Investigating the Cellular Localization of APE1

by

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B.Sc. University of Northern British Columbia, 2009

THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE
IN
MATHEMATICAL, COMPUTER, AND PHYSICAL SCIENCES
(CHEMISTRY)

UNIVERSITY OF NORTHERN BRITISH COLUMBIA

April 2012

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Abstract

Apurinic/apyrimidinic endonuclease 1 (APE1) is a well-known DNA repair enzyme with multiple functions which include redox-activation, 3'-DNA phosphodiesterase, 3'-5' DNA exonuclease, and RNase H activities. Recently the novel function of APE1 in RNA metabolism has been demonstrated. APE1 was shown to possess the ability to cleaving single-stranded RNA as well as abasic RNA. It was also shown to physically interact with proteins involved in RNA metabolism.

Previous studies have discovered the altered distribution and expression of APE1 in different cell types including cancer cells. The cytoplasmic distribution of APE1 has been shown to have correlation with aggressive carcinomas and poor prognoses for patients. This has led to an increasing number of studies on the undefined extra-nuclear roles of APE1 and APE1's potential implications in cancer development. In this thesis, I aimed (i) to confirm the importance of nuclear localization signal (NLS) of APE1 at the N-terminus, (ii) to discover the role of single nucleotide change of APE1 human population variants in its sub-cellular distribution, (iii) to assess the cellular localization of APE1 with processing bodies and stress granules under cellular stresses, and (iv) to initiate immuno-fluorescence analysis of APE1 in breast cancer tissue. Our results revealed that APE1 indeed has an important NLS of 1-20 amino acids at the N-terminus and APE1 human population variants showed nuclear localizations identical to the wild-type APE1. There was no co-localization of APE1 with PBs and/or SGs implying that APE1 is unlikely to be involved in mRNA processing that is carried out in PBs and SGs. We observed re-distribution of the ND20-APE1-GFP upon cellular stresses in HepG2 cells and this phenomenon has highlighted possible degradation of the cytoplasmic APE1 upon cellular stress. This finding
is also consistent with previous discovery of the five critical lysine residues that are responsible for ubiquitination and APE1 degradation upon cellular stress.

In summary, this thesis confirmed the role of the NLS in APE1 cellular localization. This is the first study to reveal the cellular localization of human population variants of APE1 and the N-terminal deletion variants of APE1 human population variants. This research has discovered degradation of the cytoplasmic APE1 upon cellular stress via, possibly, ubiquitination of the five lysine residues at the N-terminus region of 21-40 amino acids.
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# Glossary

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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>APE1</td>
<td>apurinic/apyrimidinic DNA endonuclease 1</td>
</tr>
<tr>
<td>TIA-1</td>
<td>T-cell antigen binding protein 1</td>
</tr>
<tr>
<td>Dcp1a</td>
<td>decapping enzyme 1a</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>mRNP</td>
<td>mRNA protein complex</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
</tr>
<tr>
<td>NMD</td>
<td>nonsense-mediated mRNA decay</td>
</tr>
<tr>
<td>SMD</td>
<td>staufen-1-mediated mRNA decay</td>
</tr>
<tr>
<td>NSD</td>
<td>nonstop decay</td>
</tr>
<tr>
<td>NGD</td>
<td>no-go decay</td>
</tr>
<tr>
<td>PB</td>
<td>processing body</td>
</tr>
<tr>
<td>SG</td>
<td>stress granule</td>
</tr>
<tr>
<td>GSNO</td>
<td>S-nitroso glutathione</td>
</tr>
<tr>
<td>LMB</td>
<td>leptomycin B</td>
</tr>
<tr>
<td>CHX</td>
<td>cycloheximide</td>
</tr>
<tr>
<td>EPO</td>
<td>epoxomycin</td>
</tr>
<tr>
<td>DQZ</td>
<td>diaminoquinazoline</td>
</tr>
<tr>
<td>AP site</td>
<td>apurinic/apyrimidinic site</td>
</tr>
<tr>
<td>NIR</td>
<td>nucleotide incision repair</td>
</tr>
<tr>
<td>BER</td>
<td>base excision repair</td>
</tr>
<tr>
<td>CRD</td>
<td>coding region determinant</td>
</tr>
<tr>
<td>NLS</td>
<td>nuclear localization signal</td>
</tr>
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</table>
NES  nuclear export signal
ND20  20 amino acids of N-terminus deleted APE1 variant
ND25  25 amino acids of N-terminus deleted APE1 variant
ND30  30 amino acids of N-terminus deleted APE1 variant
ND35  35 amino acids of N-terminus deleted APE1 variant
ND41  41 amino acids of N-terminus deleted APE1 variant
pWT-APE1 plasmid of pEGFP-WT-APE1
pND20 plasmid of pEGFP-ND20
pND25 plasmid of pEGFP-ND25
pND30 plasmid of pEGFP-ND30
pND35 plasmid of pEGFP-ND35
pND41 plasmid of pEGFP-ND41
SNP single nucleotide polymorphism
PCR polymerase chain reaction
FBS fetal bovine serum
GFP green fluorescent protein
RFP red fluorescent protein
mRFP monomeric red fluorescent protein
DTT dithiothreitol
SDS sodium dodecyl sulfate
TEMED tetramethylethylenediamine
APS ammonium persulfate
PBS phosphate buffered saline
Acknowledgements

I would like to thank my supervisor, Dr. Chow H. Lee, for his guidance, advice, and support throughout my undergraduate research experiences and this MSc. Thesis. I would also like to thank my supervisory committee members, Dr. Andrea Gorrell and Dr. Kuo-Hsing Kuo for their insightful advice and support. I would also like to thank past and present Dr. Lee’s laboratory members, Dr. Maggie Li for her endless attention, time, and technical support, Joseph, Mavis, Conan, Tavish, Mark, Chris, Randi, Dustin, Shirley, Gerrit, and Manbir for their friendship and feedbacks. Finally, I would like to thank my family for their mentoring and support throughout my studies and friends, Matt, Travis, and Owen for putting their precious time on editing my thesis chapters.
Candidate's Publications Relevant to this Thesis

Article


Abstract

American Association of Cancer Research 2011 Annual Meeting (Poster Presentation)
Chapter 1

Introduction

The mechanism of mRNA stability or turnover in cells is one of the critical points in the regulation of gene expression. Until recently, two exonucleolytic mRNA degradation pathways, including 5'-3' and 3'-5' exonucleolytic degradations, were considered as primary pathways. In contrast, the discovery of endoribonucleases that cleave mRNA molecules internally has enlightened an alternative mRNA turnover mechanism. Among these newly discovered endoribonucleases, an enzyme known as apurinic/apyrimidinic DNA endonuclease 1 (APE1) has recently been discovered to possess an endoribonuclease activity against c-myc messenger RNA (mRNA) in vitro and also possibly in vivo (Barnes et al. 2009). The objective of this Master thesis was to determine the cellular localization of APE1 and the potential changes in its endoribonuclease activity in relation to its sub-cellular localization.

This thesis concerns identifying the cellular localization of an endoribonuclease. Hence, I will first review mRNA degradation and its importance in gene expression. This is followed by discussion on different mammalian mRNA degradation pathways. The biochemical functions of APE1 including its discovery as an endoribonuclease and its role in RNA metabolism will also be highlighted. Finally, localization of APE1 in different cell types including cancers will be discussed.

1.1 mRNA degradation and gene expression

mRNA stability or turn-over is an integral control point in the regulation of gene expression in virtually all eukaryotic species. The stability of various mRNAs within a cell can differ and this results in a magnitude of difference in mRNA abundance following transcriptional
repression or transcriptional induction (Ross 1995). Earlier studies in the field have focused on the significance of mRNA sequences in conferring the instability to a transcript. The most common instability elements are found within the 3’ untranslated regions (UTRs) such as in AU-rich elements, and within coding regions of exemplary mRNAs like those of c-myc and c-fos (Jing et al. 2005). Among factors that control RNA metabolism, dynamic association of mRNAs with RNA-binding proteins is known to influence the fate of mRNAs (Giorgi and Moore, 2007). Moreover, the stability of individual mRNAs can be controlled in response to a variety of stimuli, from hormones to viruses to ions, allowing for rapid changes in gene expression (Wilusz and Wilusz 2004). Accordingly, degradation and stability of mRNAs have been reviewed to be critical in the physiological and pathological processes (Bettegowda and Smith 2007). In an effort to understand the role of mRNA degradation in these processes, mRNA decay pathways and some of the responsible enzymes have been discovered.

1.1.1 mRNA surveillance control pathways

Cells have specific mechanisms to eliminate RNAs that are incorrectly processed or have improper function either because of mutations within the genes that encode them or because of mistakes made during their metabolism (Isken and Maquat 2007). This mRNA surveillance control is one of the mRNA decay pathways and mainly responsible for degrading defective or aberrant mRNAs resulting in proper protein synthesis (Garneau et al. 2007). The surveillance machineries can detect and destroy aberrant mRNAs through four different ways including nonsense-mediated mRNA decay (NMD), Staufen-1-mediated mRNA decay (SMD), nonstop decay (NSD), and no-go decay (NGD).

NMD is most well-studied mRNA surveillance pathway, which is responsible for degrading translationally abnormal RNAs that can cause premature termination of translation
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(Nicholson et al. 2010). For example, random nonsense and frame-shift mutations, programmed DNA arrangements, or errors in mRNA splicing can all result in premature termination of translation and production of truncated proteins (Isken and Maquat 2007). These products have potential to acquire dominant-negative or gain-of-function activities as well as have no function; therefore, the abnormal RNAs need to be dealt with by a surveillance system. Interestingly, NMD is also known to reduce non-functional transcripts that have assimilated transposons or retroviral sequences, and target upstream open translational reading frame or an intron within their 3′-UTR (Mendell et al. 2004 and Wittmann et al. 2006).

The mechanism of NMD decay is the following: cap-binding proteins (CPB), such as CBP80-CBP20 that bind to the 5′ caps of newly synthesized transcripts before pre-mRNA splicing, and post-splicing exon junction complex (EJC) recruit various NMD factors such as Upf1, Upf2, Upf3, SMG1, eRF1, and eRF3 (Conti and Izaurralde 2005). This process will degrade target defective mRNAs by blocking ribosome formation and recruiting mRNA decaying factors. In contrast, the competitive decay pathway of NMD named SMD targets both CBP80-CBP20 bound and eIF4E-bound mRNAs that contains STAU1-binding site and occurs independently of splicing (Kim et al. 2005). Unlike NMD, SMD down-regulates the expression of genes encoding mRNAs that contain an STAU1-binding site in their 3′-UTRs.

Another mRNA surveillance pathway is NSD, which targets non-stop mRNA that lacks in-frame termination codons possibly due to a premature polyadenylation, an abortion of transcription, or an incomplete 3′-to-5′ decay of ribosome-associated mRNAs (Akimitsu 2008). NSD in cells functions to degrade translationally dead-end templates, and to release the unproductively stalled ribosomes for the translation of other mRNAs (Van Hook et al. 2002 and Frischmeyer et al. 2002). This stalled ribosome is responsible for recruiting decapping enzymes,
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Dcp1 and Dcp2, and exosome-associated factor, Ski7, which then recruits 5'-3' exoribonuclease, Xrn1, and exosome to degrade target mRNA (Van Hook et al. 2002 and Frischmeyer et al. 2002).

The last mRNA surveillance pathway known is NGD, which targets translationally active mRNAs that block ribosome progression by forming stem-loop structures. NGD requires the recruitment and interaction of Dom34 and HBS1 to recognize stalled ribosomes, and to recruit the endoribonuclease for mRNA destruction (Doma and Parker 2006). Two cleavage products generated by the endoribonuclease are then degraded by the exosome and Xrn1 (Doma and Parker 2006). All these aforementioned mRNA surveillance pathways are involved with stepwise assembly of RNA-protein complexes, and are responsible for degradation of aberrant mRNAs.

1.1.2 General turnover by 5'-3' and 3'-5' decay pathways

The turnover of mRNA in higher eukaryotes is a highly regulated process that plays an important role in gene expression. mRNA decay pathways can be generally classified into two broad categories - exoribonucleolytic and endoribonucleolytic pathways (Parker and Song 2004 and Garneau et al. 2007). Primarily from studying yeast and mammals, two exoribonucleolytic pathways of mRNA decay, which degrade mRNA in either 5'-3' or 3'-5' directions, have been identified.

In both exonucleolytic decay pathways, a variety of mRNA deadenylases, PAN2-PAN3 (Brown et al. 1996), CCR4-NOT (Tucker et al. 2001), and PARN (Dehlin et al. 2000), usually initiate the degradation process by shortening the poly-A tail at the 3' end of the mRNA to a length of 10 to 15 nucleotides. Following this shortening of the poly A tail, mRNA is then subjected to degradation in either 5'-3' or 3'-5' directions.
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In 5'-3' degradation, a decapping of the 5' end takes place by the decapping enzyme, Dcp1 and Dcp2, with several accessory factors such as Lsm proteins (Tharun and Parker 2001), Edc proteins (Schwartz et al. 2003), and the DExC/H-box RNA helicase Dhh1 (Coller et al. 2001). They leave a 5' monophosphate susceptible to an exonucleolytic decay. The exposed transcript is subjected to cleavage by 5'-3' exoribonucleases such as Xrn1p and Rat1p in eukaryotic cells (Johnson 1997). In contrast, mRNAs can be subjected to degradation by 3'-5' decay. It is carried out by an exosome which is comprised of diverse 3'-5' exoribonucleases, several accessory proteins, and RNA helicases (Parker and Song 2004).

The relative prevalence between the two exoribonucleolytic pathways remains controversial because it appears these two pathways redundantly supplement each other (Parker and Song 2004). Knocking out components of either the 5'-3' or the 3'-5' pathway had minimal effects on the pool of mRNAs present in yeast (Houalla et al. 2006). In mammalian cells, decay intermediate analysis showed that both 5'-3' and 3'-5' pathways are involved in mRNAs that carry adenosine-uridine rich element (ARE) suggesting dynamic and flexible pathways of mRNA decay (Garneau et al. 2007).

1.1.3 Endoribonuclease decay pathways

In contrast to exoribonucleolytic pathway and mRNA surveillance pathway, endoribonucleolytic cleavage of cellular mRNAs is not well-established. This may be the most efficient means of destroying an mRNA because it produces two fragments of mRNA that are susceptible to exonucleases. Some of cellular endonucleasese that have been characterized in recent years are PMR1, IRE1, RNase L, Dicer, Ago2, hDIS3, and APE1 (Li et al. 2010 and Tomecki et al. 2010).
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PMR1 was first identified as a polysome-associated endonuclease that could destabilize certain serum protein mRNAs including albumin and vitellogenin (Cunningham et al. 2001 and Yang and Schoenberg 2004). IRE1 is known to target actively translating mRNAs that are usually targeted to the endoplasmic reticulum (ER) (Hollien and Weissman 2006). Then it works as a part of the unfolded-protein response during periods of ER stress by splicing and activating X-box-binding protein 1 (XBP1) gene (Calfon et al. 2002 and Lin et al. 2007) and degrading mRNAs that are localized to the ER (Hollien and Weissman 2006). In contrast, RNase L is involved in an antiviral response (Bisbal et al. 2007). Dicer and Ago2 are involved in the biogenesis of small RNAs, including siRNAs and miRNAs for post-transcriptional gene silencing (Kim et al. 2009a and Liu et al. 2004). Dicer is responsible for cleaving double-stranded RNAs in which its product gets loaded onto the RISC complex and processed by Ago2; thus, they play an important role in gene regulation via RNA interference mechanism (Murchison et al. 2007 and Liu et al. 2004). Lastly, APE1 was recently discovered to have an endoribonucleolytic activity against c-myc mRNA in vitro (Kim et al. 2009).

Endoribonucleases are highly specific and/or regulated and they appear to be only activated when needed (Garneau et al. 2007). So far, there are still many questions that need to be answered for a better understanding of their mechanisms and cellular functions.

1.1.4 mRNA degradation locales

Lastly, mRNA degradation locales such as P bodies (PBs) and stress granules (SGs) are additional determinants of mRNA degradation.

