damage, BJ, A549 (wild-type p53) and H1299 (p53^{-/-}) cells were incubated with cisplatin for indicated time periods (Figure 5-1B). The nuclear concentration of XPA started to increase no later than 4 hrs after cisplatin addition in BJ and A549 cells while to decrease in the cytosol. The nuclear translocation of XPA in H1299 cells occurred later than in BJ and A549 cells; however, significant nuclear import of XPA was observed after 8 hrs of incubation with cisplatin. With 20 hrs of exposure to cisplatin cytoplasmic XPA protein started to recover to pre-exposure levels in A549 and BJ cells, even though the DNA-damage induced nuclear accumulation of XPA continued to the 24-hour time point. Different from BJ and A549 cells, the increasing of cytoplasmic XPA protein started after 24 hours of incubation with cisplatin, although a similar increasing of the nuclear XPA was observed. In agreement with the previous reports (167, 196),

cisplatin also induced overexpression and nuclear accumulation of p53 in A549 and BJ cells (Figure 1B).

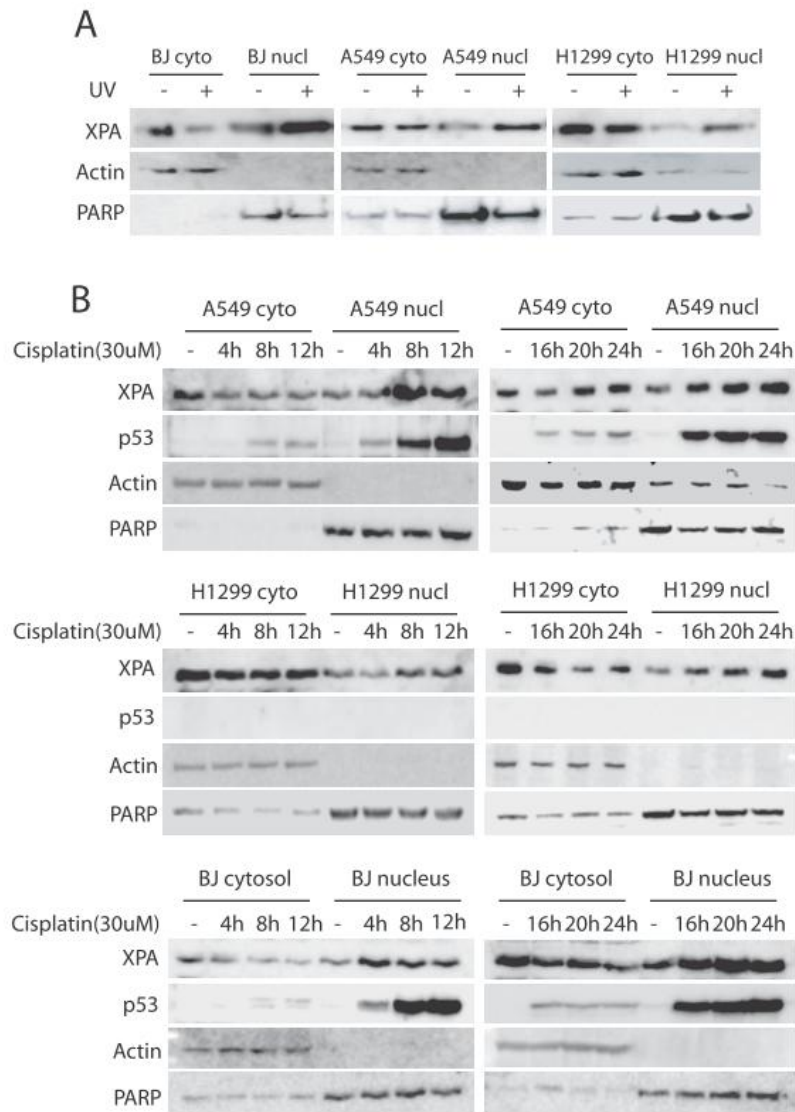


Figure 5-1. UV-C irradiation- and cisplatin-induced DNA damage induce nuclear import of XPA A, the normal foreskin cell line BJ, human lung adenocarcinoma epithelial cell line A549, and p53-deficient non-small cell lung carcinoma cell line H1299 were subjected to 20 J/m² UV-C irradiated followed by a 2-hr recovery. Fractionation and western blotting were performed to assess the subcellular localization of XPA. B, the chemotherapeutic agent cisplatin also induced cytoplasmic-to-nuclear translocation of XPA. Normal primary cell line BJ, lung cancer cell line A549 and H1299 were incubated with DMSO (mock treatment) or 30 uM cisplatin for indicated time points. Fractionation and western blotting were performed to assess the expression and

intracellular localization of XPA. PARP and β -actin were assessed to check nuclear and cytoplasmic protein loading, respectively.

Neither Chk1 nor MK2 is Required for Damage-induced XPA Nuclear Import

Our recent study demonstrated that UV-induced XPA nuclear import is dependent on ATR/p53 signaling pathway in the p53-proficient cancer cells. However, the data in Figure 5-1 showed no defects of XPA nuclear import in p53-deficient cancer cells H1299. Since both MK2 and Chk1 kinase can transmit damage signals from ATR independent of p53 to arrest cell cycle progression, it of interest to determine whether Chk1 and/or MK2 are required for the UV-induced nuclear import of XPA in H1299 cells. As shown in Figure 5-2A, no difference in XPA nuclear translocation was observed between the control siRNA and the Chk1 silencing cells, indicating that Chk1-mediated signaling is not required for the nuclear import of XPA. Similar result was also obtained for cells with MK2 knockdown by siRNA (Figure 5-2B). These results were further confirmed using two selective kinases inhibitors, UCN-01 and SB203580. UCN-01 inhibits both Chk1 and MK2 activities while SB203580 mainly targets the p38/MK2 pathways. These inhibitors were incubated with H1299 cells to stop the signal transductions mediated by Chk1 and/or MK2. Consistent with the siRNA knockdown results, inhibition of the two kinases did not change the UV-induced XPA nuclear import (Figure 5-2C).

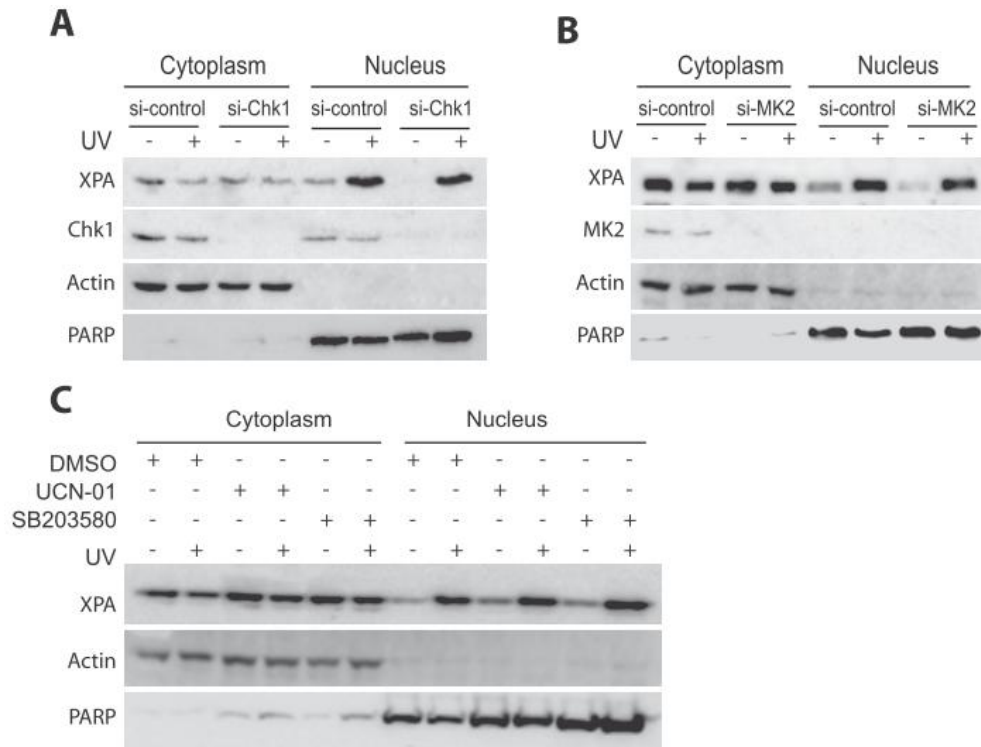


Figure 5-2. The DNA damage-induced XPA nuclear import is transmitted by unknown alternative pathways in the p53-deficient lung carcinoma cell H1299: Chk1 and/or MK2 are not required. A, knockdown of ATR downstream checkpoint Chk1 has no effects on the XPA nuclear accumulation. H1299 cells were transfected with control siRNA or Chk1 siRNA. After 48hrs, transfected cells were mock or 20 J/m² UV-C treated and further incubated for 2 hr to let the cells recover. The subcellular location of XPA was assessed with fractionation and western blotting. B, transient knockdown of MK2 kinase had no effects on the XPA nuclear translocation in the H1299 cells. Control siRNA or MK2 siRNA treated H1299 cells were mock-treated or irradiated with 20 J/m² UV-C followed by a 2-hr recovery. Fractionation and western blotting were employed to analyze the location of XPA and the expression of MK2. C, A549 cells were pretreated with DMSO, 250 nM of UCN-01 or 10 uM of SB203580 for 1hr in culture. Cells were then mock-treated or irradiated with 20 J/m² UV-C followed by a 2 hr recovery in the presence of the inhibitors. Cell fractionation and western blotting were performed to assess the translocation of XPA following UV-C irradiation.

ATR and/or ATM are not Required for Damage-Induced XPA Nuclear Import

The observation that Chk1 and MK2 were not required for damage-induced XPA nuclear import in H1299 (p53^{-/-}) cells implies that ATR may not be involved in regulation of nucleotide excision repair in p53 deficient H1299 cells. To test the hypothesis, ATR and ATM were depleted in H1299 cells. As shown in Figure 5-3, ATR and ATM were depleted by siRNA transfections individually (Figure 5-3A), or simultaneously (Figure 5-3B). However, the UV-induced cytosol-to-nucleus translocation of XPA was not affected, as indicated by the equal amount of decreasing XPA in cytosol and increasing XPA in the nucleus between the control siRNA- and ATR/ATM siRNA-transfected cells. The result indicates that neither ATR nor ATM was required for the damage-induced XPA nuclear import.

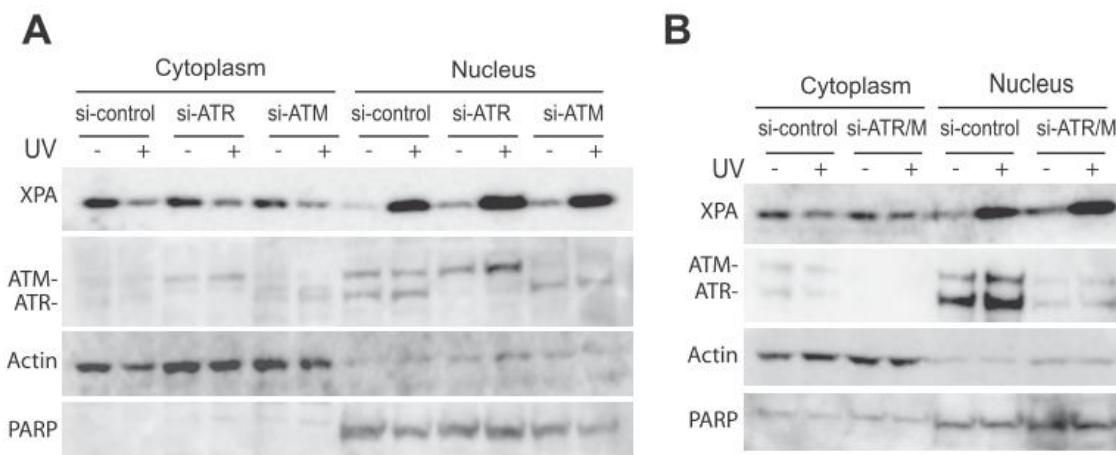


Figure 5-3. UV-induced XPA nuclear import is not regulated by ATR and/or ATM in the p53-deficient lung carcinoma cell (H1299)

A, silencing of the major cell cycle checkpoints ATR or ATM did not affect the UV-induced XPA import. In the control siRNA, ATR siRNA, and ATM siRNA transfected cells, UV-induced nuclear translocation of XPA was assessed using cell fractionation followed western blotting, after mock or 20 J/m² of UV-C irradiation and a 2-hr recovery period. B, ATR and ATM were simultaneously depleted using transient siRNA transfections. Fractionation and western blotting

assay were employed to assess the UV-induced nuclear import of XPA after 2 hr recovering from mock or a 20 J/m² UV-C irradiation.