Processing bodies (PBs) are granular cytoplasmic foci that have concentrated components of the 5'-3' mRNA-decay pathway. These components include general mRNA decay factors, NMD factors, AU-rich element-mediated mRNA decay (AMD) factors, and miRNA-induced
mRNA factors (Anderson and Kedersha 2006, Eulalio et al. 2006, Garneau et al. 2007, and Kulkarni et al. 2010). They are tightly linked to the decay of many short-lived mRNAs that contain a premature termination codon (Kulkarni et al. 2010) and AREs in their 3' UTR (Franks and Lykke-Anderson 2007). They also degrade target mRNAs of miRNAs (Liu et al. 2005). They are thought to have an equilibrium of formation and dissociation according to the amount of mRNA that is subjected to silencing, translational arrest, or rapid decay (Kulkarni et al. 2010).

Because mRNAs in PBs are also in a dynamic equilibrium with polysomes (Teixeira et al. 2005) and mRNA-decay intermediates can be found in PBs (Sheth and Parker 2003), researchers consider PBs as the site of mRNA turnover.

In contrast, SGs are often referred to as an mRNA silencing foci. Although PBs and SGs share some components and can interact physically, they are known to have an independent regulatory system, sets of different markers, and separate functions (Kedersha and Anderson 2009). Interestingly, SGs are formed in response to a stress-induced translational arrest and are more closely linked to translation and the sorting of specific mRNAs. They are formed under certain environmental stresses such as in heat shock, oxidative stress or energy deprivation (Anderson and Kedersha 2008). Under these stressors, the translation of housekeeping genes is arrested and un-translated mRNA accumulates in SGs (Nonhoff et al. 2007). This creates a high local concentration of mRNA protein complexes (mRNPs) which in turn may increase their reactions in SGs or decrease their reactions in the cytosol (Buchan and Parker 2009). The aggregation of molecules and factors may also help to protect unemployed mRNAs from deleterious molecules and subsequent decay. Altogether these have potentials to alter the translation and degradation of the mRNA but they are yet to be carefully studied (Buchan and Parker 2009).
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1.2 Biochemical functions of APE1

Human APE1 is a 35.5kDa protein and it is often referred as APEX1 or Ref-1. It is the mammalian ortholog of Escherichia coli Xth (Exo III) and is known as a multi-functional protein that plays an important role in DNA repair upon cellular stress and DNA damage and in redox reactions (Tell et al. 2009). In this section, the main functions of APE1 will be discussed. This includes 3' DNA phosphodiesterase, 3'-5' DNA exonuclease, nucleotide incision repair (NIR), transcriptional repressor, and RNase H activities.

1.2.1 Established functions of APE1 in the nucleus

1.2.1.1 Abasic DNA incision activity

DNA damage is continuously generated under physiological conditions due to the intrinsic instability of nucleic acid or because of the attacks by endogenous chemical species, most notably reactive oxygen or nitrogen species formed during mitochondrial respiration (Lindahl 1993, and Lindahl and Barnes 2000). One of the most frequent forms of DNA damage is the apurinic/apyrimidinic (AP) site and these AP sites are the product of both spontaneous and damage-induced base loss (Figure 1) (Loeb and Preston 1986). AP sites are repaired through the DNA base excision repair (BER) pathway and APE1 plays an important role in recognizing the AP site and cleaving the site to generate 3'-OH termini and 5'-deoxyribose phosphate termini (Izumi et al. 2000).
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Figure 1. Structures of adenine, abasic DNA and adenosine base. The AP site is product of DNA damage that occurs spontaneously under physiological conditions.

Owing to its important role in the BER pathway, the active site and essential residues involved in the abasic DNA cleaving activity have been studied extensively (Gorman et al. 1997, Mol et al. 2000, and Beernink et al. 2001). The essential residues identified are Asn68, Asp70, Asp90, Glu96, Tyr171, Arg177, Asp210, Asn212, Met270, Asp283, Asp308, and His309 in which most of them are involved in a hydrogen bonding network. A summary of the proposed role of each residue in AP site catalysis is presented in Table 1. Even though the catalytic mechanism of abasic DNA incision activity of APE1 and the essential residues were discovered, there are still debatable details which did not reach a conclusion (Gorman et al. 1997, Mol et al. 2000, and Beernink et al. 2001). Therefore, further work is required to gain a full story of the abasic DNA endonuclease catalytic mechanism of APE1.
Table 1. Summary of proposed roles of critical residues of APE1 in abasic DNA incision activities.

<table>
<thead>
<tr>
<th>Critical Residues</th>
<th>Proposed role in AP site catalysis</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asn68</td>
<td>Forms hydrogen bonding network</td>
<td>Gorman et al. 1997</td>
</tr>
<tr>
<td></td>
<td>Binds to the divalent metal ion in the metal binding site A</td>
<td>Gorman et al. 1997</td>
</tr>
<tr>
<td>Asp70</td>
<td>Binds to the AP site</td>
<td>Barzilay et al. 1995</td>
</tr>
<tr>
<td></td>
<td>Co-ordinates Mg$^{2+}$</td>
<td>Barzilay et al. 1995</td>
</tr>
<tr>
<td>Lys98</td>
<td>Forms a hydrogen bonds to the carboxyl group of Asp70</td>
<td>Gorman et al. 1997 and Timofeyeva et al. 2011</td>
</tr>
<tr>
<td></td>
<td>Helps induced fit during the enzyme action</td>
<td>Gorman et al. 1997 and Timofeyeva et al. 2011</td>
</tr>
<tr>
<td>Glu96</td>
<td>Metal ion binding site (Mg$^{2+}$)</td>
<td>Gorman et al. 1997</td>
</tr>
<tr>
<td></td>
<td>Interacts with the phosphate group</td>
<td>Gorman et al. 1997</td>
</tr>
<tr>
<td></td>
<td>Helps nucleophilic attack and stabilizes the leaving group</td>
<td>Gorman et al. 1997</td>
</tr>
<tr>
<td>Tyr171</td>
<td>Forms hydrogen bonding network</td>
<td>Gorman et al. 1997 and Beernink et al. 2001</td>
</tr>
<tr>
<td></td>
<td>Co-ordinates Mg$^{2+}$</td>
<td>Gorman et al. 1997 and Beernink et al. 2001</td>
</tr>
<tr>
<td>Arg177</td>
<td>Delivers a hydrogen bond to the AP site 3'-phosphate</td>
<td>Gorman et al. 1997 and Beernink et al. 2001</td>
</tr>
<tr>
<td></td>
<td>Locks APE1 onto abasic DNA</td>
<td>Gorman et al. 1997 and Beernink et al. 2001</td>
</tr>
<tr>
<td>Asp210</td>
<td>Acts as the Lewis base</td>
<td>Erzberger and Wilson 1999</td>
</tr>
<tr>
<td></td>
<td>Protonates the 3'-leaving group</td>
<td>Erzberger and Wilson 1999</td>
</tr>
<tr>
<td>Asn212</td>
<td>Interacts with oxygen on the phosphate backbone</td>
<td>Mundle et al. 2009</td>
</tr>
<tr>
<td></td>
<td>Orient bond for incision</td>
<td>Mundle et al. 2009</td>
</tr>
<tr>
<td>Met270</td>
<td>Locks APE1 onto abasic DNA</td>
<td>Mundle et al. 2009</td>
</tr>
<tr>
<td>Asp283</td>
<td>Forms a hydrogen bond with His309</td>
<td>Mundle et al. 2009</td>
</tr>
<tr>
<td></td>
<td>Stabilizes the positive charge that develops upon proton abstraction.</td>
<td>Mundle et al. 2009</td>
</tr>
<tr>
<td></td>
<td>Coordinates metal ions</td>
<td>Mundle et al. 2009</td>
</tr>
<tr>
<td>Asp308</td>
<td>Stabilize the transition state</td>
<td>Erzberger and Wilson 1999</td>
</tr>
<tr>
<td></td>
<td>Facilitate the dissociation of the product.</td>
<td>Erzberger and Wilson 1999</td>
</tr>
<tr>
<td>His309</td>
<td>Acts as the general base</td>
<td>Mundle et al. 2009</td>
</tr>
<tr>
<td></td>
<td>Abstracts a proton from a water molecule to generate the active site nucleophile</td>
<td>Mundle et al. 2009</td>
</tr>
</tbody>
</table>

In BER pathway, there are enzymes that directly act on DNA and other protein factors that are involved in recruitment of other necessary components for the pathway. For instance, it
requires a DNA glycosylase which cleaves a specific modified or damaged base and generates the AP site (Tell et al. 2009). APE1 which cleaves the AP site, DNA polymerase b which replaces the missing nucleotide, and DNA ligase which carries out the ligation reaction are also required (Tell et al. 2009). On the other hand, there are proteins that recruit other BER proteins and increasing overall BER pathway such as XRCC1, proliferating cell nuclear antigen (PCNA), and poly(ADP-ribose) (Caldecott et al. 1996). Interestingly, APE1 also acts as a recruitment protein in the BER pathway by interacting directly or indirectly with other BER enzymes such as glycosylases (OGG-1, MYH, MPG), DNA polymerase b, XRCC1 and FEN1 (Tell et al. 2009). These interactions are also known to benefit the BER pathway through activation of the recruited BER enzymes (Tell et al. 2009). Therefore, APE1 is considered as a key component in the BER pathway.

1.2.1.2 Redox activity

In cells, maintaining the intracellular redox balance is very important for all aerobic organisms because they are always exposed to the reactive oxygen species (ROS) from an oxidative stress. Most ROS produced in cells are the by-products of respiration and ionizing radiation (Tell et al. 2009). Therefore, cells have developed their own system to control the intracellular amount of ROS by implementing antioxidant enzymatic and non-enzymatic systems such as glutathione, catalase, peroxidases etc. (Tell et al. 2005). APE1 has been identified as a nuclear redox protein which activates the DNA binding activity of several transcription factors such as nuclear factor-kappaB (NF-κB), early growth response protein-1 (Egr-1), p53, HIF-1α, CREB, AP-1 and paired box-containing (Pax) proteins (Xanthoudakis and Curran 1992a and 1992b, Huang and Adamson 1993, Gaiddon et al. 1999, and Nishi et al. 2002). There are two components that are considered as functional domains for the APE1 redox activity. They are the
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N-terminal region between residues 35 and 127, called redox co-activator, and one cysteine residue, Cys65, located in the DNA-binding domain (Tell et al. 2002 and Tell et al. 2009). APE1 provides a redox-dependent mechanism for regulation of the aforementioned target transcription factors (Georgiadis et al. 2008). It is carried through the action of these functional domains which are maintained in the reduced state (Georgiadis et al. 2008). Researchers have proposed that a conformational change is needed for APE1 to expose the critical residue, Cys65, to confer its redox activity (Georgiadis et al. 2008 and Manvilla et al. 2011). S-glutathionylation of Cys99 and subsequent conformational change to abolish abasic DNA incision activity supports this hypothesis (Kim et al. 2011a).

Recently, redox chaperone activity of APE1 has been reported, suggesting that APE1 binds to target transcription factors and carries out its redox activity by using other reducing molecules such as glutathione and thioredoxin rather than by APE1 itself (Ando et al. 2008 and Schlotterer et al. 2010). This redox-chaperone activity is thought to be mediated by direct interactions between APE1 and target transcription factors and it does not require high concentrations of APE1 as does its redox activity (Ando et al. 2008). In contrast, very recent study supports APE1 as a unique redox protein by itself. This study has identified three cysteine residues (C65, C93, and C99) as necessary and sufficient for APE1’s redox activity (Luo et al. 2011). The residues can form disulfide bonds between them and they also can form disulfide bonds with thioredoxin upon oxidation (Luo et al. 2011). Therefore, it still remains unknown exactly how APE1 carries out its essential redox activity in vivo and further work is clearly required.
1.2.2 Other functions of APE1

Besides the two major functions discussed above, APE1 is known to have other functions which includes 3' DNA phosphodiesterase, 3'-5' DNA exonuclease, nucleotide incision repair (NIR), transcriptional repressor, and RNase H activities.

Firstly, APE1 has 3' DNA phosphodiesterase activities that can remove 3'-blocking phosphate groups to produce 3'-OH (Suh et al. 1997 and Evans et al. 2000). These 3'-blocking groups are generated by DNA glycosylases NEIL1 and NEIL2 (Izumi et al. 2003). APE1 also removes deoxyribonucleoside analogs via its 3'-5' exonuclease activity because these deoxyribonucleoside analogs that are formed by complex glycosylases and by radiation are capable of blocking repair pathway (Flaherty et al. 2002). All of these activities are estimated to have weaker activities than its abasic incision activity by 33-fold for 3'-5' exonuclease activity to a 100-fold for 3' DNA phosphatase (Wilson et al. 1995) and 3' DNA phosphodiesterase activities (Krokan et al. 2000).

Other than those nuclease activities of APE1, APE1 has been implicated in NIR pathway. It has been suggested that APE1 can cleave the sugar-phosphate backbone of a damaged nucleotide and produce a break with 3'-OH and 5'-phosphate (Ischenko and Saparbaev 2002). The 3'-OH end gets repaired with DNA polymerase that adds a new nucleotide to fill the gap and the 5'-phosphate end gets processed by FEN1 (Ischenko and Saparbaev 2002). The optimal conditions for the NIR pathway is very different than that of abasic DNA incision activity (Gros et al. 2004) which suggests NIR-related activities of APE1 are determined by different domains and different mechanisms (Gros et al. 2004).

APE1 has also been suggested to play a role in negatively regulating the Rac1/GTPase to prevent oxidative stress (Ozaki et al. 2002) and to regulate vascular tone and endothelial nitric oxide production (Jeon et al. 2004). It was first discovered in the promoter of the human
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parathyroid hormone (PTH) gene and later in the APE1 promoter itself (Okazaki et al. 1994 and Izumi et al. 1996). APE1 is considered as a transcriptional repressor as a component of a trans-acting complex through binding to negative calcium responsive elements (N-CaRE) (Okazaki et al. 1994). This is supported by another study done by Bhakat et al. (2003) which showed calcium-induced APE1 acetylation at lysine residues 6 and 7 and this acetylated APE1 has an increased capability of binding nCaRE elements. This APE1 acetylation can turn specific functions of APE1 on and off; therefore, it suggests the important role of specific post-translational modifications of APE1 as switches. Post-translational modifications are considered as important mechanisms in regulating the multiple functions of APE1 and interactions of proteins with APE1 (Bhakat et al. 2003).

Lastly, APE1 is known to possess RNase H activity (Barzilay et al. 1995) as well as RNA binding ability in association with ribosomes in the cytoplasm of different cell types (O'Hara et al. 2009). It also has the ability to interact with other protein factors involved in RNA metabolism such as YB-1 (Chattopadhyay et al. 2008), RNA polymerase II (Sengupta et al. 2011) and hnRNP-L (Xanthoudakis et al. 1994). Recently, researchers have discovered the ability of APE1 to cleave abasic ssRNA molecules (Berquist et al. 2008) and c-myc mRNA (Barnes et al. 2009) providing support for the notion that APE1 is an ideal candidate for a novel RNA cleansing process. These roles of APE1 in RNA metabolism will be further discussed in more detail in the following section.
1.3 APE1 in RNA metabolism

APE1 serves multiple functions in the cell. Previously known functions include endonuclease activity on abasic DNA, redox activation of transcription factors implicated in apoptosis and cell growth, as well as 3' phosphodiesterase, 3'-5' exonuclease, and RNase H activities. There have been a few skeptical discoveries of association of APE1 with other factors that are involved in RNA metabolism and RNase H activities of APE1 in the 1990s (Xanthoudakis et al. 1994 and Barzilay et al. 1995). And recently there have been increasing studies on the novel functions of APE1 in controlling RNA metabolism. This phenomenon is expected because it opens up the new areas of studying multiple functions of APE1 in translational research. Up to date, there are three components that support this new role of APE1 in RNA metabolism: (i) physical association of APE1 with other factors that are involved in RNA metabolism, (ii) discovery of abasic RNA incision activity and its role in rRNA control, and (iii) discovery of endoribonuclease activity of APE1 and its role in the control of mRNA abundance.