Discussion

DNA damage checkpoints and DNA repair are two major types of cellular DNA damage response pathways and a close coordination between these two pathways is believed to be crucial for maintaining the genome integrity and stability in cells. Our recent studies indicated that ATR-dependent checkpoint pathway is required for regulation of the DNA damage-induced XPA translocation from cytoplasm to nucleus (74, 134). Interestingly, the results from this study indicate that although this ATR dependence occurs in p53-proficient normal and cancer cells treated with cisplatin or UV, it does not happens in p53-deficient lung cancer cells such as H1299 cells. This was further confirmed by the independence of XPA nuclear import on Chk1 and p83MARK/MK2, two major downstream substrates of ATR checkpoint signaling. Given the fact that XPA is a crucial factor of NER, XPA-deficient cells exhibit the highest UV sensitivity among other NER factors (122), and XPA nuclear import is essential for the activity of NER(74, 134), our findings suggest that NER is independent of ATR-dependent checkpoint in the p53-deficient cancer cells. In addition, XPA nuclear import, thus likely NER, was also found independent of ATM checkpoint in the p53-deficient cancer cells. This implies that all the well-known major DNA damage checkpoints appear to have no effect on NER in the p53-deficient cancer cells. In spite of this, the fact that XPA nuclear import is a DNA damage-induced event indicates that it is a regulated event in cells. We propose that an alternative checkpoint pathway may be responsible for the regulation. Taken together, our results suggest that the cellular DNA damage responses to cisplatin treatments are different in p53 proficient (A549) and deficient (H1299) cells.

One of the major challenges in treating cancer patients with cisplatin is the drug's side-effects: it introduces DNA damage to cancer cells while also causing damage to normal cells (140). One solution for this problem is to identify mechanistic differences of DNA damage responses between normal and cancer cells. In the current study, effort was made to define the unique mechanism of DNA damage responses in p53-deficient lung cancer cells as p53 is the most commonly mutated gene in human cancers, particularly the lung cancer. Thus three cell lines including the human normal primary cell line BJ, the p53-proficient lung cancer cell line A549, and the p53-deficient lung cancer cell line H1299 were treated with cisplatin or UV-C irradiation. A pronounced cytoplasm-to-nucleus translocation of XPA was observed in each of these cell lines (Figure 5-1), which is consistent with our previous observations on other types of cells treated with UV. Importantly, however, different from p53-proficient cells the damage-induced XPA import is not dependent on ATR or other major known checkpoints in the p53-deficient lung cancer cells-H1299 (Figure 5-2 and Figure 5-3). These observations reveal a mechanistic difference of DNA damage responses between p53-proficient and p53-deficient lung cancer cells. Evidently, identifying the novel regulation mechanism responsible for the damage-induced XPA import in the p53-deficient cancer cells deserves further investigation in the future.

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CHAPTER 6

CYTOPLASMIC ATR LACKING CHECKPOINT KINASE ACTIVITY IS A BAX-INHIBITING ANTIAPOPTOSIS PROTEIN AT MITOCHONDRIA

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Abstract

ATM and ATR are the two major DNA damage checkpoint kinases. Intriguingly, unlike ATM whose deficiency promotes carcinogenesis, inhibition of ATR has been shown to suppress UV carcinogenesis in mice. We hypothesized that ATR might contain an antiapoptotic activity unrelated to its checkpoint function. Indeed, here we show that besides its nuclear checkpoint functions, ATR in the cytoplasm is an antiapoptotic protein. Upon UV damage, cytoplasmic ATR translocates to mitochondria, blocking the recruitment of proapoptotic Bcl-2-associated X (Bax) protein to mitochondria and preventing the loss of mitochondrial membrane potential ($\Delta\Psi$) and apoptosis. Consistently, Bax-depletion significantly reduced the effect of ATR on $\Delta\Psi$. Remarkably, the cytoplasmic ATR exhibits no checkpoint kinase activity, a hallmark function of nuclear ATR. Inhibition of ATR's kinase activity or silencing of ATRIP and Chk1 failed to affect Bax relocalization to mitochondria. Furthermore, knocking down p53 did not change the role of ATR in mitochondrial $\Delta\Psi$, indicating that the antiapoptotic role of ATR is independent of the proapoptotic p53. Our results reveal a novel checkpoint-independent antiapoptotic function of ATR at mitochondria in the cellular response to UV damage.

Introduction

The human genome is constantly exposed to endogenous and exogenous DNA-damaging agents which may cause genomic instability and cancer. To counter these threats, cells have evolved multiple systems to signal DNA damage and to facilitate repair (1, 3, 5). Such responses include DNA damage checkpoints, activation of transcriptional programs, DNA repair and apoptosis. As the two major DNA damage checkpoint kinases from the phosphoinositide 3-kinase-related protein kinases (PIKK) family, ATM (ataxia telangiectasia mutated) and ATR (ATM and Rad3-related) sense DNA damage, activate checkpoints, arrest cell cycle progression and facilitate DNA repair to restore DNA integrity (1, 7, 9, 10). Alternatively, apoptosis is activated to eliminate damaged cells if DNA damage is too severe (8, 11). ATM is primarily activated by DNA double-strand breaks (DSBs), while ATR is activated by recognizing the stretches of replication protein A (RPA)-coated single-stranded DNA (ssDNA) generated from DNA damage-induced replication stress or during the repair of certain types of DNA damage (9, 10, 21). TopBP1 binds to the surface of the ATR–ATRIP (ATR-interacting protein) complex to activate ATR (63, 197). In addition to their specificities for different types of DNA insults, ATM and ATR also demonstrate coordinate and overlapping functions in DNA damage responses (DDR) (198, 199).

Given the critical roles of ATM and ATR in DDR, it is believed that deficiency of ATM- or ATR-dependent checkpoint pathways may lead to genome instability and, thus, to cancer (198, 200). Indeed, *ataxia telangiectasia* (AT) patients have a high propensity for cancer, and knockout of ATM and/or BRCA1 promoted cancer (201-204). In contrast to ATM, ATR is essential for viability of mice and the cells from ATR-knockout mice died of apoptosis (25, 85, 100). Furthermore, inhibition of ATR enhanced apoptosis through a p53-independent mechanism

(58, 101-105) regardless of ATR's kinase activation in some cancer cells (105), and suppressed UV-induced carcinogenesis (102, 103). Also interestingly, a body of evidence from human epidemiologic and mouse model studies has demonstrated that caffeine consumption significantly decreased the risk of cancer (24, 106-116). Notably, caffeine is an inhibitor of cell cycle checkpoint pathways (111), but the inhibition may be independent of the kinase activities of ATM or ATR (117). Together the current evidence suggests that unlike ATM, ATR may contain an antiapoptotic activity and inhibition of ATR suppresses carcinogenesis. However, the molecular mechanism remains unknown. Given the potential antitumor activity of ATR inhibition, ATR has drawn attention as a promising target for anticancer chemotherapy, further underscoring the importance for understanding the mechanism.

Since all its currently known functions, particularly as a checkpoint regulator, occur in the nucleus, ATR has been considered as a nuclear protein. However, the p53-independent apoptotic activity triggered by inhibition of ATR does not appear consistent with its role in cell cycle checkpoint regulation since loss of checkpoints leads to uncontrolled cell growth, genome instability or a p53-dependent DNA damage-induced apoptosis. We thus reasoned that ATR may contain a checkpoint-independent antiapoptotic function. To test this hypothesis, we investigated the cytoplasmic functions of ATR. We found that cytoplasmic ATR was an antiapoptotic protein which bound to mitochondria upon UV damage and inhibited the UV-induced recruitment of Bax to mitochondria. Importantly, this antiapoptotic function of ATR also was independent of its checkpoint activities.

Materials and Methods

Cell Culture, UV irradiation, Drugs, and Antibodies

All cells except ATR^{+/+} and ATR^{flox/-} cells (a gracious gift from Dr. David Cortez, Vanderbilt University) were either purchased from American Type Culture Collection (Manassas, Virginia) or Coriell Cell Repositories (Camden, New Jersey). Cells were maintained in D-MEM supplemented with 10% FBS (SH3039603 from Thermo Scientific) and 1% penicillin-streptomycin, and grown at 37°C, 5% CO₂. UV-C irradiation was performed using a 254 nm lamp at a fluence of 0.83 J/m²/sec. Camptothecin (C9911) and hydroxyurea (H8627) were purchased from Sigma Chemical Co. Stock solutions of camptothecin (CPT) and hydroxyurea (HU) were made in DMSO and H₂O, respectively, before addition to cell culture medium. The ATR kinase inhibitor, NU6027, was purchased from EMD Millipore (189299) and was used at a final concentration of 10 μM in cell culture medium. For western blotting and immunofluorescence microscopy primary goat polyclonal antibodies against ATR (sc-1887), ATRIP (sc-33410), and heat shock protein 70 (HSP70) (sc-1060), mouse monoclonal antibodies against DNA-PK (sc-135886), p53 (sc-6243), ubiquitin (sc-166553) or PARP (sc-8007) were purchased from Santa Cruz Biotechnology Co. Mouse monoclonal antibody against mitochondria heat shock protein 70 (MHSP70) (MA3-028) was purchased from Thermo Scientific. Rabbit polyclonal antibodies against ATR (A300-137A) and ATM (A300-299A) were purchased from Bethyl Laboratories, Inc, and were used in most of the western blotting. Mouse monoclonal antibody against lamin A/C (4777), rabbit polyclonal antibodies against Chk1 phosphorylated at serine 345 (2348), and p53 phosphorylated at serine 15 (9284) were purchased from Cell Signaling Biotechnology Co. Rabbit polyclonal anti-sumo2/3 (ab81371) and anti-sumo1 (ab5316) antibodies were purchased from Abcam. Antibody kits that recognize Bcl-2

proapoptotic family proteins were purchased from Cell Signaling Biotechnology Co. (9942). To verify that the DNA damage-induced and antibody-detected cytoplasmic slower bands were truly ATR in our western blotting, a different ATR antibody was purchased from Cell Signaling Technology (2790) and used to detect ATR. A FITC-conjugated primary mouse anti-actin antibody was obtained from Sigma Chemical Co. The anti-actin, anti-HSP70, anti-PARP and anti-lamin A/C antibodies were used in western blots to confirm successful subcellular fractionations and to normalize protein loadings.

Immunoblotting

Cells were harvested by scraping or trypsin digestion, and re-suspended in lysis buffer (50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1x protease inhibitor cocktail [Roche]). 2X SDS loading buffer then was added to the lysates and the mixtures were heated at 100°C for 10 min to denature proteins. After heating the samples in SDS-PAGE; gradient gels of 3-8% acrylamide (Invitrogen) were used for maximum resolution of the slower migrating cytoplasmic ATR band. Proteins were transferred from the gels onto PVDF membranes. The membranes then were blocked with 5% nonfat milk and probed with specific primary and secondary antibodies. Chemiluminescent signal was captured using a Fuji Film camera, and western blot images were processed with Multi-Gauge 3.0 software.

RNAi and Plasmid Transfections

p53 siRNA duplexes were purchased from Santa Cruz (sc-29435). siRNA duplexes targeting other genes were synthesized by Genepharma using the following sequences: ATR siRNA, sense strand 5'-CCUCCGUGAUGUUGCUUGATT-3' and antisense strand 5'-UCAAGCAACAUCACGGAGGTT-3'; ATM siRNA, sense strand 5'-CAUACUACUCAAGACAUTT-3' and antisense strand 5'-

AAUGUCUUUGAGUAGUAUGTT-3'; ATRIP siRNA, sense strand 5'-
AGAGAAACUGUCCAAUATT-3' and antisense strand 5'-
UAAUUGGAACAGUUUCUCUTT-3'; Chk1 siRNA, sense strand 5'-
ACAGUAUUUCGGUAUAAUATT-3' and antisense strand 5'-
UAUUAUACCGAAAUACUGUTT-3'; Bax siRNA, sense strand 5'-
GACGAACUGGACAGUAACATT-3' and antisense strand 5'-
UGUUACUGUCCAGUUCGUCTT-3'. The siRNA transfection reagent was purchased from Polyplus Transfection and the transfections were performed as described previously. Typically, cells were harvested at 48- or 72-hour post-transfection. Plasmids for transient expression of human wild-type ATR were gifts from Dr. Stephen J. Elledge at Harvard Medical School. The transfection of ATR plasmids into HCT-116 ATR^{fllox/-} was done with jetPEITM transfection reagent (Polyplus) by following the company's instructions.

Cytoplasmic and Nuclear Protein Extraction

Subcellular fractionation was performed using the Proteo JETTM cytoplasmic and nuclear protein extraction kit (Fermentas) by following the manufacturer's procedure. Briefly, 10 volumes of cell lysis buffer (with 1x protease inhibitors) were added to 1 volume of packed cells. After a short vortexing and incubation on ice for 10 min, cytoplasm was separated from nuclei by centrifugation at 500xg for 7 min at 4°C. Isolated nuclei were washed once or twice with 500 µL of the nuclear washing buffer and then collected by centrifugation. The collected nuclear pellets were re-suspended in ice-cold nuclear storage buffer, and 1/10 volume of the nuclear lysis reagent was added to lyse nuclei with rotation for 15 min at 4 °C. The nuclear lysate was collected by centrifugation at 20,000xg for 15 min at 4 °C. In all of the fractionation experiments,

western blotting of β -actin and PARP were assessed to check the quality of fractionation and protein loading.

Mitochondria Isolation

Mitochondrial isolation was performed using several mitochondrial extraction kits (Qiagen and Thermo) according to the manufacturer's instructions. The Qiagen kit benefits from an additional density gradient purification step. Briefly, cells were suspended in lysis buffer to disrupt the plasma membrane. Plasma membranes and compartmentalized organelles, such as endoplasmic reticulum, mitochondria, and nuclei, remained intact and were collected by centrifugation at 1000xg for 10 min. The resulting pellet was resuspended in disruption buffer, repeatedly passed through a narrow-gauge needle (21 gauge), and re-centrifuged at 1000xg for 10 min to pellet nuclei, cell debris, and unbroken cells. The supernatant that contains mitochondria was re-centrifuged at 6000xg for 10 min to pellet mitochondria. After removal of the supernatant, mitochondria were lysed with 1X SDS loading buffer and analyzed by SDS-PAGE and western blotting.

Immunofluorescence Microscopy

For immunofluorescence microscopic detection of cellular proteins, cells were grown on coverslips before the initiation of experimental treatments. After UV-irradiation or treatments with chemical DNA damaging agents for indicated times, cells were fixed with 4% paraformaldehyde in PBS and blocked with 15% BSA in PBS for 1hr at room temperature. Proteins then were detected with primary antibodies and fluorescent-conjugated secondary antibodies (Invitrogen). Cells on coverslips were coated with prolong gold antifade reagent containing DAPI (Invitrogen) before microscopic examination using 100X magnification.

Mitochondrial Membrane Potential Assays

MitoTracker red CMXRos (M7512 from Life Sciences) is a red-fluorescent dye that stains mitochondria in live cells and its accumulation is dependent upon an intact mitochondrial membrane potential. The staining of live cells is done by following their instructions. Briefly, cells were seeded at a confluence of 50% one day ahead of the DNA damage treatments. To prepare a stock solution, MitoTracker red CMXRos was dissolved in DMSO to a final concentration of 1 mM in a darkened room and used immediately. Right before usage, this dye was diluted to 200 nM in fresh growth medium without FBS. After incubation for the desired time periods following DNA damage treatments, the media was removed and prewarmed (37°C) staining solution was added to the cells and incubated for 30 minutes in the dark. Then, both the adherent and non-adherent cells were harvested, washed with PBS, and analyzed in an Accuri C6 flow cytometer.

The JC-1 Mitochondrial Membrane Potential Assay Kit was purchased from Cayman Chemical. The procedure for labeling cells was done by following the company's instructions. The fluorescence of this dye was recorded by an Accuri C6 flow cytometer.

In Vitro Kinase Activity Assay

The *in vitro* kinase assay with ATR was performed as described previously. Briefly, endogenous ATR was immunoprecipitated from cytoplasmic or nuclear extracts of A549 cells irradiated with 40 J/m² UV-C with a 2-hr post-irradiation recovery period. The ATR captured on protein G beads (53128 from Thermo Scientific) was washed thrice with PBS containing 0.05% NP40, followed by one wash with kinase buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 10 mM MgCl₂, 10 mM MnCl₂, 2 mM DTT, 10% glycerol, 1X protease and phosphatase inhibitors, and 0.5 mM ATP]. Then, the immunoprecipitates were resuspended in 20 uL of kinase buffer

containing 10 uCi of [γ - 32 P] ATP and 0.5 μ g of human GST-p53 protein (P05-30BG from Signal Chem). The kinase reaction was incubated at 30 °C for 30 minutes and stopped by the addition of SDS loading buffer. Proteins were separated by SDS-PAGE and the radiolabeled proteins were visualized by gel scanning using a PhosphorImage scanner (Fuji, Stamford, CT). Immunoprecipitated endogenous ATR and the amount of GST-p53 in each sample were confirmed by western blotting.

Flow Cytometry-Based Apoptosis Assays

To measure the percentage of cells in the sub-G1 cell phase, propidium iodide solution was purchased from Sigma-Aldrich to stain nuclear DNA as described previously. Briefly, cells were grown in dishes to reach 70-80% confluence, trypsin-harvested, fixed with 70% ethanol at 4°C, and then pelleted and re-suspended in PBS containing 20 μ g/mL of propidium iodide and 100 U RNase for 30 minutes at 37°C. After staining 30,000 cells were counted using an Accuri C6 flow cytometer to measure the DNA content.

Apoptosis Analysis with Annexin V

Phosphatidylserine exposure on the outer leaflet of the plasma membrane was detected by the FITC-Annexin V apoptosis detection Kit II (Invitrogen V13242) according to the manufacturer's instructions. Briefly, cells were pelleted following treatment and washed with PBS. Cells were then re-suspended in binding buffer at 1×10^6 cells/ml, mixed with FITC-Annexin V and incubated at room temperature for 15 minutes in the dark. The Annexin V-positive cells were analyzed by flow cytometry.

Statistical Analysis

The statistical analysis of samples was performed with a two-tailed student's t-test, and a p-value of less than 0.05 was considered as significant.

Results

ATR Has a Role in Suppressing Mitochondria-Mediated Apoptosis Pathways

To determine whether cytoplasmic ATR has effects on mitochondria-mediated apoptosis induced by UV irradiation, mitochondrial membrane potential ($\Delta\Psi$) was measured in HCT-116 ATR^{+/+} cells and HCT-116 ATR-deficient cells (ATR^{fllox/-}) (72) (Figures 6-1A, 6-1B and 6-1C). Mitochondrial membrane potential reflects the pumping of hydrogen ions across the inner membrane during electron transport and oxidative phosphorylation. Loss of $\Delta\Psi$ is one of the events during apoptosis (205, 206). The loss of $\Delta\Psi$ leads to increased membrane permeability and the release of mitochondrial components such as cytochrome c (205, 206). To measure $\Delta\Psi$, fluorescent dyes MitoTracker red CMXRos and JC-1 were used to measure $\Delta\Psi$ integrity. These dyes passively diffuse across the plasma membrane and accumulate in active mitochondria, dependent upon the organelle's membrane potential. As shown in Figures 6-1A-1C, the ATR^{fllox/-} cells experienced a significantly more rapid loss of $\Delta\Psi$ than the ATR^{+/+} cells, especially for recovery times longer than 8 hours (Figures 6-1B and 6-1C). To confirm that the effect of ATR on mitochondrial $\Delta\Psi$ is part of apoptosis, apoptotic cells were measured by annexin V staining and by counting cells in sub-G1 phases (Figures 6-1D and 6-1E). Indeed, significantly more ATR^{fllox/-} cells went into apoptosis than the wild type cells. The role of ATR in regulating $\Delta\Psi$ was further demonstrated by the siRNA knockdown of ATR in A549 cells (Figure 6-1F). In contrast, knockdown of ATM had no significant effect on $\Delta\Psi$ as compared to the control in cells treated with either CPT (camptothecin) or UV (Figure 6-1F).

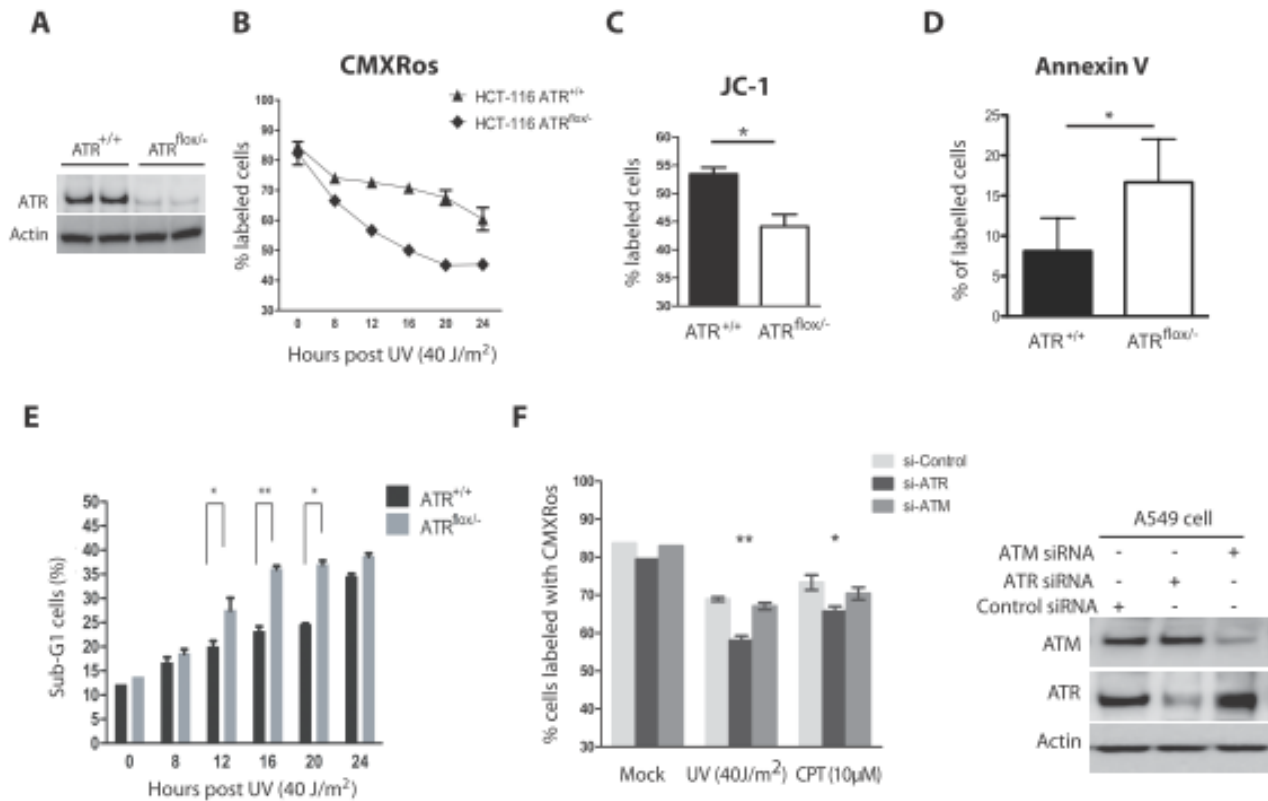


Figure 6-1. ATR deficiency/silencing lead to loss of mitochondrial membrane potential and promote apoptosis induced by DNA damage

A, Western blotting assessed the expression of ATR in the HCT-116 ATR^{+/+} vs. ATR^{flox/-} whole cell lysates. B, The HCT-116 and ATR^{flox/-} cells were mock- or UV-C irradiated, allowed to recover for the indicated time periods, labeled with MitoTracker red CMXRos and analyzed by flow cytometry ($\geq 30,000$ cells measured). The quantitative data in the plot are from three independent experiments. C, More of the HCT-116 ATR^{flox/-} cells lost mitochondrial $\Delta\Psi$ than the ATR^{+/+} cells as measured by flow cytometry analysis of JC-1 labeled cells 16-hours post-UV. Asterisk above bars indicates that a significant difference was found between the data from three independent experiments. D, Flow cytometer measurement of cells labeled with FITC-Annexin V in the HCT-116 ATR^{+/+} and ATR^{flox/-} cells (data generated by Moises Serrano); the values are an indicator of apoptotic cell death 16-hours post-UV. E, DNA content of each cell was measured by flow cytometer analysis at the indicated time points of UV recovery. Percentage of sub-G1 cells (with DNA content less than 2C) was calculated by dividing the number of cells in sub-G1 by the total number of propidium iodide positive cells. F, A549 cells were transiently transfected with control, ATR or ATM siRNAs, and then mock, UV or CPT treated. At 16 hours

post-induction, cells were labeled with MitoTracker red CMXRos and analyzed with flow cytometry. The asterisks means statistical differences were observed when compared with the control siRNA group (*, $p \leq 0.05$; **, $p \leq 0.01$). The western blot in the right panel is a confirmation of the silencing efficiency of the siRNAs in A549 cells at 48-hours post-transfections.

Since p53 is well-known for its role in regulating DNA damage-induced apoptosis, we next examined whether the role of ATR in maintaining $\Delta\Psi$ depends on p53. As shown in Figure 6-2, more ATR^{flox/-} cells lost $\Delta\Psi$ than the ATR^{+/+} cells with a p53 knockdown background (ATR^{+/+}, si-p53 cells versus ATR^{flox/-}, si-p53 cells) (Figure 6-2). In contrast, depletion of p53 in ATR^{+/+} cells (ATR^{+/+}, si-p53) significantly increased the number of cells with intact $\Delta\Psi$ as compared with control siRNA-transfected ATR^{+/+} cells (ATR^{+/+}, si-control) (Figure 6-2). This result is consistent with the well-established proapoptotic role of p53. Taken together, these results suggest that the antiapoptotic role of ATR is independent of p53.