1.3.1 Physical association of APE1 with proteins involved in RNA metabolism

The first evidence of the involvement of APE1 in RNA metabolism was discovered by Barzilay et al. (1995) where the RNase H activity of APE1 was demonstrated. However, this alone was not able to convince other researchers who were very skeptical about APE1's new role. It was the next discovery of the interaction and association of APE1 with ribosome that slowly led scientists to view APE1 differently (Duguid et al. 1995). So far, APE1 has been found to interact with a number of other protein factors involved in RNA metabolism and they can be divided into five groups of proteins that fall into different areas of RNA metabolism (Table 2).
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The first group of proteins, hS3 and YB-1, are involved in recognition of damaged nucleic acids including RNA (Table 2). Both proteins are known to bind to 8-oxoguanine (8-oxo-G) residues, ssRNA, and RNA molecules. They are thought to protect RNA molecules from degradation or recruit other proteins for repair (Hedge et al. 2004 and Chattopadhyay et al. 2008). Proteins involved in pre-mRNA maturation or splicing are the next group. These include hnRNP-L, PRP19, and MEP 50 (Table 2). Since hnRNP-L exists as a complex of hnRNPs and hnRNAs (Pinol-Roma et al. 1989 and Dreyfuss et al. 1993), it has been of no surprise when APE1 was discovered to have an association with RNA polymerase II which synthesizes hnRNAs in the nucleus (Sengupta et al. 2011). This RNA polymerase II involved in RNA synthesis of pre-mRNA, snRNA, and microRNA is the third group of protein associated with APE1 (Table 2). Proteins that are involved in rRNA metabolism or ribosome biogenesis (NPM1, RSSA, and RLA0) are the fourth group (Table 2). Lastly, PRPS1 and PRPS2 that are involved in general ribonucleotides metabolism and synthesis are the fifth group (Table 2).
Table 2. List of APE1 interacting proteins found in the literature.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Functions</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteins involved in</td>
<td></td>
<td></td>
</tr>
<tr>
<td>recognition of</td>
<td></td>
<td></td>
</tr>
<tr>
<td>damaged RNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hS3</td>
<td>Binding to 8-oxo-G residues of RNA</td>
<td>Hedge et al. 2004</td>
</tr>
<tr>
<td></td>
<td>Binding to ssRNA</td>
<td></td>
</tr>
<tr>
<td>YB-1</td>
<td>Binding to ssRNA and RNA molecules</td>
<td>Chattopadhyay et</td>
</tr>
<tr>
<td></td>
<td>Protection of mRNA from degradation</td>
<td>al. 2008</td>
</tr>
<tr>
<td></td>
<td>Binding to 8-oxo-G containing RNAs</td>
<td></td>
</tr>
<tr>
<td>Proteins involved in</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pre-mRNA maturation or</td>
<td></td>
<td></td>
</tr>
<tr>
<td>splicing</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hnRNP-L</td>
<td>Pre-mRNA splicing</td>
<td>Kuninger et al. 202</td>
</tr>
<tr>
<td></td>
<td>Regulation of chromatin modification</td>
<td>2</td>
</tr>
<tr>
<td>PRP19</td>
<td>DNA double-strand break (DSB) repair</td>
<td>Vascotto et al. 20</td>
</tr>
<tr>
<td></td>
<td>Pre-mRNA splicing</td>
<td>09</td>
</tr>
<tr>
<td>MEP50</td>
<td>Component of the 20S PRMT5 containing methyltransferase complex, which</td>
<td>Vascotto et al. 20</td>
</tr>
<tr>
<td></td>
<td>modifies specific arginines to dimethylarginines in several spliceosomal</td>
<td>09</td>
</tr>
<tr>
<td></td>
<td>Sm proteins</td>
<td></td>
</tr>
<tr>
<td>Protein involved in</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RNA synthesis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RNA polymerase II</td>
<td>Catalysis of DNA transcription</td>
<td>Sengupta et al. 20</td>
</tr>
<tr>
<td></td>
<td>Synthesis pre-mRNA and most snRNA and microRNA.</td>
<td>11</td>
</tr>
<tr>
<td>Proteins involved in</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rRNA metabolism or</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ribosome biogenesis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NPM1</td>
<td>Assembly and transport of ribosomal protein</td>
<td>Vascotto et al. 20</td>
</tr>
<tr>
<td></td>
<td>Control of centrosome duplication</td>
<td>09</td>
</tr>
<tr>
<td></td>
<td>Regulation of the tumor suppressor ARF</td>
<td></td>
</tr>
<tr>
<td>RSSA</td>
<td>Member of the ribosomal protein S2P family</td>
<td>Vascotto et al. 20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>09</td>
</tr>
<tr>
<td>RLA0</td>
<td>Acidic ribosomal protein, rich in hydrophobic amino acid residues</td>
<td>Vascotto et al. 20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>09</td>
</tr>
<tr>
<td>Proteins involved in</td>
<td></td>
<td></td>
</tr>
<tr>
<td>general ribonucleotides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>metabolism and synthesis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PRPS1</td>
<td>Catalysis of the phosphoribosylation of ribose 5-phosphate to 5-phosphoribosyl-1-pyrophosphate, which is necessary for the de-novo and salvage pathways of purine and pyrimidine biosynthesis</td>
<td>Vascotto et al. 20</td>
</tr>
<tr>
<td>PRPS2</td>
<td>Ribose metabolism</td>
<td>Vascotto et al. 20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>09</td>
</tr>
</tbody>
</table>

Altogether, these interactions and associations of APE1 with different protein factors involved in RNA metabolism suggest a direct involvement of APE1 in RNA metabolic pathways. However, it is still unclear which specific activities of APE1 can be exerted with the association of these proteins and how these affect the overall metabolism of RNAs.
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1.3.2 Discovery of abasic RNA incision activity and its role in rRNA control

The second evidence of the involvement of APE1 in RNA metabolism was first highlighted by Berquist et al. (2008) when they showed APE1’s ability to cleave abasic ssRNA molecules. However, this alone was insufficient to prove APE1 as a novel RNA cleansing protein since researchers was not able to address whether the formation of abasic RNA molecules is a spontaneous or guided process and whether the damaged RNAs were significant enough to cause defect in general protein synthesis (Berquist et al. 2008).

Interestingly, the N-terminal region of APE1 was discovered to interact with NPM1 and regulate rRNA metabolism within nucleoli (Vascotto et al. 2009). The five lysine residues, K24, K25, K27, K31 and K32, are involved in the interaction of APE1 with both RNA and NPM1 (Fantini et al. 2010). By binding to the N-terminal region of APE1 required for stable RNA binding, NPM1 reduces abasic ssRNA incision activity of APE1. In contrast, disruption of the APE1-NPM1 interactions increases the activity of APE1 (Tell et al. 2010). It was also found that oxidized rRNA such as 8-oxo-G rRNA was accumulated in APE1-depleted cells and caused impaired translation, lowered intracellular protein content and decreased cell growth rate (Vascotto et al. 2009). It was postulated that after APE1 cleaves the damaged rRNA, exosome complex and XRN1 exoribonuclease may degrade the resulting RNA fragments (Tell et al. 2010).

The aforementioned findings demonstrated that APE1 can recognize and cleave abasic RNA, affect cell growth through its involvement in rRNA control mechanism, play a role as a novel RNA cleansing enzyme, and affect gene expression through post-transcriptional mechanisms (Vascotto et al. 2009 and Tell et al. 2010).
1.3.3 Discovery of endoribonuclease activity of APE1 and its role in the control of mRNA half-life

The third evidence for the involvement of APE1 in RNA metabolism was discovered by Barnes et al. (2009) who identified APE1 as an endoribonuclease that specifically cleave in between the single stranded regions of UA, CA, and UG dinucleotides at a specific coding region of c-myc mRNA termed the coding region determinant (CRD) \textit{in vitro}. The knock-down of APE1 in HeLa cells led to an increased steady-state c-myc mRNA levels and half-life (Barnes et al. 2009), suggesting that APE1 may control c-myc mRNA degradation in cells. In addition, another study also showed APE1 can cleave CD44 mRNA, micro RNA (miR-21 and miR-10b), and three RNA components of SARS-corona virus (orf1b, orf3, spike) \textit{in vitro} (Kim et al. 2010). They also reported that APE1 can reduce the ability of the Dicer enzyme to process pre-miRNAs \textit{in vitro} (Kim et al. 2010); however, its activity against all these RNA substrates \textit{in vivo} are yet to be demonstrated.

It was suggested that the RNA cleaving activity of APE1 utilizes the same active site as the abasic DNA incision activity since because both E96A and H309N APE1 mutants were almost inactive in cleaving the RNA template (Barnes et al. 2009). Further site-directed mutagenesis studies on APE1 active site using N68A, D70A, Y171F, D210N, F266A, D308A, and H309S confirmed that APE1 shares the same active site for both abasic DNA incision activities and RNA cleaving activity (Kim et al. 2011b). However, these APE1 mutants retained their ability to bind the RNA substrate, required 2'-OH on the sugar for cleavage, and did not require presence of a divalent ion unlike abasic DNA incision activity of APE1 (Kim et al. 2011). Therefore, it was postulated that although APE1 shares the same active site, they may have different mechanisms for cleaving RNA, abasic ssRNA, and abasic DNA.
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These results demonstrate APE1 as an endoribonuclease that has the potential to influence the stability of mRNAs, microRNAs, and SARS-corona virus RNAs and provide further support for its role in the control of RNA metabolism.

1.4 Role of APE1 in cancer

1.4.1 Possible role of APE1 in cancers

APE1 is a multi-functional enzyme that has abasic DNA incision, redox regulation, 3' DNA phosphatase, 3' DNA phosphodiesterase, 3'-5' DNA Exonuclease, NIR, transcriptional repressor, and RNase H activities. The abasic DNA incision activity of APE1 in BER pathway is very well studied and this activity is one of the main functions of APE1.

Currently, chemotherapy and radiation therapy are selected as one of the main ways to treat cancers and these are based on creating DNA damage which leads to an impaired cell signaling and eventual death (Helleday et al. 2008). For normal cells, this DNA repair pathway is required for spontaneous oxidative stresses that all aerobic organisms are exposed to. However, any defect in a specific DNA repair pathway in cancer cells can lead to an abnormally efficient DNA repairs and reduce the effect of cancer therapy (Bapat et al. 2009). APE1, an important component of BER pathway, is known to contribute to the maintenance of genetic integrity by repairing abasic DNA and regulating apoptosis by activating transcription factors. Therefore, defect in APE1 activities or altered expressions of APE1 are associated with cancer proliferation and can influence the sensitivity of the tumor cells to therapeutic reagents (Bapat et al. 2009). Some of examples are the correlations demonstrated between high APE1 levels and the resistance of head and neck cancers (Koukourakis et al. 2001), glioma cells (Bobola et al. 2004) and medulloblastoma (Bobola et al. 2005), osteosarcoma (Wang et al. 2004), and primitive
neuroectodermal tumors (Bobola et al. 2004) to chemo/radiotherapy. Another good example illustrating this point is targeting the DNA repair activity of APE1 as a cancer treatment strategy when patients were treated with clinically relevant DNA damaging chemotherapeutic agents such as bleomycin, carmustine, temozolomide (TMZ), and gumericabine (Madhusudan et al. 2005, and Luo and Kelley 2004). All of these resulted in a reduced APE1 activity, decreased cell proliferation, and increased apoptosis and hypersensitivity. Many studies have also shown an increased hypersensitivity of APE1 knock-down cancer cell lines to chemotherapeutic agents. Providing further support for these findings were studies in animal models using anti-sense oligonucleotides (reviewed in Fishel and Kelley 2007).

1.4.2 Relationship of APE1 human population variants and cancers

Interestingly, researchers have found the correlation of APE1 polymorphisms with many diseases including cancers (Wilson et al. 2011). Polymorphism of APE1, high frequencies of inherited DNA sequence variants, is found in the human population. The DNA polymorphism of APE1 has been under intensive investigation because of the association of polymorphic APE1 genes with the development of cancers of the bladder (Figueroa et al. 2007), breast (Rossner et al. 2006), esophagus (Zhai et al. 2009), lung (Li et al. 2011) and cervix-uterine (Cheng et al. 2009). The major sequence variants identified in APE1 gene and their function changes are summarized and shown in Figure 2.

Sequence variants that are located on the surface of the APE1 endonuclease domain were predicted to be damaging to its function (Hadi et al. 2000). D148E APE1 is the population variant with the highest observed frequency of ~46% (Tomkins et al. 2000). D148E was predicted to have no impact on the structure or function such as abasic DNA incision activity but the cancer cells carrying this mutation showed hypersensitivity to ionizing radiation (Hu et al. 2001). Other studies have shown the association of D148E polymorphism to various cancer risks in the human population but there are also studies which showed no such associations (Hung et al. 2005, Jiao et al. 2006, Bartsch et al. 2007, Lo et al. 2009, Deng et al. 2010, and Ji et al. 2011). There is no identified associated risk for Q51H but I64V has been associated with a decreased lung cancer risk (Zienolddiny et al. 2006).
Abasic DNA endonuclease activity of population variants of APE1 has been previously reported. L104R, E126D, R237A, and D283G were found to exhibit about 40-60% reductions in specific abasic DNA cleaving activity whereas no defect in endonuclease activity was found in D148E, G241R, and G306A variants (Hadi et al. 2000). In contrast, recent work by Dr. Lee's lab discovered that D148E, Q51H, I64V, G241R, R237A, and G306A, exhibited 76-85% reductions in endoribonuclease activity against a specific coding region of c-myc RNA (Kim et al. 2012). Interestingly, both L104R and E126D variants exhibited endoribonuclease activity which is distinct from the WT-APE1 (Kim et al. 2012). Further studies on APE1 polymorphisms in an effort to discover their effects on protein structure, nuclease activities, redox (REF-1) regulatory function, intracellular distribution should be undertaken in order to gain a better understanding of the correlation of APE1 polymorphisms and cancer risks.

1.4.3 Relationship of cellular localization of APE1 and cancers, and proposed functions of APE1 in each cellular compartment

Most reports demonstrate that APE1 localizes to the nucleus to carry out its abasic DNA incision and redox regulatory activities (Tell et al. 2005). However, there are number of immunohistochemical studies on APE1 localization reporting differences in APE1 sub-cellular localization in different cell types including cancers (Duguid et al. 1995, Kakolyris et al. 1997, Kakolyris et al. 1998a, Kakolyris et al. 1998b, Kakolyris et al. 1999, Puglisi et al. 2001, Kelley et al. 2001, and Di Maso et al. 2007). Altered localization of APE1 from nucleus to cytoplasm shown by stains in various tumors has been well-documented (Evan et al. 2000 and Tell et al. 2005). This cytoplasmic expression of APE1 is correlated with more aggressive carcinomas and a poor prognosis for the patients (Tell et al. 2010). This has led to the speculation of yet undefined extra-nuclear roles of APE1 and its potential implications in cancer development. In
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this section I will review mechanisms that are known to control APE1 localization and the cytoplasmic localization of APE1 in regards to its functions and associations with cancers.

1.4.3.1 Mechanisms that control APE1 localization

APE1 like any other cellular proteins gets translated in the cytoplasm and then translocated to the nucleus. This nuclear localization of APE1 is controlled by the nuclear localization signal (NLS) at its N-terminus. The NLS is composed of first 20 amino acids and is an unstructured sequence which uses karyopherin a1 and a2 for its translocation (Jackson et al. 2005). This region of APE1 is also important for abasic RNA binding and is involved in protein-protein interactions that are devoted to redox-mediated transcriptional co-activation activity (Vascotto et al. 2009). It was proposed that Granzyme A cleaves APE1 at Lys31 and this results in APE1 missing the first 33 amino acid residues (ND33-APE1) (Fan et al. 2003). In turn this alters APE1 accumulation within the nucleus (Fan et al. 2003 and Jackson et al. 2005). There is also a finding which reported proteolysis occurring at residue Asn33 rather than Lys31 (Chattopadhyay et al. 2006). The proteolysis at the 33rd residue of APE1 may be a general molecular device for redirecting APE1 to mitochondria (Tell et al. 2001) and this is supported by the discovery that showed mitochondrial APE1 (mtAPE1) as a truncation product of APE1. The truncated APE1 does not have the first 33 N-terminal residues in bovine liver, mouse liver, and NIH 3T3 cell line (Chattopadhyay et al. 2006). Given that mtAPE1 lacks the N-terminal domain which aids in BER pathway by enhancing abasic DNA binding and interactions with other repair protein, it is considered not to be involved in interactions with other proteins for DNA repair in the mitochondria (Mitra et al. 2007).