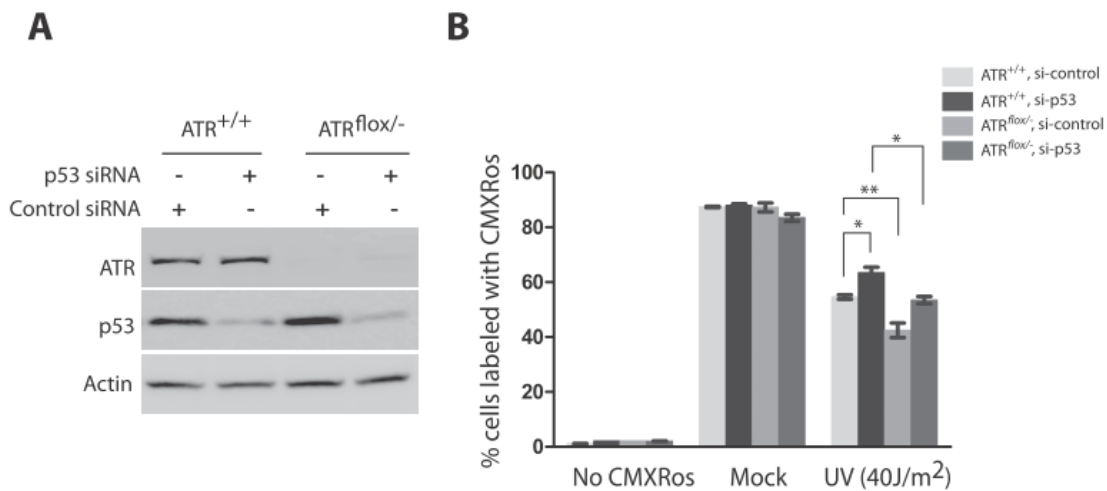


Figure 6-2. p53 is not required for the mitochondrial functions of ATR

A, HCT-116 ATR^{+/+} and ATR^{flox/-} cells were transfected with control siRNA or with p53 siRNA. The expression of ATR and the efficiency of p53 knockdown were confirmed by western blotting (left). B, At 16-hour post-irradiation, cells were labeled with MitoTracker red CMXRos

and assessed by flow cytometry. A horizontal bracket indicates that a statistic difference was observed between the two indicated groups ($*$, $p \leq 0.05$; $**$, $p \leq 0.01$). As expected, a significant reduction in the number of cells with intact $\Delta\Psi$ was found for $ATR^{lox/-}$ cells versus $ATR^{+/+}$ cells transfected with control siRNA, suggesting that ATR prevents cells from apoptosis. Similar results were obtained for cells with a p53 knockdown background ($ATR^{+/+}$, si-p53 versus $ATR^{lox/-}$, si-p53), indicating that the antiapoptotic role of ATR at mitochondria is independent of p53. In contrast, comparison between $ATR^{+/+}$ cells transfected with control siRNA ($ATR^{+/+}$, si-control) and p53 siRNA ($ATR^{+/+}$, si-p53) indicates that p53 depletion increased the number of cells with intact $\Delta\Psi$, suggesting that p53 promotes apoptosis following UV irradiation.

ATR Inhibits the UV-induced Bax-Localization to Mitochondria through a Kinase-Independent Mechanism

In order to determine the mechanisms for the ATR's role in regulating mitochondrial $\Delta\Psi$, we examined the potential involvement of Bcl-2 family proteins as well as p53, the central players in the regulation of mitochondrial membrane permeability and apoptosis (8, 11, 43, 44, 47). In addition to p53, these proteins included both proapoptotic and antiapoptotic proteins: Bax, Bcl-2-associated death promoter (Bad), BH3 interacting-domain death agonist (Bid), Bcl-2 homologous antagonist killer (Bak), Bcl-2-like protein 11 (Bim), B-cell lymphoma 2 (Bcl-2), and induced myeloid leukemia cell differentiation protein (Mcl-1). As shown in Figure 6-3A, the ATR deficiency resulted in a significant increase of Bax localization to mitochondria in response to DNA damage in comparison with the ATR-proficient cells. In contrast, the loss of ATR had no effect on mitochondrial localization of other factors including p53. The p53 protein also serves as a control in this determination: p53 was previously shown to localize to mitochondria to promote apoptosis (45, 47, 207). This DNA damage-induced mitochondrial translocation of p53 also was observed in Figure 6-3A.

Since ATR, a PIKK kinase, is an essential cell cycle checkpoint protein that phosphorylates hundreds of downstream proteins in responses to DNA damage (7, 60, 69), it is of great interest to determine whether the antiapoptotic activity of ATR at mitochondria is dependent on the kinase/checkpoint activity of ATR. Surprisingly, as shown in Figure 6-3B, inhibition of ATR kinase activity by the ATR kinase-specific inhibitor NU6027 did not lead to Bax-mitochondria translocation in ATR^{+/+} cells as it happened in ATR^{flox/-} cells. The inhibition of ATR kinase activity was confirmed by the attenuated phosphorylations of p53 at Ser15 and Chk1 at Ser345 in response to NU6027 treatment (Figure 6-3C). This result suggested a kinase-independent role of ATR as an antiapoptotic protein at mitochondria in response to DNA damage. Furthermore, since ATRIP plays an essential role in ATR checkpoint activation (21, 72), ATRIP was depleted by siRNA to inactivate ATR kinase activity (Figure 6-3E). Similarly, Chk1, the major kinase substrate of ATR, also was depleted by siRNA (Figure 6-3E). As shown in Figure 6-3D, cells depleted with ATRIP or Chk1 failed to promote the mitochondrial recruitment of Bax in contrast to silencing of ATR.

This kinase-independent role of ATR in mitochondria led to a question of whether the cytoplasmic ATR functions differently from the nuclear ATR as a kinase. To address this question, an *in vitro* ³²P-labeling assay was conducted to measure the kinase activity of cytoplasmic and nuclear ATR towards recombinant p53, a downstream kinase substrate of ATR (7, 69, 152). As shown in Figure 6-3F, the immunopurified cytoplasmic ATR from A549 cells with or without UV-irradiation failed to transfer the phosphate group to p53. In contrast, the immunopurified nuclear ATR phosphorylated p53 normally. These results suggest that the cytoplasmic ATR lacks the hallmark activity of ATR as a kinase. Also interestingly, we found that ATRIP is present almost exclusively in the nucleus and little in the cytoplasm (Figure 6-3G).

Silencing of ATRIP reduced the amount of nuclear ATR but did not affect the DNA damage-induced formation of cytoplasmic ATR (Figures 6-3E and 6-3G). Together these data indicate that the antiapoptotic function of ATR is independent of its checkpoint kinase activity, and implies that the cytoplasmic ATR may be responsible for the antiapoptotic function at mitochondria.

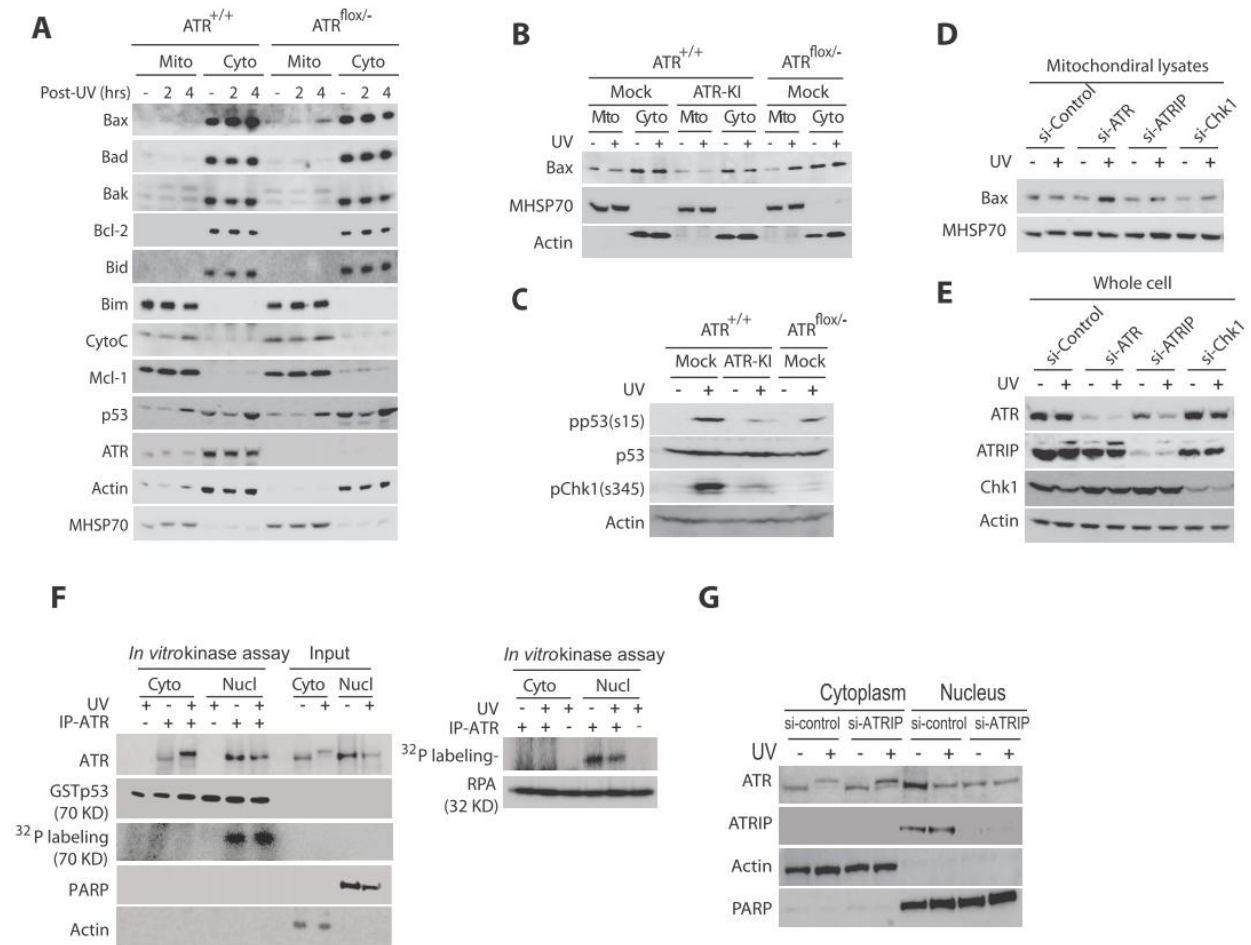


Figure 6-3. Cytoplasmic ATR which lacks checkpoint kinase activity inhibits the UV-induced Bax translocation to mitochondria

A, HCT-116 ATR^{+/+} and ATR^{flox/-} cells were mock or 40J/m² of UV-C treated and allowed to recover for the indicated times. Mitochondria (Mito) and cytosolic (Cyto) fractions were isolated from cytoplasm, loaded onto SDS-PAGE and apoptotic proteins were assessed by western blotting. MHSP70 and actin were checked for quality of the fractionation and for protein loading.

B, HCT-116 ATR^{+/+} and ATR^{fllox/-} cells were mock (DMSO) or ATR inhibitor (ATR-KI; 10 μM NU6027) treated for 1-hour before irradiation with 40 J/m² of UV and allowed to recover for 4 hours. Cytoplasm from each cell treatment was fractionated into mitochondria (Mito) or cytosol (Cyto) and the association of Bax with each fraction was monitored by western blotting. C, The same treatment of cells were performed as in B. Phosphorylation of p53 on serine 15 and Chk1 on serine 345 were monitored by western blotting to measure the checkpoint kinase activity of ATR. D, and E, HCT-116 ATR^{+/+} cells, 72-hours after transfection of ATR, ATRIP, or Chk1 siRNAs, were mock or 40 J/m² of UV irradiated and allowed a 4-hours recovery. The efficiency of the knockdowns is shown in E and the amount of Bax that was associated with isolated mitochondria is shown in D. F, A549 cells were mock- or UV-treated (40J/m²) followed by a 2-hour recovery. Cytoplasmic (Cyto) and nuclear lysates (Nucl) were collected as described in the methods, endogenous ATR was immunoprecipitated, and *in vitro* phosphorylation of exogenous GST-p53 or RPA was carried out to assess the checkpoint kinase activity of ATR (whole cytoplasmic ATR compared with one third of nuclear ATR) in the presence of [γ -³²P] ATP (middle panel). Western blotting confirmed the presence of ATR and equal loading of GST-p53 and RPA. Presented are representative data from at least three independent experiments. G, The occurrence of cytoplasmic ATR is independent of ATRIP. A549 cells were transfected with control or ATRIP siRNAs for 48 hours before mock or 40 J/m² UV irradiation and a 2-hour recovery. The cells then were separated into nuclear and cytoplasmic fractions for analysis of ATR and ATRIP protein distributions.

The Antiapoptotic Role of ATR Depends on Bax.