There also may be a nuclear export signal (NES) of APE1 that regulates APE1 translocation from the nucleus to the cytoplasm. The nuclear accumulation of APE1 after
treatment with the nuclear export inhibitor Leptomycin B was observed and it suggested the presence of a NES that is near a Leu-rich region at the C-terminus (Jackson et al. 2005). They noted three leucine residues, Leu291, Leu292, and Leu295 residues, which are exposed in the surface of the APE1 3D structure and modification of these residues might have caused nuclear accumulation (Jackson et al. 2005). Others have suggested that the two cysteine residues of APE1, Cys93 and Cys310, can undergo S-nitrosation in response to a nitric oxide stimulation and this can lead to the export of APE1 in a CRM1-independent process (Qu et al. 2007). It has been suggested that APE1 has a NES at the 64-80 amino acid residues and along with these two cysteine residues, they are responsible for re-localization of APE1 to the cytoplasm (Qu et al. 2007).

Lastly, the interaction with specific nuclear proteins can be a possible mechanism for APE1 nuclear distribution. Jung et al. (2007) found that the nuclear localization of APE1 was regulated by the GAFF45a nuclear protein expression. However, further study on the downstream mechanism of this nuclear import of APE1 is required.

1.4.3.2 Cytoplasmic localization of APE1, its proposed functions, and its relationship with cancers

The cytoplasmic localization of APE1 has been noted in cells that have high metabolic or proliferactive rates such as spermatocytes, thyrocytes, lymphocytes, heptocytes, and hippocampal cells (Duguid et al. 1995, Kakolyris et al. 1998, Kakolyris et al. 1997, Rivkees and Kelley 1994, Tell et al. 2000, and Wilson et al. 1996). Other cell types are known to exhibit both nuclear and cytoplasmic localization of APE1 and these include normal thyroid cells stimulated by TSH, transformed thyroid cell, mucosal and parietal cells of the stomach, cerebellar Purkinje cells, adrenal cortical cells, and some cervical cells (Tell et al. 2000, Kakolyris et al. 1998a, and Rivkees and Kelley 1994). The cytoplasmic APE1 is thought to have two main roles in the
cytoplasm. Firstly, as a mtDNA repair device in mitochondria upon oxidative stress (Rivkees and Kelley 1994 and Wilson et al. 1996). Secondly, as an ER stress response device where it induces apoptosis (Fan et al. 2003). Maintaining newly synthesized transcription factors in a reduced state prior to their transport to the nucleus is another proposed role for cytoplasmic APE1 (Mitra et al. 2006).

Recently, APE1's role in RNA metabolism has been highlighted (Barnes et al. 2009 and Tell et al. 2010). The involvement of the rRNA and mRNA control mechanisms may explain the cytoplasmic localization of APE1. Therefore, it appears that sub-cellular localization of APE1 may be governed by a strictly regulated process and the biological relevance of the APE1 localization clearly requires further investigations.

Researches have reported complex localization of APE1 in various tumors and these have been well-reviewed (Evan et al. 2000 and Tell et al. 2005). The nuclear/cytoplasmic and cytoplasmic expressions of APE1 in these tumors are also found to be correlated with more aggressive carcinomas and a poor prognosis for the patients. For instance, the cytoplasmic localization of APE1 in hepatocellular carcinoma (HCC) was associated with a significant lower degree of differentiation and with a shorter survival time (Di Maso et al. 2007). Similarly in epithelial ovarian cancer, correlations between the cytoplasmic localization of APE1 and low tumor differentiation, significantly higher stage, and lower survival rate were found (Sheng et al. 2011). All these findings suggest that cytoplasmic localization of APE1 is associated with tumor progression and APE1 may be used as a prognostic marker for aggressive cancers.
1.5 Research objectives

Although, there is increasing number of studies that investigated the possible link between the role of APE1 and its cytoplasmic localization no consensus has been reached. This has led to the speculation of yet undefined extra-nuclear roles of APE1 and its potential implications in cancer development. As a result, the role of APE1 in mRNA and rRNA metabolism has been recently highlighted (Tell et al. 2010). Recent works has shown that APE1 is capable of cleaving abasic RNA (Berquist et al. 2008) and physically associate with a nuclear protein NPM1, known to be involved in ribosomal RNA processing (Vascotto et al. 2009). This finding also supports the identification of APE1 as an endoribonuclease that may play a role as a cytoplasmic regulator of RNA metabolism (Barnes et al. 2009). In order to further understand the involvement of APE1 in RNA metabolism in the cytoplasm and the possible biological significance, the following research objectives were established.

The first objective was to confirm the cellular localization of wild-type and N-terminal deletion variants of APE1. In addition, the objective was to identify the cellular localization of APE1 human population variants. To accomplish this, we generated the APE1-GFP fusion proteins in order to visualize them in live cells of HeLa and HepG2. Visualization of the wild-type and N-terminal deletion variants of APE1 were expected to confirm the role of nuclear localization signal of APE1 located in the N-terminus. Also, we intended to assess the cellular localization of APE1 human population variants. To date, there has been no reports on the localization of APE1 human population variants nor was there reports on localization of APE1 ND20-population mutants. Therefore, our results from the analysis of the expression of APE1-population variants-GFP in HeLa and HepG2 cells are expected to reveal if there is a relationship between their sub-cellular localization and role in diseases.
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The second objective is to identify the cellular localization of wild-type, N-terminal deletion, and human population variants of APE1 under cellular stress. We were particularly interested in assessing the possible co-localization of APE1 with processing bodies and stress granules which are well-established mRNA degradation locales. The results are expected to reveal the re-distribution of APE1 under stress conditions and the involvement of APE1 in RNA metabolism carried out in the processing bodies and stress granules. We also preliminarily investigated APE1 distribution in the breast cancer tissue and its co-localization with processing bodies and stress granules. The results are expected to provide an insight of APE1 distribution in the breast cancer.
Chapter 2

Cellular localization of wild-type, N-terminus deleted APE1, and population variants

2.1 Introduction

This chapter presents the experiments conducted to determine the cellular localization of wild-type APE1, N-terminal deletion variants, and population variants of APE1. To date, there has been no report on localization of APE1 population variants nor were there reports on localization of APE1 ND20-population variants. Therefore, it is intriguing to evaluate the localization of APE1 and its population variants in HeLa and HepG2 cells.

2.2 Methodology

This section describes the methods employed in investigating the cellular localization of wild-type APE1, N-terminal deletion variants, and population variants of APE1 in HeLa and HepG2 cells.

2.2.1 Primer design

The following primers were designed to amplify APE1 cDNA from pFLAG-CMV-5.1 (4.7Kbp) vector (Table 3). Restriction sites of EcoRI and BamHI were incorporated for cloning of APE1 cDNA into pEGFP-N1 vector. These primers were generated by Integrated DNA Technologies (IDT) and were dissolved in autoclaved water to yield the concentration of 300 pmol/μl.
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Table 3. Sequences of primers used to amplify APE1 cDNA. Underlined text indicate where mutations were introduced.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>APE1-EcoRI-F</td>
<td>5'-ACC A GAA TTC AT ATG CCG AAG CGT GGG AAA-3'</td>
</tr>
<tr>
<td>APE1-ND20-EcoRI-F</td>
<td>5'-ACC A GAA TTC ATG CCA GAG GCC AAG AAG AGT AAG A-3'</td>
</tr>
<tr>
<td>APE1-ND41-EcoRI-F</td>
<td>5'-ACC A GAA TTC ATG CCA GCC CTG TAT G-3'</td>
</tr>
<tr>
<td>APE1-BamHI-R-no stop</td>
<td>5'-ACC A GGA TCC ATC AGT GCT AGG TAT GGG TGA TGA TGA G-3'</td>
</tr>
</tbody>
</table>

2.2.2 PCR amplification of WT APE1 and ND variants

To prepare cDNA for sub-cloning APE1, the pFLAG-CMV-5.1-APE1 was used as the template for PCR. A total reaction mixture of 25 µl contained 100 ng of pFLAG-CMV-5.1 plasmids, 1 µl of Phusion DNA polymerase (Finnzymes), 0.2 mM dNTPs, 1X Phusion HF reaction buffer (Finnzymes), 0.4 pmoles of forward and reverse primers. The mixture was put into the PCR MiniCycler PCR machine (MJ Research Inc., Watertown, MA) which was programmed to incubate the mixture at 95°C for 2 min, and for 30 cycles of 30 sec at 95°C, 30 sec at 55°C, and 1 min at 72°C. It was then incubated for 10 min extension at 72°C and held at 4°C. Once the PCR was finished, the mixture containing amplified cDNA was added with 2 µl of agarose loading dye and run in 1 % agarose gel for 1 hour at 100V in 0.5X TBE buffer. The PCR fragment was visualized under UV light and excised using a scalpel. The PCR fragment was purified using QIAEX II Gel Extraction Kit (Qiagen Inc., Valencia CA) and following the manufacturer's protocol.

2.2.3 Restriction Digestion of PCR products and plasmids

The purified PCR fragments of APE1 cDNA was digested using EcoRI and BamHI. The pEGFP vector was linearized using the same enzymes. A 20 µl digestion reaction contained 1-3
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µg of plasmids or PCR products, 1 µl of EcoRI and BamHI (10U/µl), 1x reaction bufer (ReACT 3 from Invitrogen), 0.1 µg/µl BSA, and made up to volume with autoclaved water. The digestion was carried at 37°C for 1 hour. After digestion, 1 µl of 1 kb DNA ladder (Invitrogen), all of linearized samples, and 1 µl of uncut plasmids were loaded onto 1% agarose gel to check for completion of the reaction. The gel was run for 1 hour at 100 V in 0.5X TBE buffer and visualized under UV light and the bands were excised. Each DNA band was purified using QIAEX II Gel Extraction Kit (Qiagen Inc., Valencia CA).

2.2.4 Sub-cloning WT-APE1 and APE1 ND variant cDNAs into pEGFP plasmid

The purified digested APE1 cDNA and pEGFP vector were ligated in a reaction mixture of 20 µl containing 1x Ligation Buffer (Invitrogen), 4 µl of linearized pEGFP vector, 8 µl of cut APE1 cDNA, 1 µl of T4 DNA ligase (Invitrogen), and made up to volume with autoclaved water. The reaction was incubated at room temperature for 2 hours.

Tubes of 100 µl DH5α bacteria from -80°C freezer were thawed on ice for 5 min. All of the 20 µl ligation reaction was transferred to each tube of bacteria and the bacteria were incubated for 25 min on ice. They were heat-shocked for 90 sec at 42°C in the water bath and then cold-shocked for 2 min on ice. To each tube, 500 µl of LB was added and it was incubated for 30 min at 37°C in the water bath. Each transformed bacteria was plated onto the agar plate containing appropriate antibiotic, Kanamycin (25 µg/ml), and was incubated at 37°C incubator overnight.

2.2.5 Mini plasmid preparation to isolate pWT-APE1, pND20, and pND41

A single colony was picked from the plate and was grown overnight at 37°C with shaking in a 50 ml falcon tube. The tube contained 10 mL of LB and the appropriate antibiotic, Kanamycin (25 µg/ml). The next day, the culture was split in 7 eppendorf tubes and was
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centrifuged for 2 min at 12,000 rpm. The cells were pelleted and the supernatant was removed by pipetting. The pEGFP-APE1 (pWT-APE1), pEGFP-ND20 (pND20), and pEGFP-ND41 (pND41) vectors were then purified using QIAprep® Spin Miniprep Kit (250) (Qiagen, Hilden, Germany) and following the manufacturer's protocol. The concentrations of purified plasmids pWT-APE1, pND20, and pND41 vectors were measured using the NanoDrop 1000 UV/Vis (Thermo Scientific, Wilmington, DE). Plasmid sequences were confirmed by DNA sequencing at Macrogen Inc. (Seoul, Korea). The data can be found in the Appendix.

2.2.6 Generation of APE1 population variants using pWT-APE1 vector

To generate population APE1 variants from pWT-APE1 vector, the following primers were used in site-directed mutagenesis (Table 4). These primers were synthesized by Integrated DNA Technologies (IDT) and were dissolved in autoclaved water to a final concentration of 300 pmol/μl. The pWT-APE1 vector containing cDNAs of APE1 was amplified via PCR using each set of primers.

Table 4. Sequences of forward and reverse primers to generate population APE1 variants using pWT-APE1 vector. Bolded texts indicate where the mutations were introduced.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>I64V-F</td>
<td>5' GCC ACA CTC AAG GTC TGC TCT TGG AAT-3'</td>
</tr>
<tr>
<td>I64V-R</td>
<td>5' -ATT CCA AGA GCA GAC CTT GAG TGT GGC-3'</td>
</tr>
<tr>
<td>L104R-F</td>
<td>5' -TCA GAG AAC AAA CGA CCA GCT GAA CTT-3'</td>
</tr>
<tr>
<td>L104R-R</td>
<td>5' -AAG TTC AGC TGG TCG TTT GTT CTC TGA-3'</td>
</tr>
<tr>
<td>E126D-F</td>
<td>5' -CCT TCG GAC AAG GAC GGG TAC AGT GCC-3'</td>
</tr>
<tr>
<td>E126D-R</td>
<td>5' -GCC ACT GTA CCC GTC TTT GTC CGA AGG-3'</td>
</tr>
<tr>
<td>D148E-F</td>
<td>5' -TAC GGC ATA GGC GAA GAG GAG CAT GAT-3'</td>
</tr>
<tr>
<td>D148E-R</td>
<td>5' -ATC ATG CTC CTC TCC GCC TAT GCC GTA-3'</td>
</tr>
<tr>
<td>G306A-F</td>
<td>5' -TCC AAG GCC CTC GCC AGT GAT CAC TGT-3'</td>
</tr>
<tr>
<td>G306A-R</td>
<td>5' -ACA GTG ATC ACT GCC GAG GCC CTT GGA-3'</td>
</tr>
<tr>
<td>D308A-F</td>
<td>5' -GCC CTC GCC AGT GCT CAC TGT CCT AT-3'</td>
</tr>
<tr>
<td>D308A-R</td>
<td>5' -G GAG CCG TCA CGA GTG ACA GGA TAG-5'</td>
</tr>
</tbody>
</table>
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2.2.7 Site directed mutagenesis to generate pWT-APE1 population variants and pND20-population variants

To generate pAPE1 population variants, the pWT-APE1 vector was amplified via PCR using each set of primers for each population variants of APE1. The pND20 vector was used for the generation of pND20-population variants. A total reaction mixture of 50 µl contained 100 ng of either pWT-APE1 or pND20 plasmids, 1 µl of Phusion DNA polymerase (Finnzymes), 0.2 mM dNTPs, 1xPhusion HF reaction buffer (Finnzymes), 0.4 pmoles of forward and reverse primers. The mixture was put into the PCR MiniCycler PCR machine (MJ Research Inc., Watertown, MA) which was programmed to incubate the mixture at 95°C for 2 min, and for 18 cycles of 45 sec at 95°C, 45 sec at 55°C, and 10 min at 72°C. Then it was incubated for 20 min extension at 72°C and hold at 4°C to finish generating vectors. Once the PCR was finished, the mixture was added with 5 µl of agarose loading dye and run in 1% agarose gel for 1 hour at 100 V in 0.5X TBE buffer. The PCR fragment was visualized under UV light and excised. The PCR fragment was purified using QIAEX II Gel Extraction Kit (Qiagen Inc., Valencia CA).

The purified vectors were transformed with 100 µl DH5α bacteria by heat-shock and plated on Kanamycin (25 µg/ml) plates. Three colonies for each vector were picked. Plasmids were isolated using the QIAprep® Spin Miniprep Kit (250) (Qiagen, Hilden, Germany) and following the manufacturer's protocol. Then the concentrations of purified vectors were measured using the NanoDrop 1000 UV/Vis (Thermo Scientific, Wilmington, DE). Plasmid sequences were confirmed by DNA sequencing performed at Macrogen Inc. (Seoul, Korea).
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2.2.8 Assessing the localization of WT-APE1-, ND20-, ND41-, and population variants-GFP

Cell culture and reagents

HeLa and HepG2 cells were cultured in a tissue culture flask (T25CN) (Sarstedt, Newton, NC) with 10 ml of Minimum Essential Medium (MEM) (Invitrogen, Carlsbad, CA) supplemented with 10 % fetal bovine Serum (FBS). The cells were incubated under 5 % CO₂ and at 37 °C. Maintenance of cells were done by splitting the cells in 1:10 ratio into a fresh T25 flask approximately after three days of growth or when the cells have reached a confluency of 80-90 %. To plate cells on the 2-chambered cover-glass slide, cells in the T25 flask were washed with 2 ml of PBS pH 7.4 (Invitrogen, Carlsbad, CA) and subsequently treated with 1 ml of 0.25 % Trypsin-EDTA (Invitrogen, Carlsbad, CA) and incubated at 37 °C and 5 % CO₂ for 2 min. Then, 9 ml of MEM was added to the cells. Cells were separated by pipetting up and down. To determine cell count, about 30 μl of cells were taken and mixed with equal volume of Trypan Blue dye (Sigma) and the number of cells in the 16-square quadrant of the Bright Line hemacytometer (Hausser Scientific, Horsham, PA) was counted. From the average number of the cells counted, the cell concentration in the T25 flask was determined and the cells were diluted to 2.5 x 10⁴ cells/ml and were plated at 2 ml onto each chamber. The plated cells were grown for 24 hours at 37 °C and 5 % CO₂ before transfection.

Transfection

Before transfection, the media was replaced with 1.5 ml of fresh MEM containing 10% FBS. A mixture of 1.2 μl of Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA) and 148.8 μl of OPTI-MEM (Invitrogen, Carlsbad, CA) was prepared and incubated at room temperature for 5 min. Another mixture of 0.75 μg (for HeLa cells) and 1 μg (for HepG2 cells) of each vector and 150 μl of OPTI-MEM was prepared and incubated at room temperature for 5 min. The
mixture of 150 µl OPTI-MEM and Lipofectamine 2000 Reagent was added to the 150 µl of the vector and OPTI-MEM mixture, shaken vigorously, and incubated at room temperature for 20 min. The final 300 µl mixture was added to each chamber of the cover-glass slide. The cells were grown for a further 48 hours at 37 ºC and 5 % CO₂ before they were viewed under the FluoView™ FV1000 Confocal Microscope (Olympus America INC., Merville, NY).

**Confocal Microscopy**

Before viewing the cells, media was changed to fresh media and the living cells were viewed under the microscope. Images were collected using the microscope in sequential mode using a 20X dry lens (lens specification, UPlanFI 20x / NA 0.50 ∞/-) at subsequent zoom-in mode of 1X or 2X using Multi-line Ar laser (457 nm, 488 nm, 515 nm, Total 30 m). Images were exported from the FV1000 confocal software into a JPEG file with a resolution of 1024 X 1024. No image manipulation was performed to generate the figures.

**2.3 Results and Discussion**

**2.3.1 Cellular localization of WT-APE1-, ND20-, and ND41-GFP in HeLa and HepG2 cells**

Upon exposure of WT-APE1-GFP in both HeLa and HepG2 cells, the fluorescence was localized to the nucleus (Figure 3A and 3D). This was expected and consistent with previous reports (Ramana et al. 1998; Duguid et al. 1995) as APE1 has well established roles in the nucleus such as abasic DNA cleaving activity. Therefore, when the generated plasmids of WT-APE1-GFP were transiently expressed in both HeLa and HepG2 cell lines, the fusion protein WT-APE1-GFP showed nuclear localization (Figure 3A and 3D).

Various sub-cellular localization of APE1 has been documented in the literature (Duguid et al. 1995; Kakolyris et al. 1997; Kakolyris et al. 1998a; Kakolyris et al. 1998b; Kakolyris et al.
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1999; Puglisi et al. 2001; Kelley et al. 2001; Maso et al. 2007). This localizations have been an enigmatic phenomena. Interestingly, many DNA repair proteins including APE1 are known to be present in both the nucleus and cytoplasm. This is due to the proteins being transiently targeted to the nucleus and mitochondria in response to DNA damage signals (Mitra et al. 2002). Jackson et al. (2005) found that APE1 has two known independent nuclear localization signals. A canonical NLS is composed of the first seven residues at the N-terminus and the amino acid stretch of residues between 8 and 13 (Jackson et al. 2005). In the absence of N-terminal region, APE1 has been known to exhibit cytoplasmic localization (Jackson et al. 2005). This finding was of interest to Chattopadhyay et al. (2006) who found that APE1 can undergo hydrolysis during boiling of the protein samples. The hydrolysis results in APE1 losing its N-terminal region of 32-33 amino acids. Accordingly, the mitochondrial APE1 (mtAPE1) is a truncated product of APE1 missing 33 N-terminal residues (Chattopadhyay et al. 2006). It was also postulated that Granzyme A cleaves APE1 at Lys31 and blocks stable interaction with other proteins for DNA repair. The blockage results in a truncated form of APE1 in mitochondria (Chattopadhyay et al. 2006). Therefore, when the ND20-GFP and ND41-GFP were expressed in both HeLa and HepG2 cells, they were localized to both the nucleus and the cytoplasm (Figure 3B-3F). This was consistent with the findings by Jackson et al. (2005). However, it was difficult to observe the mitochondrial localization of ND20-GFP and ND41-GFP since we did not implement any mitochondrial tracker. Also, determining the mitochondrial localization of APE1 was not the objective of this experiment. It should be pointed out that Chattopadhyay et al. (2006) used HEK293 human kidney cells, while we used HeLa and HepG2 cells. This may also be a contributing factor why we did not observe any localization of WT-APE1 GFP to the mitochondria.
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Figure 3. Expression of the fusion proteins, APE1-, ND20- and ND41-GFP, in HeLa and HepG2 cells. HeLa and HepG2 cells were plated on 2-chamber slides at $5 \times 10^4$ cells/ml. After one day in culture, cells were transfected with 1 µg of plasmid using Lipofectamine reagent. Cells were viewed under the confocal microscope after two days of transfection. (A, B, and C) Expressions of pWT-APE1, pND20, and pND41 in HeLa cells. (D, E, and F) Expressions of pWT-APE1, pND20, and pND41 in HepG2 cells. The scale bar is for all images.

2.3.2 Cellular localization of population variants- and ND20 population variants-GFP in HeLa and HepG2 cells

There are numerous studies investigating the possible association of the human population variants of APE1 with increased disease susceptibility (Hung et al. 2005; Jiao et al. 2006; Bartsch et al. 2007; Pardini et al. 2008; Lo et al. 2009; Deng et al. 2010; Ji et al. 2011). However, there has been no study on determining the localization of APE1 variants in cells. In this study, we took the initiative to determine the cellular localization of APE1 population variants.

When the GFP fusion proteins of I64V, L104R, E126D, D148E, G306A, and D308A were transiently expressed in both HeLa and HepG2 cells, they showed nuclear localization similar to that of observed with the WT-APE1-GFP (Figures 4 and 5). All of these population
variants are classified as non-synonymous single nucleotide polymorphisms (SNPs) because they result in a single amino acid change in a protein. This change is often associated with changes in gene expression, mRNA conformation and stability, and translational efficiency. For example, changing the Pro 111 residue to Ala in Caveolin-1 drastically changed the conformation of the protein and prevented the localization of the protein into lipid rafts; thus, preventing the functioning of caveolae (Aoki and Epand 2011). They may also play a direct role with or without other factors in the phenotypic expression of diseases or in the susceptibility of an individual to many common diseases and drug resistance (Shastry 2009). With the exception of L104R and E126D, all of the variants that we investigated were found to retain their abasic DNA incision activities. This suggests their silent effects on APE1 abasic DNA repair function (Kim 2009). Interestingly, when they were assessed for RNA cleaving activity, the variants showed significant reduction in activity as compared to the WT-APE1, with the exception of L104R and E126D which exhibited different RNA cleaving patterns (Kim et al. 2012). Therefore, we anticipated re-distribution of the population variants of APE1, especially L104R and E126D, if they were expressed in human cancer cell lines. However, all variants tested showed uniform distribution to the nucleus (Figures 4 and 5). These results show that SNPs of APE1 population variants do not play a role in APE1 redistribution. It also suggests that APE1 may play a more prominent role in the nucleus than in the other compartments of HeLa and HepG2 cells.
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Figure 4. Expression of population variants-GFP in HeLa cells. HeLa cells were plate on 2-chamber slide at 5X10^4 cells/ml. After one day in culture, cells were transfected with 1 μg of plasmid using Lipofectamine reagent. Cells were viewed under the confocal microscope after two days of transfection. Expressions of (A) pI64V, (B) pL104R, (C) pE126D, (D) pD148E, (E) pG306A, and (F) pD308A in HeLa.

Figure 5. Expression of population variants-GFP in HepG2 cells. HepG2 cells were plate on 2-chamber slide at 5X10^4 cells/ml. After one day in culture, cells were transfected with 1 μg of plasmid using Lipofectamine reagent. Cells were viewed under the confocal microscope after two days of transfection. Expressions of (A) pI64V, (B) pL104R, (C) pE126D, (D) pD148E, (E) pG306A, and (F) pD308A in HepG2.
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CHAPTER 2- CELLULAR LOCALIZATION OF WILD-TYPE, N-TERMINAL DELETION, AND POPULATION VARIANTS OF APE1

We also tested the GFP fusion proteins of ND20-population variants. To see if they exhibit any different sub-cellular localization when population variants were deliberately restricted to the cytoplasm, the variants including ND20-L104R, ND20-E126D, ND20-D148E, and ND20-G306A were tested. However, no significant difference in their localization was observed for all ND20-population variants as compared to the ND20 (Figures 6 and 7). This re-confirmed that N-terminal region of APE1 is important for nuclear localization of APE1 and that single amino acid change outside of 20 amino acids of the N-terminus does not play any role in APE1 localization.

Figure 6. Expression of ND20 population variants-GFP in HeLa cells. HeLa cells were plate on 2-chamber slide at 5X10^4 cells/ml. After one day in culture, cells were transfected with 1 μg of plasmid using Lipofectamine reagent. Cells were viewed under the confocal microscope after two days of transfection. (A) Expressions of pND20-L104R, (B) pND20-E126D, (C) pND20-D148E, (D) pND20-G306A in HeLa.
Figure 7. Expression of ND20 population variants-GFP in HepG2 cells. HepG2 cells were plate on 2-chamber slide at 5X10^4 cells/ml. After one day in culture, cells were transfected with 1 µg of plasmid using Lipofectamine reagent. Cells were viewed under the confocal microscope after two days of transfection. (A and A-1) Expression of pND20-L104R in HepG2 and its DIC overlap. (B and B-1) Expression of pND20-E126D in HepG2 and its DIC overlap. (C and C-1) Expression of pND20-D148E in HepG2 and its DIC overlap. (D and D-1) Expression of pND20-G306A in HepG2 and its DIC overlap.
Chapter 3

Localization of APE1 under cellular stress

3.1 Introduction

This chapter describes the experiments conducted to study the possible co-localization of WT-APE1, N-terminal deletion variants and population variants of APE1 with processing bodies (PBs) and stress granules (SGs). The decapping enzyme, Dcp1a, was used as a PBs marker and it was tagged with RFP for live visualization in cells. One of the factors for SGs assembly, TIA-1 tagged with monomeric RFP (mRFP), was used as a SGs marker. In addition, possible mechanism of ND20 re-distribution upon cellular stress was investigated using different cellular inhibitors such as nuclear export inhibitor, nuclear import inhibitor, protein synthesis inhibitor, and ubiquitin protease inhibitors. Biochemical analysis of exogenous APE1 with and without cellular stress was carried out to confirm the immunofluorescence study. Finally, localization of APE1 in the formalin fixed and paraffin-embedded (FFPE) breast carcinoma was investigated.

3.2 Methodology

3.2.1 Reagents and buffer preparation

The reagents shown in Table 5 and 6 were used throughout the experiments described in this chapter.
CHAPTER 3- LOCALIZATION OF APE1 UNDER CELLULAR STRESS

Table 5. List of chemicals to generate reagents used.

<table>
<thead>
<tr>
<th>Final concentration</th>
<th>Type of stress</th>
<th>Stock</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 mM Sodium Arsenite (arsenite)</td>
<td>Oxidative Stress</td>
<td>Stock: 0.5 M (0.156 g in 1 ml of water)</td>
</tr>
<tr>
<td>1 mM S-Nitroso glutathione (GSNO)</td>
<td>Nitrosative Stress</td>
<td>Stock: 0.247 M (0.025 g in 300 μl of water)</td>
</tr>
<tr>
<td>5 nM Leptomycin B (LMB)</td>
<td>Nuclear export inhibition</td>
<td>Stock: 9.25 μM (5 μg/ml supplied)</td>
</tr>
<tr>
<td>0.35 mM Cycloheximide (CHX)</td>
<td>Protein synthesis inhibition</td>
<td>Stock: 0.35 M (0.1 g in 1 ml of DMSO)</td>
</tr>
<tr>
<td>8 μM Epoxomycin (EPO)</td>
<td>Ubiquitin protease inhibition</td>
<td>Stock: 0.4507 mM (50 μg in 200 μl of water)</td>
</tr>
<tr>
<td>50 μM 2,4-Diaminoquinazoline (DQZ)</td>
<td>Nuclear import inhibition</td>
<td>Stock: 0.1 M (0.1 g in 6.243 mL of water)</td>
</tr>
<tr>
<td>8 μM MG-132</td>
<td>Ubiquitin protease Inhibition</td>
<td>Stock: 0.01 M (1 mg in 210 μl of DMSO)</td>
</tr>
</tbody>
</table>

Table 6. List of buffers to generate reagents used in this chapter.

<table>
<thead>
<tr>
<th>Buffers</th>
<th>Reagents</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X PBS (pH 7.4)</td>
<td>1.37 M NaCl, 27 mM KCl, 100 mM Na₂HPO₄, and 20 mM KH₂PO₄</td>
</tr>
<tr>
<td>10X TBS (pH 7.4)</td>
<td>0.2 M Tris-HCl and 1.5 M NaCl</td>
</tr>
<tr>
<td>Blocking buffer</td>
<td>2% Triton X-100, 2% BSA, and 1X TBS pH 7.4</td>
</tr>
<tr>
<td>Wash buffer</td>
<td>0.2% Triton X-100, 0.2% BSA, and 1X TBS pH 7.4</td>
</tr>
</tbody>
</table>

3.2.2 Assessing the localization of APE1, N-terminus deleted APE1, and APE1 population variants undergoing cellular stressors

Cell culture and transfection

HeLa and HepG2 cells were cultured and plated following the procedures outlined in Chapter 2. The plated cells were grown for 24 hours at 37°C and 5% CO₂ before transfection. For HeLa cells, 0.75 μg of each vector was used and 1 μg of each vector was used for HepG2 cells. The cells were grown for 48 hours at 37°C and 5% CO₂ until they were viewed the FluoView™ FV1000 Confocal Microscope (Olympus America INC., Merville, NY).
CHAPTER 3- LOCALIZATION OF APE1 UNDER CELLULAR STRESS

Induction of cellular stress

Before viewing the cells using the confocal microscope, the media were replaced with 1.0 ml of fresh MEM containing 10% FBS and incubated at 37°C and 5% CO₂ for at least 30 min. Cells were then exposed to a cellular stressor. For HeLa and HepG2 cells, sodium arsenite (0.5 mM arsenite for 4hr) and S-Nitroso glutathione (1 mM GSNO for 4hr) were tested. In some experiments inhibitors such as Leptomycin B (5 nM LMB for 6hr), Cycloheximide (0.35 mM CHX for 1hr), Epoxomicin (8 μM EPO for 6hr), 2,4-Diaminoquinazoline (50 μM DQZ for 6hr), and MG-132 (8 μM MG-132 for 6hr) were added and arsenite was subsequently added. Cells were then viewed under the confocal microscope following the procedures outlined in Chapter 2.