Upon DNA damage, Bax translocates from the cytosol to mitochondria as an active form to initiate apoptosis (208, 209). Active Bax forms lipid pores in the mitochondria membrane, leading to a drop of the mitochondria membrane potential (210-212). Our data suggested that ATR may regulate the mitochondrial translocation of Bax and, thus, the antiapoptotic functions of ATR would depend on Bax. To confirm this, ATR^{+/+} and ATR^{fllox/-} cells were transfected with scrambled or Bax siRNA (Figure 6-4A). Then, the capacity of ATR in maintaining an ATR-

dependent $\Delta\Psi$ (the $\Delta\Psi$ difference between ATR^{+/+} and ATR^{flox/-} cells) was measured in cells treated with UV (Figure 6-4B and 6-4C). The $\Delta\Psi$ was determined by quantification of CMXRos and JC-1 staining. Indeed, knockdown of Bax in cells significantly reduced the ability of ATR to maintain a higher $\Delta\Psi$ as compared to the cells transfected with control siRNA (Figure 6-4B and 6-4C).

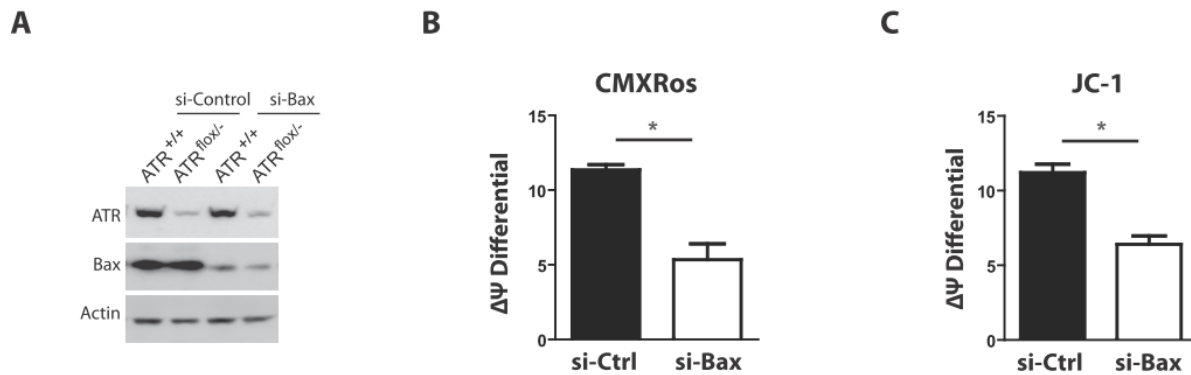


Figure 6-4. Effects of ATR's inhibition of mitochondrial membrane potential depend on Bax A, Western blotting confirms Bax silencing 48 hours after siRNA transfections in the HCT-116 ATR^{+/+} and ATR^{flox/-} cells. B, and C, HCT-116 ATR^{+/+} and ATR^{flox/-} cells were transfected with control (scrambled) or Bax siRNA for 48 hours before UV irradiation. After treatments, flow cytometry was employed to determine the percentage of cells with intact mitochondria $\Delta\Psi$ as indicated by labeling with MitoTracker Red CMXRos (panel B) or JC-1 (panel C). The capacity of ATR to regulate mitochondrial $\Delta\Psi$ was determined by calculating the difference between ATR^{+/+} cells and ATR^{flox/-} cells ($\Delta\Psi$ Differential = % ATR^{+/+} - % ATR^{flox/-}). Statistical analysis was performed by comparing the capacities of ATR in the control siRNA and Bax siRNA transfected cells. Asterisks above bar means a significant difference was found.

DNA Damage-Induced Translocation of ATR to Mitochondria

To determine the mechanism for the antiapoptotic function of ATR and whether the cytoplasmic ATR plays a role, cells were treated with UV, CPT or hydroxyurea (HU), and then the subcellular fractions were analyzed. Interestingly, a form of cytoplasmic ATR was found to

migrate slower in 3-8% gradient SDS-PAGE than the nuclear or native ATR (Figure 6-5A). This DNA damage-induced form of cytoplasmic ATR was observed in multiple human cell lines including normal primary fibroblasts (BJ) and multiple cancer cells (A549, H1299, HeLa, HCT-116, MCF-7) (data not shown). This form of ATR was detected only when cells were fractionated as described and the samples were separated in 3-8% gradient gels. We confirmed these observations by treating cells with increasing doses of UV (Figure 6-S1), or by irradiating cells with a single UV dose followed by recovery for different periods (Figure 6-S1). Meanwhile, a moderate decrease of nuclear ATR also was constantly observed (Figures 6-3F, 6-3G, 6-5A and 6-S1D). Several approaches were taken to verify that the slower-migrating band is ATR. First, the damage-induced formation of cytoplasmic ATR can be recognized by different ATR antibodies whose epitopes map to different region of ATR (Figure 6-S1C). Second, ATR^{fllox/-} cells were transfected with increasing amounts of wild-type ATR plasmids. A similar UV-induced slower-migrating form of cytoplasmic recombinant ATR was observed in the transfected ATR^{fllox/-} cells treated with UV (Figure 6-S2A). Third, this was confirmed further by mass spectrometry-based protein analysis of the two cytoplasmic ATR bands (Figures 5-S2B and 5-S2C). In this analysis, both forms of the cytoplasmic ATR were immunoprecipitated by anti-ATR antibody, visualized by Coomassie blue staining in SDS-PAGE (Figure 6-S2B) and identified by mass spectrometry (Figure 6-S2C).

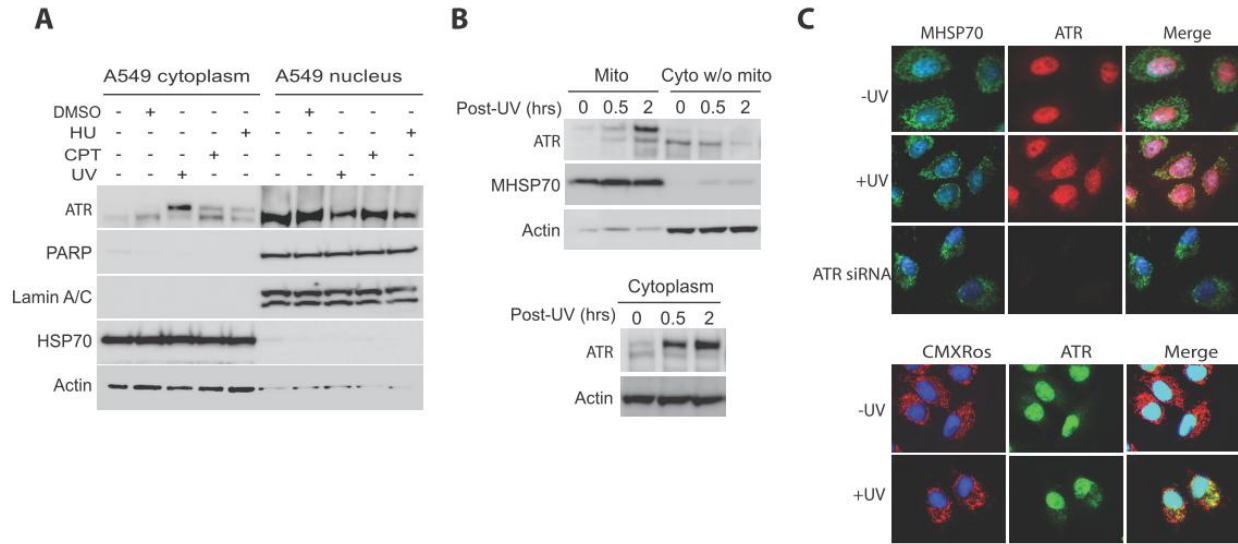


Figure 6-5. DNA damage induced a slower-migrating form of cytoplasmic ATR which localizes to mitochondria

A, A549 cells were mock treated, UV-C irradiated (20 J/m^2 , followed by 2 hour recovery), treated with CPT ($10 \mu\text{M}$ for 4 hour), or incubated with HU (2 mM for 20 hour). Fractionation and western blotting assessed the formation of cytoplasmic ATR which exhibited a slower electrophoretic mobility. Heat shock protein 70 (HSP70), actin, PARP, and lamin A/C were probed to ensure the quality of fractionation and protein loading. B, A549 cells were treated with 20 J/m^2 of UV-C and left to recover for 0, 0.5 or 2 hours. Following recovery, mitochondria were isolated as described in the Methods. Mitochondria, cytosolic lysate without mitochondria (top panel) and total cytoplasmic proteins (bottom panel) were analyzed by western blotting. The presences of MHSP70 and actin were probed as quality controls for the experiment. The UV-induced formation of cytoplasmic ATR seemed happening very fast after UV, as there was a slow migrating ATR formed when the cells were processed immediately post-UV irradiation (less than 5-minutes). C, A549 cells were seeded on coverslips one day before cells were mock- or UV-C irradiated (20 J/m^2) and allowed a 2-hour recovery. To determine if ATR localized to mitochondria, cells were co-stained with anti-ATR antibody and either MitoTracker Red CMXRos or anti-MHSP70. The fluorescence of MHSP70 and DAPI were merged for labeling of mitochondria and nuclei respectively. For quality control of staining, A549 cells were transfected with ATR siRNA to confirm that the detected anti-ATR antibody fluorescence is from ATR

exclusively. Presented images are representative data from at least three independent experiments.

Next we examined the subcellular localization of the cytoplasmic ATR. To this end, mitochondria were isolated from cells using methods described previously (213, 214). As shown in Figure 6-5B, an accumulation of slower migrating ATR occurred in the mitochondrial fraction following UV-irradiation. Correspondingly, the cytosolic fraction without mitochondria showed a dramatic reduction of the slower migrating ATR after UV-irradiation compared to the total in the cytoplasm (Figure 6-5B top vs. Bottom panels). These results strongly suggest that UV induced a translocation of slower migrating cytoplasmic ATR to mitochondria. In addition, we co-stained ATR and mitochondria in cells using mitochondrial heat shock protein 70 (MHSP70) or MitoTracker red CMXRos. As shown in Figure 6-5C, significantly more co-localization signals (yellow) were observed in the merged fluorescent images of the UV-irradiated cells than in non-irradiated cells. Knockdown of ATR confirmed that the observed ATR immunofluorescence signal was from ATR exclusively (Figure 6-5C). We also investigated the localization of DNA damage checkpoint kinase ATM in the cytoplasm. Interestingly, little ATM was detected in the cytoplasm and so was its co-localization with MHSP70 (Figure 6-S3).

Discussion

Although inhibition of ATR has been demonstrated to promote apoptosis and suppress carcinogenesis previously (58, 102-104, 144), it is generally believed that the observed effects of ATR inhibition are mediated by the hallmark checkpoint functions of ATR in the nucleus. In this study we showed that the ATR located in the cytoplasm has a checkpoint kinase-independent role in inhibiting the Bax-mediated apoptotic pathways following UV irradiation. This

antiapoptotic role of ATR was clearly independent of its checkpoint response functions. The cytoplasmic ATR did not exhibit the hallmark kinase activity of nuclear ATR. Instead, it had a direct role in regulating the mitochondrial membrane potential $\Delta\Psi$ mediated by Bax, and inhibition of ATR kinase activity had no effect on the mitochondrial $\Delta\Psi$ stabilization function of cytoplasmic ATR. Our finding reveals a novel role of ATR in the cytoplasm as an antiapoptotic protein which is directly involved in mitochondrial metabolism in cellular responses to DNA damage.

As a major DNA damage-checkpoint protein kinase, activation of ATR results in cell cycle arrest. Our results indicate that in addition to its checkpoint functions, ATR also has a direct role in suppressing mitochondria-mediated apoptosis. Remarkably, the cytoplasmic ATR is distinct from the nuclear ATR in many aspects. Unlike nuclear ATR, cytoplasmic ATR contains no checkpoint kinase activity toward p53 and RPA (Figure 6-3F). Also, different from nuclear ATR which is activated primarily by replication stress-inducing DNA damage such as UV damage, cytoplasmic ATR shows antiapoptotic activity in response to various types of DNA damage including DNA double-strand breaks induced by CPT and HU (Figures 6-1F and 6-5A). Thus, it is not surprising that cytoplasmic ATR exhibits different activity than does the kinase-active nuclear ATR. All these observations suggest that ATR has two distinct functions delivered by two different forms of ATR during DNA damage responses. Upon DNA damage, the nuclear ATR kinase is involved in activation of cell cycle checkpoints and DNA repair, while the cytoplasmic ATR stabilizes mitochondria, preventing activation of apoptosis. It is obvious that the synergetic action of these two events promotes the biological stability of the cells under the stress of DNA damage.

Also interestingly, under unstressed conditions, ATR appears to maintain a much lower level in the cytoplasm than in the nucleus. Although the cytoplasmic level of ATR might be increased via translation upon DNA damage, it appears that the increase could be more rapid by export of existing nuclear ATR. Given the large size (301 kDa) of the protein, nuclear export allows a much quicker response to DNA damage than does transcription followed by translation (Figure 6-S4A).