3.2.3 Generation of pEGFP-ND25, ND30 and ND35

The following primers were designed to amplify APE1 cDNA from pFLAG-CMV-5.1 (4.7Kbp) vector (Table 7). Restriction sites of EcoRI and BamHI were incorporated into the primers for ease in sub-cloning of APE1 cDNA into pEGFP-N1 vector. These primers were generated by Integrated DNA Technologies (IDT) and were dissolved in autoclaved water to yield a concentration of 300 pmol/μl. Sub-cloning of each amplified APE1 cDNA was put into pEGFP-N1 vector using the previously discussed methods in Chapter 2. Plasmid sequences were confirmed by DNA sequencing at Macrogen Inc. (Seoul, Korea) and the sequencing data can be found in the Appendix.
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Table 7. Sequences of primers to amplify APE1 genes from a vector. Underlined texts indicate where mutations were introduced.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>APE1-ND25-EcoRI-F</td>
<td>5'-ACC AGA ATT CAT ATG AGT AAG ACG GCC GCA</td>
</tr>
<tr>
<td></td>
<td>AAG-3'</td>
</tr>
<tr>
<td>APE1-ND30-EcoRI-F</td>
<td>5'- A CCA GAA TTC ATA TGA AAG AAA ATG ACA AAG</td>
</tr>
<tr>
<td></td>
<td>AGG CAG CAG GA-3'</td>
</tr>
<tr>
<td>APE1-ND35-EcoRI-F</td>
<td>5'- A ACA GAA TTC ATC ATG GAG GCA GCA GGA GAG</td>
</tr>
<tr>
<td></td>
<td>GGC-3'</td>
</tr>
<tr>
<td>APEl-BamHI-R-no stop</td>
<td>5'-ACC A GGA TCC ATC AGT GCT AGG TAT GGG TGA</td>
</tr>
<tr>
<td></td>
<td>TGA G-3'</td>
</tr>
</tbody>
</table>

3.2.4 Preparation of RFP-Dcpla and mRFP-TIA-1 plasmids

The Dcpla tagged with RFP was a gift from Dr. Masayuki Murata's laboratory from the University of Tokyo. The TIA-1 tagged with mRFP was a gift from Dr. Paul Anderson's laboratory from Harvard Medical School. Both plasmids were sent on filter papers containing about 100ng of plasmids. The plasmids were extracted by cutting area where the plasmids were seeded and the papers were soaked in 100 nl of auto-claved water. After incubation at room temperature for 30 min, 1 μl and 10 μl of each plasmid solution were used for transformation with 100 μl DH5α bacteria by heat-shock method and plated on Kanamycin (25 μg/ml) agar plates. Three colonies for each vector were picked and plasmids were isolated using QIAprep® Spin Miniprep Kit (250) (Qiagen, Hilden, Germany) as according to the manufacturer's protocol. Concentrations of plasmids were determined using the NanoDrop 1000 UV/Vis (Thermo Scientific, Wilmington, DE).

3.2.5 Cell fractionation

HepG2 cells were grown at the cell density of 5 X 10⁴ cells/ml on 100 mm dish for 2 days after transfection. The medium was removed and cells were washed twice with 2 ml ice-cold PBS. Cells were removed using 1 ml of ice-cold PBS and gentle scraping with cell-scaper, and transferred to a pre-chilled eppendorf tube. It was centrifuged at 2000g for 1 min and the
supernatant was removed. Cells were resuspended in 125 µl of ice-cold hypotonic buffer (10 mM HEPES pH 7.9, 10 mM KCl, 0.1% Triton X-100, and protease inhibitor cocktail (1 tablet to each 10 ml of the solution), vortexed for 15 sec, and incubated on ice for 15 min with brief mixing in between. The lysed cells were then centrifuged at 5000g for 5 min and the supernatant was transferred and labeled as cytoplasmic fraction. The pellet was resuspended in 125 µl of ice-cold hypotonic buffer and then 125 µl of SDS sample buffer (0.25 M Tris pH 6.8, 10% (w/v) SDS, 30% (v/v) glycerol, without DTT or dye). This was labeled as nuclear fraction. Another 125 µl of SDS sample buffer was added to cytoplasmic fraction and all fractions were boiled for 15-20 min. Their protein concentrations were determined using NanoDrop and then stored at -20°C until use.

3.2.6 SDS-PAGE

Two 12% polyacrylamide gels were typically made together. The lower gels (resolving gels) were made with 3.33 ml of acrylamide/ 0.8% bisacrylamide, 2 ml of 4X lower gel buffer (1.5 M Tris-base pH 8.8 and 0.4% (w/v) SDS), 2.67 ml of autoclaved water, and 16 µl of 20% (w/v) ammonium persulfate (APS), and 4.8 µl of TEMED (Sigma) and pouring into a gel apparatus (Biorad, Hercules, CA) at about 2/3 full immediately after mixing. About 300 µl of isopropanol was immediately added onto the lower gel to flatten the top surface of the gel. It was removed when the gel was solidified. The 5% stacking gel was made with 0.48 ml of 30% acrylamide/0.8% bisacrylamide, 0.75 ml of 4X upper gel buffer (0.5 M Tris pH 8.8 and 0.4% (w/v) SDS), 1.77 ml of autoclaved water, and 7.5 µl of 20% ammonium persulfate (APS), and 3 µl of TEMED (Sigma) and quickly pouring it on top of the lower gel after mixing and inserting the comb.

Up to 16 µl of each protein sample (50-80 µg) was loaded to each well and 4 µl of 5X Sample buffer with 5% (v/v) β-mercaptoethanol and then they were boiled for 5 min. As a
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reference, 10 µl of pre-stained molecular weight markers (Bio Basic, Inc., Markham, ON) was loaded to each gel. Gel was then run in 1X SDS running buffer (25mM Tris-base, 200mM Glycine, and 1% (w/v) SDS) at 200V for about 1 hour until the bromophenol blue dye reached the end of the gel.

3.2.7 Western blot analysis

Filter paper and nitrocellulose membrane were cut in equal size as the gel and were soaked in 1X transfer buffer (25 mM Tris-base, 200 mM Glycine, and 20% (v/v) methanol) for 20 min. The gel sandwich was assembled on the black side of the gel holder cassette as the following: Fiber pad → filter paper → gel → nitrocellulose membrane → filter paper → fiber pad. It was run at 100V for 1 hour and 30 min in the cold room.

After protein transfer, the membrane was washed twice with 1X PBS (pH 7.4) for 10 min. It was incubated with blocking buffer (5% skim milk, 1X PBS pH 7.4, and 0.1% Tween-20) for 1 hour at room temperature or overnight at 4°C. The membrane was washed 3 times with washing buffer (0.1% skim milk, 1X PBS pH 7.4, and 0.1% Tween-20) for 10 min each and it was incubated with 1° antibody (1:3000) for 1 hour at room temp. Another 3 washes were done with washing buffer and 2° antibody (1:5000) was added. List of all the antibodies used are presented in the Table 8. The blot was washed 3 times again with washing buffer and it was developed using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific Inc., Rockford, IL). Blot was imaged with a KODAK Image Station 4000MM PRO (Carestream Health INC., Jacksonville, FL).
Table 8. List of all the antibodies used for western blot analysis.

<table>
<thead>
<tr>
<th>Name of antibody used</th>
<th>Company</th>
<th>Catalog number</th>
<th>Dilution used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-APE1 (mouse)</td>
<td>Santa Cruz Biotech Inc.</td>
<td>Sc-17774</td>
<td>1:3000</td>
</tr>
<tr>
<td>Anti-Histone H1 (mouse)</td>
<td>Santa Cruz Biotech Inc.</td>
<td>Sc-8030</td>
<td>1:3000</td>
</tr>
<tr>
<td>Anti-Cytochrome c (mouse)</td>
<td>Abcam</td>
<td>Ab28137</td>
<td>1:3000</td>
</tr>
<tr>
<td>Anti-Mouse IgG</td>
<td>Promega</td>
<td>W402B</td>
<td>1:5000</td>
</tr>
</tbody>
</table>

The blot was stripped for subsequent antibody staining using pre-warmed (55°C) stripping solution (62.5 mM Tris pH 6.7, 2% (w/v) SDS, and 100 mM β-mercaptoethanol) for 30 min. It was washed twice with washing buffer after stripping.

3.2.8 Immunohistochemistry of breast cancer tissue

The formalin-fixed and paraffin-embedded (FFPE) breast cancer tissue sections were obtained from British Columbia Cancer Agency (BCCA) Tumor Tissue Repository. The slide underwent de-paraffinization and rehydration using a series of xylene and graded alcohol as the following orders: 1. xylene for 5 min, 2. xylene for 5 min, 3. xylene for 5 min, 4. 100% ethanol for 5 min, 5. 100% ethanol for 5 min, 6. 95% ethanol for 5 min, and 7. 70% ethanol for 5 min. The slides were then washed with 1X PBS with shaking 600 rpm for 10 min. Any excess PBS was removed by tapping and KimWipe and the tissue sections on the slides were circumscribed using an ImmEdge Pen (Vector H-4000, Burlingame, CA). They were blocked with blocking buffer for overnight at 4 ºC in a humidity chamber. The blocking buffer was removed by tapping and primary antibody incubation was done for 1 hour at room temperature in a humidity chamber. The dilution for primary antibody was 1:200 in washing buffer. We examined APE1 (Santa Cruz Biotech Inc., Santa Cruz, CA) Dcp1a (Abcam., Santa Cruz, CA), and TIA-1 (Santa Cruz Biotech Inc., Santa Cruz, CA) staining in the breast cancer tissue sections. The slides were rinsed with 1X PBS using a squeeze bottle and washed three times in 1X PBS at room temperature for 10
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min while shaking at 600 rpm. The secondary antibody was added in 1:1000 dilution in a humidity chamber. The slides were again rinsed with 1X PBS using a squeeze bottle and washed three times in 1X PBS at room temperature for 10 min while shaking at 600 rpm. DNA staining using POPO™-3 Iodide in 1:1000 dilution was incubated for 20 min in a humidity chamber followed by the rinsing and washing steps.

To mount the sections, one drop of ProLong® Gold antifade reagent (Invitrogen, Eugene, OR) was added and a coverslip was gently applied and was gently pressed to get rid of any trapped air bubbles. The edges were sealed with a clear nail polish and it was allowed to set at room temperature overnight. For long term storage, the slides were stored at 4 °C in a dark box. Each slide was visualized under the confocal microscope following the procedures outlined in Chapter 2.

3.3 Results and Discussion

3.3.1 WT-APE1-GFP transfected into HeLa and HepG2 cells undergoing cellular stress

To examine the possible co-localization of APE1 with PBs and/or SGs, we expressed the WT-APE1-GFP and the RFP-Dcp1a and/or the mRFP-TIA-1 in HeLa (Figure 8) and HepG2 (Figure 9) cells under oxidative (0.5 mM arsenite) stress or nitrosative (1 mM GSNO) stress.

In figures 8 and 9, the first column is imaged with fluorescence for WT-APE1-GFP expressed in both HeLa and HepG2 cells. For both cell types the fluorescence was found to localize in the nucleus (Figure 8A, 8D, 9A and 9D). Upon 0.5 mM arsenite (Figures 8B, 8E, 9B, and 9D) or 1 mM GSNO (Figures 8C, 8F, 9C, and 9F) treatment, distribution of the WT-APE1-GFP did not change. We also tested different concentrations of arsenite (0.25 mM, 0.5 mM, 1 mM and 2 mM) or GSNO (0.5 mM, 1 mM, and 2 mM) and different exposure time for arsenite
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(30 min, 1 hr, 2 hr, and 6 hr) and GSNO (2 hr, 4 hr, and 6 hr). However, we did not observe any re-distribution of the WT-APE1-GFP from the nucleus under the various tested conditions (data not shown).

No change in the WT-APE1-GFP distribution upon cellular stress was unexpected because previous reports showed that upon treatments with oxidative and nitrosative stressors, APE1 was found to localize to the mitochondria or cytoplasm through their immunohistochemical and biochemical analyses (Frossi et al. 2002; Qu et al. 2007). This may be explained by the fact that the localization of APE1 has not been completely characterized in all cell types and there may be variations at least amongst HeLa and HepG2 which were used in our experiments and in Raji cells (Frossi et al. 2002) and HEK293 cells (Qu et al. 2007) used by others. Varying results from different cell types can also be found in Pines et al. (2005) where an oxidative stress caused APE1 translocation into the nucleus in ARO cells.

The second column represents the RFP-Dcpl1a (Figure 8A-C and 9A-C) and the mRFP-TIA-1 (Figure 8D-F and 9D-F) in red color. The RFP-Dcpl1a was found to localize in the nucleus and as dots in the cytoplasm of both HeLa and HepG2 cells with or without 0.5 mM arsenite and 1 mM GSNO (Figure 8A-C and 9A-C). When the mRFP-TIA-1 was expressed in both HeLa and HepG2 cells without any cellular stress (Figure 8D and 9D), they were localized to the nucleus generally with one or two dots in the cytoplasm. However, when the cells were exposed to 0.5 mM arsenite and 1 mM GSNO, there were more dots representing the assembly SGs in the cytoplasm (Figure 8E-F and 9E-F). These results are consistent with previous studies on the localization of Dcpl1a and TIA-1 with or without cellular stress (Kedersha et al. 2005; Lin et al. 2007; Fujimara et al. 2008).
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The third column represents merged images of the first two columns and the last column shows the merged images of the third column with cells under trans-luminescent light (Figures 8 and 9). There was no overlap of the WT-APE1-GFP and the RFP-Dcpla and/or the mRFP-TIA-1 in the cytoplasmic dots when we examined the merged images with and without 0.5 mM arsenite or 1 mM GSNO (Figures 8 and 9). There were overlaps of the WT-APE1-GFP and the RFP-Dcpla and/or the mRFP-TIA-1 due to their nuclear expression; however, this nuclear expression of the RFP-Dcpla and the mRFP-TIA-1 do not indicate their localization with the PBs or SGs respectively (Figures 8A, 8C, 8D, 8E, 9D, and 9E). Therefore, we conclude that there was no cytoplasmic co-localization of the WT-APE1-GFP with the RFP-Dcpla and/or the mRFP-TIA-1 in either HeLa or HepG2 cells (Figures 8 and 9). This suggests that APE1 is at least not likely to be involved in mRNA processing that is carried out in PBs and SGs.
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Figure 8. Expression of WT-APE1-GFP, RFP-Dcp1a and/or mRFP-TIA-1 in HeLa cells upon treatment with 0.5 mM arsenite and 1 mM GSNO. HeLa cells were plated on 2-chamber slide at 5X10^4 cells/ml. After one day in culture, cells were transfected with 1 μg of plasmid using Lipofectamine reagent. Cells were viewed under confocal microscope two days post-transfection. (A-C) Expression of WT-APE1-GFP and RFP-Dcp1a. (D-F) Expression of WT-APE1-GFP and mRFP-TIA-1.
Figure 9. Expression of WT-APE1-GFP, RFP-Delp1a and/or mRFP-TIA-1 in HepG2 cells upon treatment with 0.5 mM arsenite and 1 mM GSNO. HepG2 cells were plated on 2-chamber slide at 5X10^4 cells/ml. After one day in culture, cells were transfected with 1 μg of plasmid using Lipofectamine reagent. Cells were viewed under confocal microscope two days post-transfection. (A-C) Expression of WT-APE1-GFP and RFP-Delp1a. (D-F) Expression of WT-APE1-GFP and mRFP-TIA-1.
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3.3.2 APE1 population variants transfected into HeLa and HepG2 cells undergoing oxidative stress

As per Chapter 2, there have been no studies on localization of human population variants of APE1 in cells. In this study, we took the initiative to determine the cellular localization of APE1 population variants upon oxidative stress (0.5 mM arsenite). The WT-APE1-GFP did not exhibit any difference in their localization upon 0.5 mM arsenite (Figure 8) and 1 mM GSNO (Figure 9) treatments. Under normal condition, we found that single amino acid change in APE1 did not change the cellular localization of APE1 (Figures 4 and 5); therefore, we did not expect to see any changes in the cellular localization of APE1 population variants under 0.5 mM arsenite and 1 mM GSNO treatment.