Another interesting observation of this study is that a new form of cytoplasmic ATR electrophoretically migrates slower than nuclear ATR in SDS-PAGE, implying that a possible posttranslational modification (PTM) to the protein may occur. Although ATR is known to undergo phosphorylation upon DNA damage (70, 119), phosphorylation would not change the electrophoretic mobility of ATR (70, 119) (Figure 6-S4B). In efforts to characterize the cytoplasmic ATR, we additionally examined the possible PTMs including glycosylation (Figures 6-S4B and 6-S4D), ubiquitinylation (Figure 6-S5A), which is required for translocation of p53 to mitochondria (213), sumoylation (Figure 6-S5A), palmitoylation (Figure 6-S5B), and prenylation (Figure 6-S5C). Unfortunately, none of these modifications was found to be responsible for the slower electrophoretic mobility of cytoplasmic ATR. N-myristoylation is also unlikely as the sequence of ATR contains no targeting amino acid for N-myristoylation, although N-myristoylation happens on BID and is required for its mitochondria membrane association and proapoptotic functions (215). Furthermore, protein translation inhibition by cycloheximide or puromycin had no effect on cytoplasmic ATR formation following UV damage (Figure 6-S4A), suggesting that the nuclear ATR is not a proteolytic product of the slower migrating cytoplasmic ATR assuming the latter to be the nascent ATR. The translation-inhibition data also indicate that, once formed, the cytoplasmic ATR is relatively stable. These results suggest that ATR might

undergo an unknown DNA damage-induced molecular modification to form the slower migrating form. Identifying the modification, though out of the scope of this study, would be an important future effort to fully understand the role of ATR in DNA damage responses.

Supplementary Figures

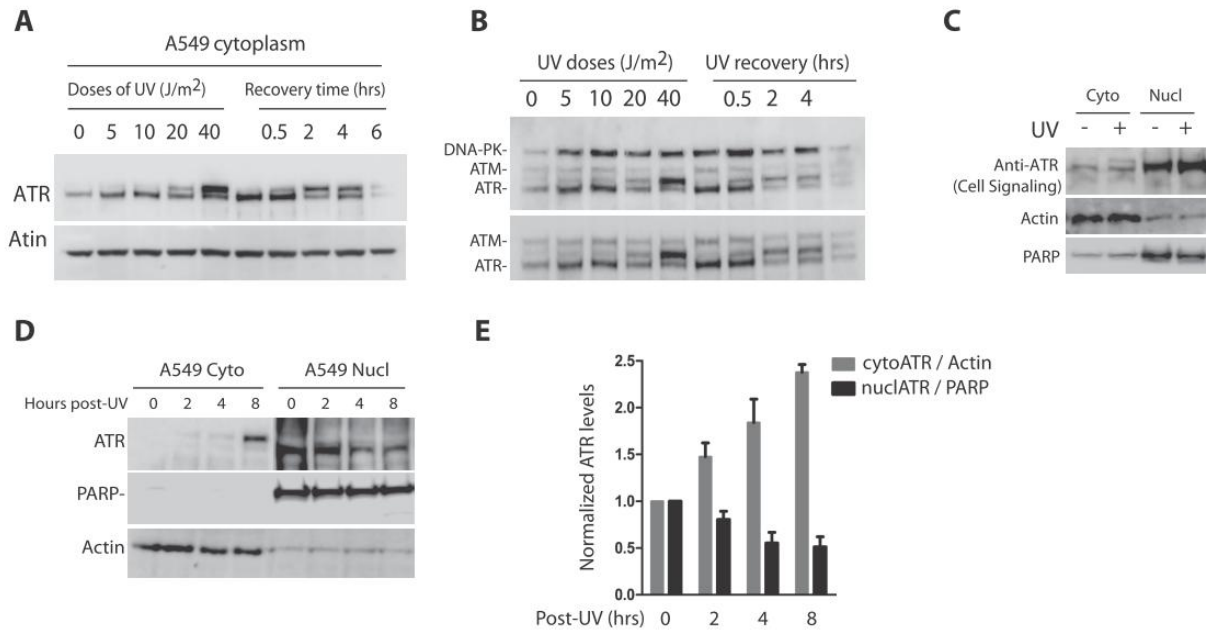


Figure 6-S1. DNA damage induced formation of a slower-migrating cytoplasmic ATR in a dose- and time-dependent manner

A, A549 cells were treated with increasing doses of UV irradiation, allowed to recovery for 2 hours; alternatively, cells were treated with 20 J/m² of UV and allowed to recover for different time periods. Cytoplasmic proteins were collected and assessed by western blotting of ATR and actin. B, Cytoplasmic ATM and DNA-PK do not exhibit electrophoretic mobility changes in response to DNA damage. The same blot as in Figure 6-S1A was successively probed with ATM and DNA-PK antibodies and shows no migration change of ATM or DNA-PK in response to DNA damage. C, A different ATR antibody was purchased from Cell Signaling which recognizes ATR at residues flanking the 1465 region (different from the Bethyl ATR antibody whose epitope maps on N-terminus of ATR at the 420 region), and confirmed the damage-induced formation of slower cytoplasmic ATR. D, The increasing total amount of cytoplasmic ATR (two bands) correlates with a decrease of nuclear ATR in DNA damage responses. A549 cells were treated with 40J/m² of UV and allowed to recover for indicated times. Cytoplasmic

and nuclear lysates were collected by fractionation and analyzed by western blotting of ATR. Nuclear PARP (cleaved PARP is an 89 KDa lower molecular weight band) and cytoplasmic actin were probed to check quality of loading. A quantitative representation of normalized ATR levels is shown in panel E.

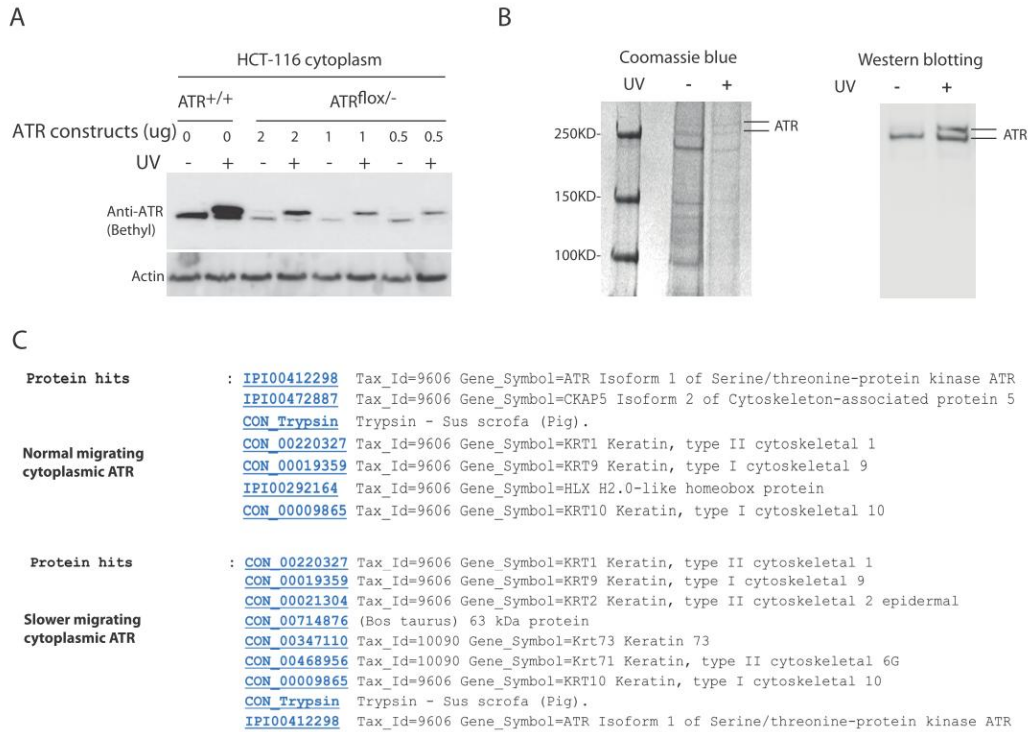


Figure 6-S2. Confirmation of the identity of cytoplasmic ATR

A, HCT-116 ATR^{+/+} or ATR^{flox/-} cells were transiently transfected with the indicated amount of pcDNA3.1 vectors containing wild-type ATR. Transfected cells were mock or UV-C irradiated (40 J/m²) followed by a 2-hr recovery. Cytoplasmic lysates were loaded onto SDS-PAGE and analyzed by western blotting of ATR. B, Preparation of samples for protein identification by mass spectrometry. A549 cells were mock or UV-C irradiated (40 J/m²) and allowed a 2-hr recovery. ATR was immunoprecipitated from cytoplasmic lysates prepared from forty 150 mm dishes of A549 cells. Proteins attached to protein G beads were released by boiling in SDS loading buffer, separated by SDS-PAGE, and stained with coomassie blue (left). The indicated ATR bands were excised and subjected to trypsin proteolysis for generating small peptide fragments amenable for protein identification by MS/MS mass spectrometry. 10% of the sample amount was loaded in SDS-PAGE and analyzed by western blotting of ATR (right). C, Mascot search results confirmed the presence of ATR in the two protein bands highlighted in panel A

(keratins are a common contamination during sample preparation for mass spectrometric analysis).

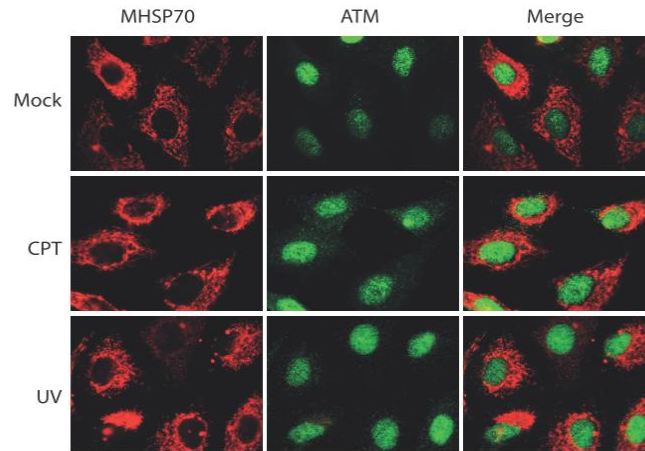


Figure 6-S3. Immunofluorescence signal of ATM does not show colocalization with mitochondria

A549 cells were mock or UV-C irradiated (40 J/m^2) with a 2-hr recovery, or treated with $10 \mu\text{M}$ CPT for 2 hr. The localization of ATM and mitochondria were resolved by immunofluorescent staining of ATM and MHSP70; staining of MHSP70 labels the mitochondria.

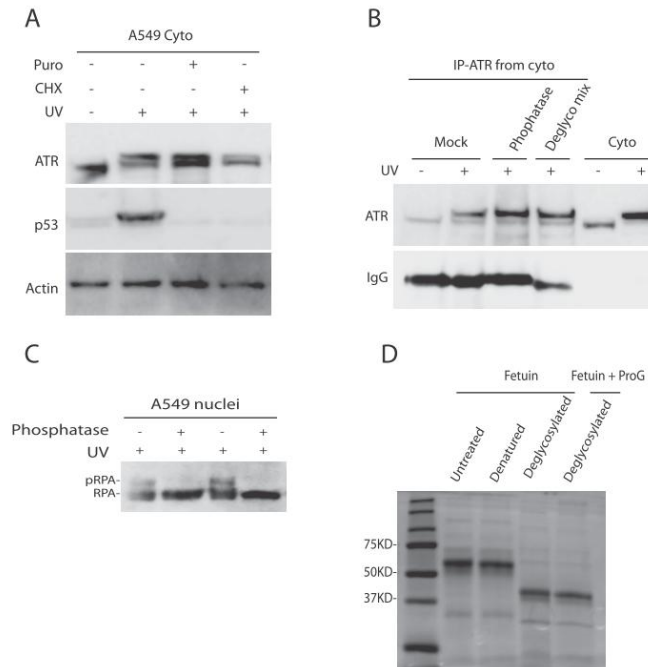


Figure 6-S4. The DNA damage-induced migratory change of cytoplasmic ATR is neither due to phosphorylation nor to glycosylation

A, A549 cells were pre-treated with 10 µg/mL of cycloheximide (CHX) or 2 µg/mL of puromycin (Puro) for 4 hours. Cells then were mock or UV-C irradiated (40 J/m²) and allowed a 2-hr recovery before cytoplasmic lysates were collected and analyzed by western blotting of ATR and p53. The lack of detectable p53 in the drug-treated samples indicates the effectiveness of the protein synthesis inhibitors. B, A549 cells were mock or UV-C irradiated (20J/m²) and allowed a 2-hr recovery. ATR was immunoprecipitated from cytoplasmic lysates. The immunoprecipitates were treated with enzyme buffer alone (Mock), lambda phosphatase or the deglycosylation enzyme mix. Western blotting was employed to assess the results. Detection of the faster-migrating IgG heavy chain indicates that the glycosylation treatment was complete. C, As a control for the phosphatase experiment in panel B, hyper-phosphorylated RPA32 was induced by UV-irradiation of duplicate A549 cell cultures before treatment of the lysates with phosphatase to confirm the complete removal of phosphate groups. The RPA32 was detected by western blotting. D, The protein fetuin which contains sialylated N-linked and O-linked glycans, was employed as a positive control for deglycosylase activity. SDS-PAGE separation and coomassie blue staining confirmed a faster migrating form of fetuin after deglycosylation reactions.