When the GFP fusion proteins of I64V, L104R, E126D, D148E, G306A, and D308A were transiently expressed in both HeLa and HepG2 cells and exposed to 0.5 mM arsenite, they showed nuclear localization (Figure 10) similar to that of observed with the WT-APE1-GFP exposed to 0.5 mM arsenite and 1 mM GSNO (Figures 8 and 9). Therefore, we did not perform further experiments to look at possible co-localization of APE1 population variants with PBs and/or SGs. These results re-confirmed that SNPs of APE1 population variants do not play any role in APE1 redistribution with or without cellular stress.
Figure 10. Expression of human population variants of APE1-GFP in HeLa and HepG2 upon treatment with 0.5 mM arsenite. HeLa and HepG2 cells were plated on 2-chamber slide at $5 \times 10^4$ cells/ml. After one day in culture, cells were transfected with 1 µg of plasmid using Lipofectamine reagent. Cells were viewed under confocal microscope two days post-transfection.

### 3.3.3 ND20-GFP transfected into HeLa and HepG2 cells undergoing cellular stress

To examine the possible co-localization of ND20 with PBs and/or SGs, we expressed the ND20-GFP, the RFP-Dcp1a and/or the mRFP-TIA-1 in HeLa (Figure 11) and HepG2 (Figure 12) cells upon 0.5 mM arsenite and 1 mM GSNO treatments.
Figure 11. Expression of ND20-GFP and RFP-Dcp1a and/or mRFP-TIA-1 in HeLa cells upon treatment with 0.5 mM arsenite and 1 mM GSNO. HeLa cells were plated on 2-chamber slide at 5X10^4 cells/ml. After one day in culture, cells were transfected with 1 μg of plasmid using Lipofectamine reagent. Cells were viewed under confocal microscope two days post-transfection. (A-C) Expression of ND20-GFP and RFP-Dcp1a. (D-F) Expression of ND20-GFP and mRFP-TIA-1.
Figure 12. Expression of ND20-GFP and RFP-Dcp1a and/or mRFP-TIA-1 in HepG2 cells upon treatment with 0.5 mM arsenite and 1 mM GSNO. HepG2 cells were plated on 2-chamber slide at 5X10⁴ cells/ml. After one day in culture, cells were transfected with 1 μg of plasmid using Lipofectamine reagent. Cells were viewed under confocal microscope two days post-transfection. (A-C) Expression of ND20-GFP and RFP-Dcp1a. (D-F) Expression of ND20-GFP and mRFP-TIA-1.
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In figures 11 and 12, the first column represents expression of the ND20-GFP in green color. When the ND20-GFP was expressed in HeLa cells, the fluorescence was found to localize to the nucleus and the cytoplasm evenly (Figures 11A and 11D). Upon 0.5 mM arsenite and 1 mM GSNO treatments, distribution of the ND20-GFP did not change in HeLa cells (Figure 11B-C and 11E-F). Interestingly, when the ND20-GFP was expressed in HepG2 cells, they were evenly localized in the nucleus and the cytoplasm without any treatment (Figure 12A and 12D). However, they exhibited more nuclear localization with 0.5 mM arsenite (Figure 12B and E) and 1 mM GSNO (Figure 12C and F) treatments. These phenomena were observed in HepG2 cells only and this may be due to the difference of APE1 behaviour in different cells types. We therefore decided to further pursue these interesting phenomena as described in sections 3.3.6 to 3.3.8.

The second column represents the RFP-Dcp1a (Figures 11A-C and 12A-C) and the mRFP-TIA-1 (Figures 11D-F and 12D-F) in red color. They exhibited nuclear distribution and as dots in the cytoplasm of HeLa (Figure 11) and HepG2 (Figure 12) cells as discussed in section 3.3.1. The third column represents merged images of the first two columns (Figures 11 and 12), and the last column shows the merged images of the third column with cells under transluminescent light (Figures 11 and 12). We found no overlap between the ND20-GFP and RFP-Dcp1a and/or the mRFP-TIA-1 in the cytoplasmic dots in cells treated with or without 0.5 mM arsenite and 1 mM GSNO (Figures 11 and 12). Therefore, we conclude there was no co-localization of the ND20-GFP with the RFP-Dcp1a and/or the mRFP-TIA-1 in HeLa (Figure 11) and HepG2 cells (Figure 12). This suggests that even if APE1 has lost its nuclear localization signal and was exposed to cellular stress, there was no co-localization with PBs and/or SGs.
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3.3.4 N-terminal deleted population variants transfected into HeLa and HepG2 cells undergoing oxidative stress

We next tested the GFP fusion proteins of ND20-population variants including ND20-L104R, ND20-E126D, ND20-D148E, and ND20-G306A in both HeLa and HepG2 to see if they exhibit any different sub-cellular localization when population variants were deliberately restricted to the cytoplasm. We found no significant difference in the localization of all ND20-population variants-GFP as compared to the ND20-GFP in HeLa cells (Figure 13). Interestingly, the ND20-population variants-GFP exhibited the same phenomena as that observed for the ND20-GFP after arsenite treatment in HepG2 cells: more nuclear localization upon arsenite treatment (Figure 13). This was expected because we found that single amino acid change outside of 20 amino acids of the N-terminus does not play any role in APE1 localization as described in chapter 2. Results from this experiment confirm that the ND20-population variants-GFP indeed behave the same way as the ND20-GFP upon cellular stress.
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Figure 13. Expression of human population variants of APE1-GFP in HeLa and HepG2 upon treatment with 0.5 mM arsenite. HeLa and HepG2 cells were plated on 2-chamber slide at 5\times10^4 cells/ml. After one day in culture, cells were transfected with 1 \mu g of plasmid using Lipofectamine reagent. Cells were viewed under confocal microscope two days post-transfection.

3.3.5 ND41-GFP transfected into HeLa and HepG2 cells undergoing cellular stress

It was previously found that a canonical NLS was located in the first seven residues at the N-terminus as well as at the amino acid stretch of residues between 8 and 13 (Jackson et al. 2005). Therefore, we expected to see no co-localization of the ND41-GFP to neither RFP-Dcp1a nor mRFP-TIA-1 but more to the nucleus in HepG2 cells upon cellular stress like the ND20-GFP.

In figures 14 and 15, the first column represents expression of the ND41-GFP in green color. We expected to see a re-distribution of the ND41-GFP to the nucleus upon cellular stress
CHAPTER 3- LOCALIZATION OF APE1 UNDER CELLULAR STRESS

in HepG2 cells like the ND20-GFP because of their similar distributions under no cellular stress. When the ND41-GFP was expressed in HeLa (Figure 14A and D) and HepG2 (Figure 15A and D) cells, the fluorescence was found to localize to the nucleus and cytoplasm evenly. However, upon 0.5 mM arsenite and 1 mM GSNO treatments, distribution of the ND41-GFP did not change in HeLa (Figure 14B-C and E-F) and HepG2 (Figure 15B-C and E-F) cells. Such differences may be due to the 21-40 amino acids at the N-terminus involved in APE1 re-distribution upon cellular stress. To find out why ND20-GFP and ND41-GFP behave differently upon cellular stress in HepG2 cells, more experiments were conducted as described in sections 3.3.6 to 3.3.8.

The second column represents the RFP-Dcp1a (Figures 14A-C and 15A-C) and the mRFP-TIA-1 (Figures 14D-F and 15D-F) in red color. They exhibited nuclear distribution and as dots in the cytoplasm of HeLa (Figure 14) and HepG2 (Figure 15) cells as discussed in section 3.3.1 and 3.3.3. The third column represents merged images of the first two columns (Figures 14 and 15), and the last column shows the merged images of the third column with cells under transluminescent light (Figures 14 and 15). There was no overlap between the ND41-GFP with the RFP-Dcp1a and/or mRFP-TIA-1 in the cytoplasmic dots with or without 0.5mM arsenite and 1mM GSNO treatments (Figures 14 and 15). Therefore, we conclude there was no co-localization of the ND41-GFP with the RFP-Dcp1a and/or the mRFP-TIA-1 in HeLa (Figure 14) and HepG2 cells (Figure 15).
Figure 14. Expression of ND41-GFP and RFP-Dcp1a and/or mRFP-TIA-1 in HeLa upon treatment with 0.5 mM arsenite and 1 mM GSNO. HeLa cells were plated on 2-chamber slide at 5X10^4 cells/ml. After one day in culture, cells were transfected with 1 μg of plasmid using Lipofectamine reagent. Cells were viewed under confocal microscope two days post-transfection. (A-C) Expression of ND41-GFP and RFP-Dcp1a. (D-F) Expression of ND41-GFP and mRFP-TIA-1.
Figure 15. Expression of ND41-GFP and RFP-Dep1α and/or mRFP-TIA-1 in HepG2 upon treatment with 0.5 mM arsenite and 1 mM GSNO. HepG2 cells were plated on 2-chamber slide at 5×10⁴ cells/ml. After one day in culture, cells were transfected with 1 μg of plasmid using Lipofectamine reagent. Cells were viewed under confocal microscope two days post-transfection. (A-C) Expression of ND41-GFP and RFP-Dep1α. (D-F) Expression of ND41-GFP and mRFP-TIA-1.
3.3.6 ND25-GFP, ND30-GFP, and ND35-GFP

To determine if there are important sequences between amino acids 21 and 40 of APE1 that may be responsible for the stress response, we generated ND25-GFP, ND30-GFP, and ND35-GFP and observed their localization with or without 0.5 mM arsenite treatment in HepG2 cells. Interestingly, all exhibited even distribution in the nucleus and in the cytoplasm regardless of 0.5 mM arsenite treatment (Figure 16). This result suggested that amino acids 21-40 at the N-terminus of APE1 are responsible for the stress response. This is so because the ND25-GFP, which only has 5 extra amino acid deletions after amino acid 20, had lost its stress response in comparison to the ND20-GFP.

Figure 16. Expression of ND25-GFP, ND30-GFP and ND35-GFP in HepG2 cells without and with 0.5mM arsenite treatment. HepG2 cells were plate on 2-chamber slide at 5X10^4 cells/ml. After one day in culture, cells were transfected with 1 µg of plasmid using Lipofectamine reagent. Cells were viewed under confocal microscope two days post-transfection. Expression of ND25-GFP (A-B), ND30-GFP (C-D), and ND35-GFP (E-F).
3.3.7 Investigation into the possible mechanism for re-distribution of ND20-GFP upon cellular stress

The re-distribution of ND20-APE1 upon cellular stress as described in the section 3.3.3 (Figure 12) may be due to: 1. nuclear export inhibition of APE1, 2. nuclear import of APE1, 3. re-distribution of existing APE1 or newly synthesized APE1, or 4. increased protein degradation in the cytoplasm. To test these possibilities, nuclear export inhibitor, nuclear import inhibitor, protein synthesis inhibitor and proteasome inhibitor were used (Table 5).

Without any stress, the ND20-GFP was evenly distributed throughout the nucleus and cytoplasm in HepG2 cells. The solvent used for two of the inhibitors, DMSO, did not have any effect on their distribution (Figure 17). As expected, both cellular stressors, 0.5 mM arsenite and 1 mM GSNO, caused the ND20-GFP to be re-distributed into the nucleus (Figure 17). For inhibitor studies, we adopted the concentrations and incubation times previously used by others as summarized in sections 3.2.1 and 3.2.2. At 5 nM LMB, 50 μM DQZ, 0.35 mM CHX, 8 μM EPO, and 8 μM MG-132, no re-distribution of the ND20-GFP was observed (Figure 17). We also tested different concentrations of each inhibitors: LMB (5, 10, 20 nM), DQZ (50 and 100 μM), CHX (0.175, 0.35, and 0.70 mM), EPO (8 and 16 μM), MG-132 (8 and 16 μM) but again we did not see any re-distribution of the ND20-GFP (data not shown).

We next investigated the effect of each inhibitor on the ND20-GFP localization upon treatment with 0.5 mM arsenite. Firstly, the protein synthesis was inhibited using 0.35 mM CHX and the cells were then exposed to 0.5 mM arsenite. This caused re-distribution of the ND20-GFP to the nucleus (Figure 17). This suggests that the re-distribution upon cellular stress is not due to newly synthesized protein that is re-distributed to the nucleus but is due to the existing proteins. Secondly, the nuclear export and import were inhibited using 5 nM LMB and 50 μM DQZ respectively, and the cells were then exposed to 0.5 mM arsenite. This also caused re-
CHAPTER 3- LOCALIZATION OF APE1 UNDER CELLULAR STRESS

distribution of the ND20-GFP to the nucleus (Figure 17). Therefore, the results suggest that the mechanism is due to neither nuclear export inhibition of the protein from the nucleus nor nuclear import of the protein to the nucleus upon cellular stress. Interestingly, when cells were treated with the proteasome inhibitors, 8 μM EPO and 8 μM MG132, and then 0.5 mM arsenite, the ND20-GFP did not show any re-distribution (Figures 17). This suggests that re-distribution of ND20-GFP was due to its degradation in the cytoplasm upon arsenite treatment. This observation is consistent with previous finding which showed that under stress condition, the ND20-GFP in the cytoplasm is discovered to be ubiquitinated and degraded by proteasome (Busso et al. 2009; Busso et al. 2011).

Ubiquitin is a protein that serves as a signal mediator in response to various cellular activities where its polymerization triggers the degradation of the ubiquitinated target protein by 26S proteasome (Char et al. 1989). APE1 is known to be ubiquitinated at the Lys residues present in the N-terminus including K24, K25, K27, K31, and K32 (Busso et al. 2009; Fantini et al. 2010; Busso et al. 2011). Busso et al. (2009) also discovered that unlike the WT-APE1, ubiquitin-APE1 were predominantly present in the cytoplasm and might get poly-ubiquitinated and degraded in the cytoplasm. Another study observed the increased level of APE1 ubiquitination upon cellular stress (Busso et al. 2011). These studies agree with what we have observed for the ND20-GFP upon cellular stress (Figures 11 and 12) and the ND25-, ND30-, ND35-, (Figure 16) and ND41-GFP (Figures 14 and 15). These results suggest that APE1 has a region of 21-40 amino acids at its N-terminus where it is responsible for the stress response through the ubiquitination pathway.
Figure 17. Expression of ND20-GFP in HepG2 cells upon treatment with various cellular stressors and inhibitors. HepG2 cells were plated on 2-chamber slides at 5X10^4 cells/ml. After one day in culture, cells were transfected with 1 μg of plasmid using Lipofectamine reagent. After two days of transfection, cells were treated with the following final concentrations of inhibitors (6 hr) before viewing under the confocal microscope: 8 μM MG-132, 8 μM EPO, 0.35 mM CHX, 5 nM LMB, and 50 μM DQZ. Subsequent 4 hr treatments with 0.5 mM arsenite were carried to look at the effect of each inhibitor on the ND20-GFP re-distribution. The line in each image is used for generating fluorescent intensity graph in figure 19.
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We obtained the intensity graphs of all the images to quantify our findings. We only included the intensity graphs of the HepG2 cells for comparison because we observed change in the distribution of ND20-GFP upon cellular stress in HepG2 cells only.

To confirm that the WT-APE1-GFP was present in the nucleus area with or without 0.5 mM arsenite or 1 mM GSNO treatments, we chose to show the intensity graphs of figure 9A-C as representatives (Figure 18). The fluorescence of the WT-APE1-GFP was mostly present in the nucleus area under normal condition (Figure 18A) and under 0.5 mM arsenite and 1 mM GSNO treatment (Figure 18B-C).