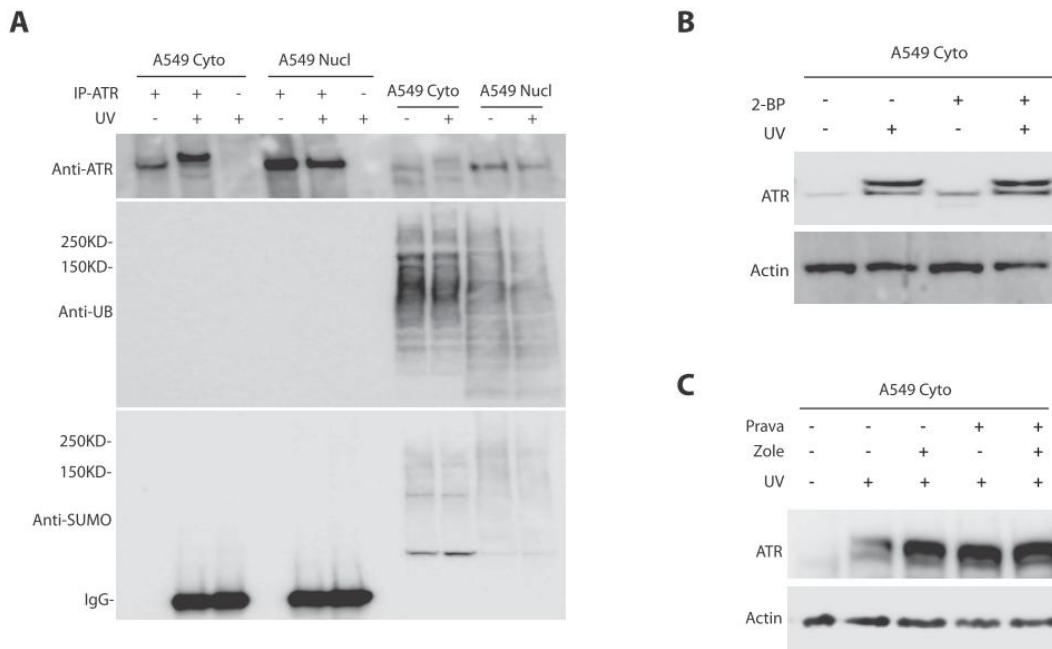


Figure 6-S5. Ubiquitination, sumoylation, palmitoylation or prenylation are not the cause of the slower migrating cytoplasmic ATR

A, A549 cells were mock or UV-C irradiated (40 J/m^2) and allowed a 2-hr recovery. ATR was immunoprecipitated from cytoplasmic or nuclear lysates, separated by SDS-PAGE, and assessed by western blotting of ubiquitin (Anti-UB), sumo (Anti-SUMO), and ATR. B, A549 cells were pre-incubated with $100 \mu\text{M}$ of 2-bromopalmitate (2-BP) for 4 hours to inhibit protein palmitoylation. Cells then were mock or UV-C irradiated (20 J/m^2) followed by a 2-hr recovery. Fractionation and western blotting assessed formation of cytoplasmic ATR. C, A549 cells were pre-incubated with $1 \mu\text{M}$ of pravastatin (Prava) and/or zoledronic acid (Zole) for 20 hr to inhibit protein prenylation. Cells then were mock or UV-C irradiated (40 J/m^2) and allowed a 2-hr recovery. Fractionation and western blotting assessed formation of cytoplasmic ATR.

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CHAPTER 7

SUMMARY AND CONCLUSIONS

Integrity of the human genome must be maintained for cells to ensure proper cellular functions and survival from one generation to the next. In cells the coordination of multiple mechanisms is required to detect and remove damaged DNA or to eliminate damaged cells in order to prevent genomic instability. Among these pathways are nucleotide excision repair (NER) and ATR-mediated DNA damage checkpoint. The NER pathway removes DNA adducts that are generated by a variety of genotoxic reagents (7, 14) such as UV-irradiation. The nuclear ATR-mediated DNA damage checkpoints survey the genome for replicative stresses and arrest cell cycle progression allowing time for repair (7, 9, 10, 14), while cytoplasmic ATR inhibits DNA damage-induced apoptotic cell death. Together, these 3 pathways are the major mechanisms that cells have evolved to handle UV-induced DNA damage in order to prevent mutation and genome instability.

A general requirement for these pathways to be fully functional is to have the DNA damage signaling and repair proteins properly modified and located at sites where they can carry out their functions (6, 7). For instance, the proper localization of DNA repair proteins to nuclear chromatin and the formation of higher order DNA-protein and protein-protein complexes at damage sites are required for DNA damage recognition and for DNA repair to occur (5-7). One such mechanism that is revealed by this dissertation is the regulation of NER by ATR via modulation of XPA phosphorylation and nuclear importation (73, 74, 78-80, 123). Functionally, XPA is believed to play roles in verifying DNA damage, stabilizing repair intermediates, and

recruiting other NER factors to the damaged DNA (76, 124-130). To accomplish these functions XPA has to enter the nuclei and gets phosphorylated in order to be fully functional in nuclear DNA repair, a process that is mediated by DNA damage checkpoint ATR (73, 74, 133, 135, 138). In addition to the requirement of proper modifications and/or localization of repair proteins to chromatin for DNA repair, the central players in mitochondrial membrane permeability and apoptosis (8, 11, 43, 44, 47, 209, 211) are needed at the mitochondria for their direct roles. Studies in this dissertation revealed a novel regulation of these central players that is mediated by cytoplasmic ATR. Our data suggest that in response to DNA damage cytoplasmic ATR localizes to mitochondria and plays a kinase-independent role in inhibiting the localization of Bax protein to mitochondria and, thus, inhibits apoptosis. A summary of the roles of ATR in regulation of NER and apoptotic cell death, which were revealed by this dissertation, are discussed by chapters as following.

Chapter 2: ATR Interaction and/or Phosphorylation of XPA is Required for DNA Repair

Recent studies have demonstrated that the ATR-dependent checkpoint pathway may coordinate with NER via regulation of XPA localization and/or phosphorylation of XPA to promote DNA repair (73, 74). To further understand whether the phosphorylation of XPA by ATR is required for removal of UV-DNA damage, as well as how the ATR-XPA interaction occurs, is biologically significant. I investigated whether ATR interaction with and/or phosphorylation of XPA were required for DNA repair (134) (presented here in Chapter 2). Using a mass spectrometry-based protein footprinting method, it was previously found in this lab that ATR interacts with a helix-turn-helix (HTH) motif in the minimal DNA-binding domain of XPA where an ATR phosphorylation site (serine 196) is located (134). A surface lysine residue of XPA, K188, was protected from biotin modification following the binding of XPA to ATR,

and when K188 was mutated to an alanine, the ability of XPA to bind to ATR was abolished. However, mutation of K188 to glutamic acid did not change the ATR-XPA complex formation, suggesting other residues on the HTH motif mediate the complex formation and that K188 may be necessary for stabilization of the α -helix domain. In cells I found that disruption of XPA-ATR interaction by a K188A mutation of XPA inhibited the ATR-dependent nuclear import and phosphorylation of XPA and reduced the ability of XPA to repair UV-DNA damage. These observations highlight the significance of XPA and ATR interaction in the regulation of NER by ATR.

Phosphorylation and the directed-nuclear import of XPA represent 2 possible mechanisms for regulation of NER by the ATR-dependent DNA damage checkpoints (Chapter 2). Cells with the phosphorylation-deficient XPA were less capable of repairing UV-induced CPDs instead of 6-4PPs, although no difference was found on its nuclear import. 6-4PPs account for ~25% of the damage generated by UV-C irradiation and can be quickly removed by NER (within 4-hours in healthy cells); while CPDs represent ~75% of total DNA lesions caused by UV-C and are repaired at a much slower rate (as long as 24-hours in many cells). Therefore, a basal XPA level in the resting nucleus, as well as the immediate ATR-directed XPA import following UV (74) could be sufficient to support the rapid removal of 6-4PPs so that no significant effects were observed. However, repair of CPDs needs more XPA molecules for a much longer period of time, and a significant delay in repair was observed due to the deficiency. Indeed, research by Lee *et al.* demonstrated that ATR enhanced XPA protein stability via phosphorylation of XPA, and the stability of XPA controlled the efficiency of UV-induced DNA repair (133). Within an earlier recovery time point (90 min after UV irradiation) than used in our studies, this even group was able to detect a subtle but significant change in 6-4PPs repair

kinetics that was affected by phosphorylation of XPA. In summary, the ATR-directed nuclear import of XPA represents a quick responding mechanism of NER for DNA repair, while phosphorylation of XPA prevents XPA degradation (133) and is responsible to maintain the persistent function of the NER.

Chapter 3: XPA-Mediated Regulation of Global Nucleotide Excision Repair by ATR Is p53-Dependent and Occurs Primarily in S-Phase

After we had published our initial work on the regulation of NER via posttranslational modification and directed-nuclear import of XPA by ATR, another group reported a requirement of ATR in maintaining NER activity primarily during S phase in human cells (15, 75). This S-phase regulation of NER by ATR leads to questions whether the ATR-directed nuclear import of XPA upon DNA damage also is cell cycle specific and downstream targets of ATR are involved in the UV-induced XPA import. Therefore, the study described in Chapter 3 was aimed to investigate ATR-directed XPA nuclear import by identifying the downstream mediators involved in the ATR kinase-dependent XPA nuclear import and the cell cycle-dependent DNA repair. We examined whether any of the major downstream checkpoint substrates of ATR, such as p53, Chk1, or MK2, are involved in the regulation of the UV-induced XPA nuclear import. We found that not only was the p53 protein itself necessary, but also, the transcriptional function of p53 and the DNA damage signaling via p53-ser15 phosphorylation were important for UV-induced XPA nuclear import. The effect of p53-ser15 phosphorylation appears to be similar as that of the p53 inhibitor pifithrin- α (167-169), which inhibited the transcriptional activity of p53 and the UV-induced XPA nuclear import.

With our strategies a cell cycle-specific regulation of NER by ATR is unveiled as in Chapter 3. Our results suggest that the UV-induced XPA nuclear import happens predominately

in S phase, while the XPA nuclear import in G1 and G2 phases is largely independent of UV irradiation and ATR. In addition, the level of UV-induced Ser15-phosphorylation of p53 was lower in G-1 and G2-phase cells than in S-phase cells. Coincidentally, DNA repair of UV-induced CPDs was much more efficient in S phase than in G1 phase. Interestingly, it was previously demonstrated that p53 deficiency had a negative impact on GG-NER but not on TC-NER (165, 166). Given the indispensable role of XPA in both GG-NER and TC-NER, the observation of p53's requirement for the UV-induced XPA nuclear import in S-phase cells implies that TC-NER may predominately occur in other cell cycle phases in a p53-independent manner.

Chapter 4: UV-Induced Nuclear Import of XPA Is Mediated by Importin-Alpha4 in an ATR-Dependent Manner

Due to its indispensable role in human NER, including both global genome and transcription-coupled NER subpathways, XPA may serve as a potential target for sensitization to cancer chemotherapy (e.g. cisplatin) via manipulation of available nuclear XPA, either at the transcriptional or posttranscriptional level (73, 78-80, 123). So far we have demonstrated that DNA damage-induced nuclear import of cytoplasmic XPA for NER in S-phase cells is a DNA damage checkpoint-dependent process mediated by ATR and p53 (Chapter 3). It remains unresolved to us how XPA is imported into the nucleus through the nuclear pore complex (NPC). Understanding of this XPA nuclear importation could be important because targeting protein trafficking is a potential strategy to improve the sensitivity of cancer cells to chemotherapeutic agents (137). The goal of the work presented in Chapter 4 was to identify the transporters that are responsible for the UV-induced nuclear import of XPA. The observation that the nuclear import of XPA depends on its NLS in cells excludes the possibility that XPA was coimported with other

proteins containing a NLS or imported by “alternative import mechanisms” (more than one mechanism) (136).

Our data also indicate that the same NLS is used by different adaptors, importin- α 4 and/or importin- α 7, for transporting XPA through the NPC. Although both importin- α 4 and importin- α 7 are required for XPA nuclear import, the requirement of importin- α 4 is mediated by ATR in DNA damage-dependent manner while that of importin- α 7 is not. Interestingly, the NLS of XPA could not be efficiently recognized by importin- α 4 in the absence of UV-irradiation. This is likely due to the masking of the XPA NLS by the binding of other cytoplasmic factors (187, 189). This hypothesis is validated by the observation that importin- α 4 immunoprecipitated from both UV- and mock-treated cell lysates could bind recombinant XPA protein *in vitro*. Elucidation of the details as to which cytoplasmic factors might mask the NLS and thus regulate this interaction merits future investigation. The binding of importin- α 4 to XPA is largely induced by UV-irradiation within ½ hour after exposure. This suggests that the efficient nuclear import of XPA may occur as early as ½ hour post-UV-irradiation which is consistent with our previous report (74). In contrast, substantial XPA-importin- α 7 interaction was observed in the absence of DNA damage and, thus, the DNA damage appeared to have no effect on the interaction. Given that a small portion of XPA is present in the nucleus even without DNA damage, a possible scenario is that importin- α 7 could be involved in the nuclear import of XPA independent of DNA damage while importin- α 4 could participate in the damage-dependent import of XPA. Additionally, we have shown that the DNA damage-induced nuclear import of XPA is cell cycle dependent, primarily occurring in S phase (135). In G2-phase cells, XPA is localized to the nucleus regardless of DNA damage (135). Thus, it is possible that importin- α 7 is mainly

responsible for nuclear import of XPA in G2 phase while importin- α 4 is for XPA nuclear import during S phase.

Finally, XPA binding protein 1 is a GTP-binding protein that was identified as an interacting partner with XPA in the yeast 2-hybrid system; it was proposed as the GTPase involved in XPA nuclear import (172). However, our results show that siRNA knockdown of XAB1 had no effect on the UV-induced nuclear import of XPA. This inconsistency could be due to the different environments within human cells and the yeast model system.

Chapter 5: Differential DNA Damage Responses in p53 Proficient and Deficient Cells: Cisplatin-Induced Nuclear Import of XPA Is Independent on ATR Checkpoint in p53-Deficient Lung Cancer Cells (138)

One of the major challenges in treating cancer patients with cisplatin is the drug's side-effects: it introduces DNA damage to cancer cells while also causing damage to normal cells (140). One solution for this problem is to identify mechanistic differences of DNA damage responses between normal and cancer cells. In the current study effort was made to define the unique mechanism of DNA damage responses in p53-deficient lung cancer cells as p53 is the most commonly mutated gene in human cancers, particularly the lung cancer. Thus 3 cell lines including the human normal primary cell line BJ, the p53-proficient lung cancer cell line A549, and the p53-deficient lung cancer cell line H1299 were treated with cisplatin or UV-C irradiation. A pronounced cytoplasm-to-nucleus translocation of XPA was observed in each of these cell lines, which is consistent with our previous observations on other types of cells treated with UV. Importantly, however, different from p53-proficient cells the damage-induced XPA import is not dependent on ATR or other major known checkpoints in the p53-deficient lung cancer cells-H1299 (Figure 5-2 and Figure 5-3). These observations reveal a mechanistic

difference of DNA damage responses between p53-proficient and p53-deficient lung cancer cells. This finding provides a possibility to specifically target this unknown pathway to sensitize the cisplatin-induced killing effects in p53-deficient cancer cells by inhibiting the DNA damage induced nuclear import of XPA. Evidently, identifying the novel regulation mechanism responsible for the damage-induced XPA import in the p53-deficient cancer cells deserves further investigation.

Chapter 6: Cytoplasmic ATR Lacking Checkpoint Kinase Activity is a Bax Inhibiting Antiapoptosis Protein at Mitochondria

ATR has been shown to have antiapoptotic functions (58, 101-105, 144) and has drawn attention as a promising target for anticancer chemotherapy. However, it was not clear to us how ATR carries out its intrinsic antiapoptotic roles. We reasoned that ATR may have a role in the mitochondria-mediated apoptotic pathways. As a novel finding (Chapter 6), our results revealed a kinase-independent antiapoptotic function of ATR through inhibition of the Bax-mediated mitochondrial pathway during cellular responses to DNA damage. Our results indicate that, in addition to its checkpoint functions, ATR also has a direct role in suppressing mitochondria-mediated apoptosis. ATR inhibits Bax-mediated loss of mitochondrial membrane potential, and the role of ATR in stabilizing mitochondrial $\Delta\Psi$ does not depend on its kinase activity. Unlike nuclear ATR, cytoplasmic ATR lacks checkpoint kinase activity toward p53 or RPA. Additionally, nuclear ATR is activated primarily by replication stress-inducing DNA damage such as UV damage, whereas cytoplasmic ATR shows antiapoptotic activity in response to various types of DNA damage such as DNA double-strand breaks induced by CPT. These observations suggest that ATR has 2 distinct functions delivered by the 2 different forms of ATR during DNA damage. The nuclear ATR kinase is involved in activation of cell cycle checkpoints

and DNA repair while the cytoplasmic ATR stabilizes mitochondria, preventing activation of apoptosis. The synergetic action of these 2 events promotes the biological stability of the cells under the stress of DNA damage (Figure 7-1). Also, in unstressed conditions, ATR appears to be in a much lower level in the cytoplasm than in the nucleus. Although the cytoplasmic level of ATR might be increased via translation when DNA is damaged, our data appear to support an export of existing nuclear ATR. Indeed, given the large size (301 kDa) of the protein, nuclear export allows a much quicker response to DNA damage than does transcription followed by translation.

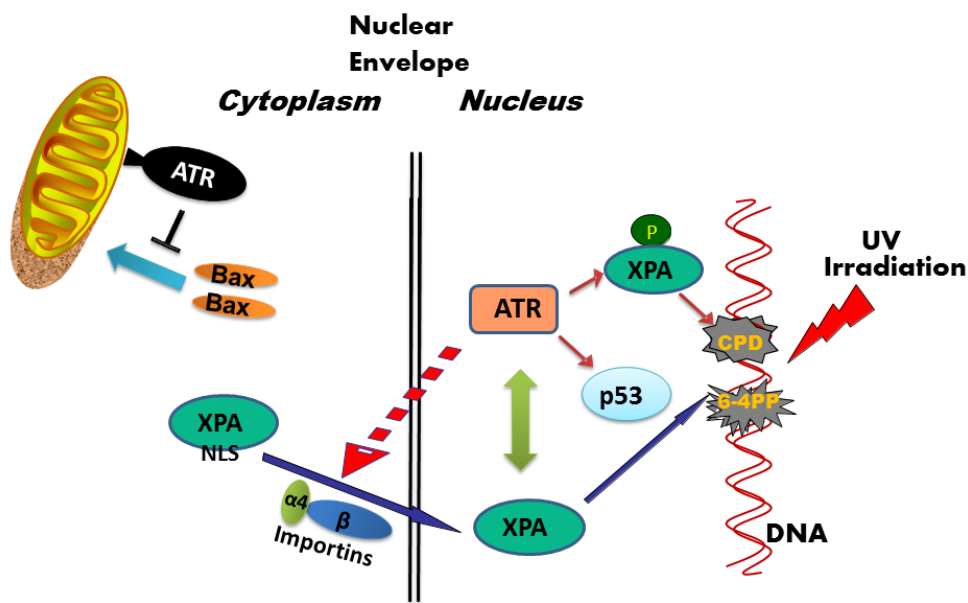


Figure 7-1. A model for the major conclusions in this dissertation

Another interesting observation of this study (Chapter 6) is that a new form of cytoplasmic ATR electrophoretically migrates slower than nuclear ATR in SDS-PAGE, implying that the occurrence of a possible posttranslational modification (PTM) to the protein. Although ATR is known to undergo phosphorylation upon DNA damage (70, 119), phosphorylation does not change the electrophoretic mobility of ATR (70, 119). In efforts to

characterize the slower migrating cytoplasmic ATR, we examined the possible PTMs including glycosylation, ubiquitinylation (which is required for translocation of p53 to mitochondria (213)), sumoylation, palmitoylation, and prenylation. Unfortunately, none of these modifications were found to be responsible for the slower electrophoretic mobility of cytoplasmic ATR. N-myristoylation is also unlikely as the sequence of ATR contains no target amino acid for N-myristoylation, although N-myristoylation happens on BID (BH3 interacting-domain death agonist) and is required for its mitochondria membrane association and proapoptotic functions (215). Furthermore, protein translation inhibition by cycloheximide or puromycin had no effect on the formation of the cytoplasmic ATR following UV damage, which suggests that the nuclear ATR is not a proteolytic product of the slower migrating cytoplasmic ATR, assuming the latter to be the nascent ATR. The translation-inhibition data also indicate that, once formed, the cytoplasmic ATR is relatively stable. These results suggest that ATR might undergo an unknown DNA damage-induced molecular modification. The formation of the mitochondria ATR, as well as the mechanism by which ATR relocates to mitochondria, may represent a novel mechanism that cells have evolved to inhibit DNA damage-induced apoptosis. Identifying the modification, although out of the scope of this study, would be an important future effort to fully understand the role of ATR in DNA damage responses.

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APPENDICES

APPENDIX A

ABBREVIATIONS

(6-4)PP, (6-4) photoproduct
9-1-1, Rad9-Rad1-Hus1 complex
AT, ataxia telangiectasia
ATM, Ataxia telangiectasia mutated
ATP, adenosine triphosphate
ATR, Ataxia telangiectasia mutated and RAD3-related
ATRIP, ATR interacting protein
Bad, Bcl-2-associated death promoter
Bak, Bcl-2 homologous antagonist/killer
Bax, Bcl-2-associated X protein
Bcl-2, B-cell lymphoma 2
Bid, BH3 interacting-domain death agonist
Bim, Bcl-2-like protein 11
CS, Cockayne syndrome
CSA, Cockayne syndrome A
Chk1, checkpoint kinase-1
Chk2, checkpoint kinase-2
CHX, Cycloheximide
CPD, cyclobutane pyrimidine dimer
CPT, camptothecin
Cyto, cytoplasm
CytoC, cytochrome C
DAPI, 4',6-diamidino-2-phenylindole
DBD, DNA binding domain
DDB, damaged DNA binding protein

DDR, DNA damage response
DMEM, Dulbecco's modified Eagle's medium 132
DMSO, Dimethyl sulfoxide
DNA-PKcs, DNA-dependent protein kinase catalytic subunit
DTT, dithiothreitol
EDTA, ethylenediaminetetraacetic acid
ERCC1, excision repair cross complementation group 1
FAT, FRAP/ATM/TRAP
FBS, fetal bovine serum
FANCD2, Fanconi anemia group D2
GFP, green fluorescent protein
GGR, global genome repair
GST, Glutathione S-transferase
HEAT, huntingtin, elongation factor 3, protein phosphatase 2A and yeast TOR1
HSP70, heat shock protein 70
HTH, helix-turn-helix
HU, hydroxyurea
IP, immunoprecipitation
kDa, kilodalton
Mcl-1, myeloid cell leukemia sequence 1
MHSP70, mitochondria heat shock protein 70
Mito, mitochondria
MK2, MAPKAP Kinase-2
MS, mass spectrometry
MS/MS, tandem mass spectrometry
mTOR, mammalian target of rapamycin
NF-kB, nuclear factor-kB
NER, nucleotide excision repair
NLS, nuclear localization signal
NPC, nuclear pore complex
PAGE, polyacrylamide gel electrophoresis

PBS, phosphate buffered saline
PDB, Protein Data Bank 133
PI, propidium iodide
PIKK, phosphatidylinositol 3-kinase-related kinase
PARP, poly ADP ribose polymerase
PTM, posttranslational modification
PVDF, polyvinylidene difluoride
RHINO, RAD9, HUS1, RAD1-interacting nuclear orphan protein
RNAi, RNA interference
RPA, replication protein A
SDS-PAGE, sodium docecyl sulfate-polyacrylamide gel electrophoresis
siRNA, small interfering ribonucleic acid
ssDNA, single-stranded DNA
SUMO, small ubiquitin-like modifier
TCR, transcription coupled repair
TFIIH, transcription factor II H
TopBP1, DNA topoisomerase 2-binding protein 1
TTD, trichothiodystrophy
UB, ubiquitin
UTR, untranslated region
UV, ultraviolet
UV-C, ultraviolet C
XAB1, XPA binding protein1
XP, Xeroderma pigmentosum
XPA-G, Xeroderma pigmentosum complementation group A-G
XPC-HR23B, Xeroderma pigmentosum complementation group C-human homolog of Rad23B
 $\Delta\Psi$, mitochondrial membrane potential

APPENDIX B
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- 2012: Environmental Mutagen Society Student and New Investigator Travel Award
- 2012: research seminar: DNA damage checkpoint protein Ataxia telangiectasia and Rad3-related (ATR): diseases and novel protein functions
- 2008 - 2012: platform and/or poster presentations in the Annual Midwest DNA Repair Symposia
- 2009 - 2011: East Tennessee State University Graduate Student Travel Awards
- 2009 - 2010: research grants recipient from East Tennessee State University School of Graduate Studies