![Figure 18. Intensity graphs the WT-APE1-GFP in HepG2 cells with or without cellular stress. Intensity of green fluorescence from GFP and their scales were obtained from the confocal microscope software and cannot be changed. (A) Intensity of the WT-APE1-GFP of Figure 9A without cellular stress, (B) Intensity of the WT-APE1-GFP of Figure 9B with 0.5 mM arsenite, and (C) Intensity of the WT-APE1-GFP of Figure 9C with 1 mM GSNO respectively. The line in each image is used for generating fluorescent intensity graph.](image-url)
We confirmed the distribution of ND20-GFP with or without cellular stress and inhibitors in HepG2 cells (Figure 19). We selected to show the intensity graphs of Figure 17. Under normal conditions, the ND20-GFP was distributed to the cytoplasm and the nucleus (Figure 19A) unlike the WT-APE1-GFP showing high intensity peak at the nucleus region (Figure 18A). Upon 0.5 mM arsenite and 1 mM GSNO treatments, the high intensity peak at the nucleus region was observed (Figure 19C-D) which agreed with our findings. There were no changes in the ND20-GFP intensity distribution under the inhibitors that we used such as 0.35 mM CHX, 5 nM LMB, 50 µM DQZ, 8 µM EPO, and 8 µM MG-132 (Figure 19E-I). This re-confirmed that the inhibitors alone did not have any effects on the distribution of ND20-GFP. The intensity graphs of the ND20-GFP treated with either 0.35 mM CHX, 5 nM LMB, or 50 µM DQZ and then 0.5 mM arsenite exhibited high intensity peak at the nucleus regions supporting our earlier findings (Figure 19J-L). Lastly, evenly distributed intensity graphs of the ND20-GFP treated with either the proteasome inhibitors, 8 µM EPO or 8 µM MG132, and then 0.5 mM arsenite were observed (Figure 19M-N). This also supports our earlier findings that the re-distribution of the ND20-GFP may be due to ubiquitination and degradation of the protein in the cytoplasm.
Lastly, to confirm that the ND41-GFP was evenly distributed with or without 0.5 mM arsenite or 1 mM GSNO treatments, the intensity graphs of figure 15D-F were used as representatives (Figure 20). The fluorescence of the ND41-GFP was evenly distributed under...
normal condition (Figure 20A) and under 0.5 mM arsenite and 1 mM GSNO treatments (Figure 20B-C). These supported our earlier finding of the loss of stress response in the ND41-GFP.

Figure 20. Intensity graphs the ND41-GFP in HepG2 cells with or without cellular stress. (A) Intensity of the ND41-APE1 of figure 15D without cellular stress, (B) Intensity of the ND41-GFP of figure 15E with arsenite, and (C) Intensity of the ND41-GFP of figure 15F with GSNO respectively. The line in each image is used for generating fluorescent intensity graph.

3.3.8 Biochemical analysis of stress response of APE1 in HepG2 cells

To confirm our live imaging studies on the stress response of APE1 in HepG2 cells, we performed biochemical analysis. To do this, we isolated nuclear and cytoplasmic fractions from HepG2 cells with or without 0.5 mM arsenite treatment. Histone H1 was used as the nuclear fraction marker and cytochrome c was used as the cytoplasmic marker (Figure 21 lanes 2, 3, 4, and 5). When the WT-APE1-GFP was expressed in HepG2 cells, it showed almost equal amount
CHAPTER 3- LOCALIZATION OF APE1 UNDER CELLULAR STRESS

of the fusion protein in both nuclear and cytoplasmic fractions in normal setting (Figure 21 lane 1). This was supported by the ratio of 1.012 cytoplasmic/nuclear exogenous APE1 (Figure 22). We were able to distinguish exogenous APE1 versus endogenous APE1 using anti-APE1 antibody because of their size differences of ~60kDa for the GFP-tagged APE1 and ~35kDa for the endogenous APE1. The presence of exogenous and endogenous APE1 in the cytoplasmic fractions was unexpected because we did not observe any cytoplasmic WT-APE1-GFP with and without stress when viewed under confocal microscope (Figures 8 and 9). This discrepancy may be due to the presence of dying cells which were not included into microscopy evaluation because of their abnormal morphology even though they had WT-APE1-GFP expression. These abnormal cells generally produced higher fluorescent signals as compared to normal cells and it is now suspected that high expression of the plasmid in these cells eventually led to their change in morphology and death. Therefore, these dying cells have likely contributed to the cytoplasmic WT-APE1-GFP detected in the western blot analysis because all cells in the 100 mm dish were harvested.

Although the re-distribution of the WT-APE1-GFP to the cytoplasm was not observed under confocal microscope, more WT-APE1-GFP was present in the cytoplasmic fraction when the cells were treated with arsenite (Figure 21 lane 4). This was supported by the ratio of 1.945 cytoplasmic/nuclear exogenous APE1 (Figure 22). This also agrees with the results reported by Qu et al. (2007) in which translocation of APE1 to the cytoplasm upon nitrosative stress in HepG2 cells was observed in their western blot analysis. Interestingly, they did not report any immunofluorescence study in HepG2 cells. Rather, they reported immunofluorescence experiment using HEK293 cells (Qu et al. 2007). This brings up a suspicion that they too might
have observed contradicting results as we did for immunofluorescence study and western blot analysis in HepG2 cells.

Both the ND20-GFP and the ND41-GFP exhibited more cytoplasmic localization in normal setting with a greater cytoplasmic proportion of the ND41-GFP (Figure 21 lane 1). When the stress was added, the ND20-GFP exhibited slight decrease in the cytoplasmic proportion of the fusion proteins whereas the ND41-GFP did not show any change (Figure 21 lane 4). These were confirmed by the ratios of 1.637 (no stress) and 1.412 (with 0.5 mM arsenite) for the cytoplasmic/nuclear ND20-GFP and 2.058 (no stress) and 2.003 (with 0.5 mM arsenite) for the cytoplasmic/nuclear ND41-GFP (Figure 22). These results are consistent with previous immunofluorescence studies described in sections 3.3.3 and 3.3.5.

![Western blot analysis of the nuclear and cytoplasmic fractions of HepG2 cells with and without arsenite treatment](image)

**Figure 21. Western blot analysis of the nuclear and cytoplasmic fractions of HepG2 cells with and without arsenite treatment.** The concentration of total protein was normalized before loading them onto the gel. The blots were stripped and re-probed. The WT-APE1-GFP, the ND20-GFP, and the ND41-GFP were detected using anti-APE1 mouse antibody. Histone H1 was used as the nuclear fraction marker. Cytochrome c was used as the cytoplasmic fraction marker.
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Figure 22. Changes in nuclear and cytoplasmic proportions of exogenous APE1 with and without arsenite treatment in HepG2 cells. The intensity of each western blot band was obtained from three replicates of western blot analyses. Intensity ratio less than 1 means more nuclear APE1 than cytoplasmic APE1, intensity ratio greater than 1 means more cytoplasmic APE1 than nuclear APE1, and intensity ratio equal to 1 indicates equal amount of nuclear and cytoplasmic APE1.

3.3.9 Localization of APE1 in FFPE breast cancer tissues: A preliminary investigation

We examined the cytoplasmic expression of APE1 in breast FFPE carcinoma tissues, specifically looking for evidence of co-localization of APE1 within PBs and SGs.

Tissues were stained with APE1 antibody and PoPo-3 which stains DNA. Green color represents APE1 staining (Figure 23 first column) and red color represents the DNA staining (Figure 23 second column). Merged images of APE1 and DNA staining is shown in the third column and the fourth column shows merged images of the third column with tissues under trans-luminescent light (Figure 23). Both nuclear (yellow arrows) and cytoplasmic (white arrows) expression of APE1 were observed in breast FFPE carcinoma tissues (Figure 23). There were
also differences in the expression of APE1 within one section depending on the cell types such as breast cells (Figure 23 first row) and duct lining cells (Figure 23 second row). However, with very little histology background, it was difficult to distinguish normal breast cells and cells at different stages of cancer. Therefore, future immunohistochemistry studies may be required to compare normal breast tissue and different stages of breast carcinoma sections.

Figure 23. Nuclear and cytoplasmic expression of APE1 in breast FFPE carcinoma tissue using confocal microscopy. APE1 was stained with alexa fluorophore 488 (AF 488) and the nucleus was stained with PoPo-3™-3 Iodide. The nuclear localization of APE1 was highlighted by yellow arrows (fat) and white arrows (thin) for the cytoplasmic localization of APE1.

Studies with APE1 and Dcplα or TIA-1 were carried out in order to look for co-localizations. First of all, the immuno-stainings of PBs and SGs were not successful (data not shown). It may be due to their small sizes and low abundance in cells that make them difficult to visualize in tissue sections. Other tissue samples such as prostate or lung carcinomas may show different amount of PBs and SGs that we may be able to visualize. Another possibility may be the dissociation or destruction of PBs and SGs when the tissues were fixed with formalin resulting in an altered protein antigenicity. Formalin fixation has three well-known disadvantages
CHAPTER 3- LOCALIZATION OF APE1 UNDER CELLULAR STRESS

such as slow fixation time, slow quenching of enzymatic activity causing RNA degradation, and extensive molecule modification affecting protein antigenicity (Chu et al. 2005). In order to view PBs and SGs in the breast carcinoma sections, an antigen retrieval pre-treatment before antibody incubation steps may be necessary. We may suggest an alternative fixation technique such as ultrasound-facilitated tissue preservation that is found to provide a more rapid and uniform molecular preservation (Chu et al. 2005).
Chapter 4
General Discussion

4.1 General overview

The mechanism controlling the mRNA stability or turnover is one of the key points in the regulation of gene expression in all eukaryotic species. To date, mRNA surveillance control pathways (Section 1.1.1), general 5'-3' and 3'-5' decay pathways (Section 1.1.2), endoribonuclease decay pathways (Section 1.1.3), and mRNA locales decay pathways (Section 1.1.4) have been discovered. APE1 is a multifunctional DNA repair protein with a broad range of activities including 3' DNA phosphodiesterase, 3'-5' DNA exonuclease, nucleotide incision repair (NIR), transcriptional repressor, and RNase H activities (Section 1.2). Its role in RNA metabolism has been highlighted recently due to the following key evidence: (i) physical association of APE1 with other factors that are involved in RNA metabolism (Section 1.3.1), (ii) discovery of abasic RNA incision activity of APE1 and its role in rRNA control (Section 1.3.2), and (iii) discovery of endoribonuclease activity of APE1 and its role in the control of mRNA abundance (Section 1.3.3). Owing to its multiple roles in cells, APE1 has been found to be associated with cancers and was targeted as an effort towards developing cancer treatments (Section 1.4.1). Interestingly, polymorphism of APE1 has been discovered to be associated with many diseases including cancers (Section 1.4.2).

Altered localization of APE1 from the nucleus to the cytoplasm has been observed in various tumors and has been correlated with more aggressive carcinomas and poor prognoses for patients (Section 1.4.3). Although, there is an increasing number of studies that have investigated the possible link between the role of APE1 and its altered localization, no consensus has been
CHAPTER 4 – GENERAL DISCUSSION

reached. The main goals of this research were (i) to confirm the cellular localization of wild-type and N-terminal deletion variants of APE1, (ii) to identify the cellular localization of human population variants of APE1, and (iii) to identify the cellular localization of wild-type, N-terminal deletion, and human population variants of APE1 under cellular stress. Overall, we found that the N-terminal region of APE1 is important for its nuclear localization and that single amino acid change outside of this 20-amino acid sequence does not play any role in APE1 localization. We discovered that APE1 is unlikely to be involved in mRNA processing that is carried out by the cytoplasmic locales such as PBs and SGs. We have also discovered that the N-terminal deletion variant of APE1 gets degraded in the cytoplasm upon oxidative or nitrosative stresses via ubiquitination and degradation by the proteasome.

4.2 Cellular localization of wild-type, N-terminal deletion variants, and human population variants of APE1

The primary objective of this phase of the investigation was to confirm the presence of a NLS of APE1 and its role in the localization of APE1 in cancer cell lines. In addition, the objective was to identify the cellular localization of APE1 human population variants. The population variants analyzed were I64V, L104R, E126D, D148E, G306A, and D308A. The effect of single nucleotide changes of APE1 in its localization was also investigated by forcing APE1 to localize in the cytoplasm through the generation of its N-terminus-deleted population variants.

4.2.1 Roles of N-terminus region of APE1

The sub-cellular distribution of APE1 is found to be predominantly nuclear which is consistent with its major abasic DNA incision and redox regulatory activities (Tell et al. 2005). Increased expression of APE1 has been associated with different tumorigenic processes (Tell et
al. 2005). In particular, a higher intracellular expression and cytoplasmic localization of APE1 have been described in lung, ovarian, thyroid, and breast cancers (Tell et al. 2005), in which they correlate with higher tumor aggressiveness and poorer prognoses of the patients (Tell et al. 2010). Jackson et al. (2005) have identified a canonical nuclear localization signal (NLS) residing in the first seven residues at the N-terminus and in the stretch between amino acids 8 and 13. The N-terminal deletion variants exhibited a cytoplasmic distribution (Jackson et al. 2005). This highlighted the importance of NLS of APE1 in sub-cellular distribution of APE1 in cells; however, the exact mechanisms regulating APE1 distribution are still unknown. The results from the expression of WT-APE1-, ND20-, and ND41-GFP in both HeLa and HepG2 cells confirmed the role of the NLS in APE1 distribution (Sections 2.3.1 and 2.3.2). In these results there were almost equal nuclear and cytoplasmic distributions of APE1 suggesting that the nuclear distribution of ND20- and ND41-GFP in cells was not completely abolished upon loss of its NLS (Sections 2.3.1 and 2.3.2). It suggests and supports the existence of more complex mechanisms for the cytoplasmic distribution of APE1.

Another important role of the N-terminus region of APE1 has been suggested by previous studies. In different cell types APE1 was found to localize also in mitochondria where it is involved in mtDNA repair activity (Chattopadhyay et al. 2006). This mitochondrial distribution of APE1 is thought to be due to the proteolytic removal of the first 31-33 amino acids at the N-terminus, which would promote nuclear exclusion of the protein (Chattopadhyay et al. 2006). Recently, the discovery of a mitochondrial targeting sequence within region 289-318, which is masked by the N-terminal domain, suggested the need for a specific and regulated unfolding-refolding of the protein to ensure proper localization (Li et al. 2010).
CHAPTER 4 – GENERAL DISCUSSION

Along with its role in mitochondrial distribution, the N-terminus region of APE1 is thought to be responsible for modulating stable protein-protein interactions for DNA repair (Chattopadhyay et al. 2006). This is supported by the recent work which demonstrated the proteolytic removal of the N-terminus region of APE1 leading to its functional inactivation in vivo without affecting its half-life (Vascotto et al. 2011). The N-terminus region is also thought to be involved in the regulation of different APE1 functions in an indirect way (Section 1.3). One example is APE1's function in activation of transcription factors including p53 and Egr-1 suggesting APE1 is involved in controlling cell cycle arrest and apoptotic programs (Pines et al. 2005). An additional hypothesis, which is under the evaluation of Dr. Tell's group, is that the truncated APE1 may play an active role in apoptotic triggering; however, this needs a further investigation (Vascotto et al. 2011). In conclusion, N-terminus region of APE1 plays a role in APE1 sub-cellular distribution and protein-protein interaction; however, further work is needed to identify the exact and complex mechanism of APE1 distribution.

4.2.2 Roles of single nucleotide polymorphisms (SNPs) of APE1

Another possible factor influencing changes in APE1 distribution is single amino acid changes in APE1. A C65S mutation is a very good example and is found to be involved in redox-assisted folding of APE1 in vivo which affects the mitochondrial localization of the protein (Vascotto et al. 2011). This is possible because the C65S mutation is found to alter the folding process of APE1 and controls its interaction with PDIA3 (Vascotto et al. 2011). It is hypothesized that C65S may induce conformational change associated with an increased affinity toward PDIA3 and this may reduce the fraction of APE1 available to translocate into the mitochondria (Vascotto et al. 2011). It may also be due to the loss of redox-dependent
conformational change required to efficiently respond to oxidative stress causing decreased translocation into mitochondria (Vascotto et al. 2011).

APE1 is known to exist as multiple human population variants and researchers have found the correlation of APE1 polymorphisms with many diseases including cancers (Wilson et al. 2011). However, there has been no study in determining the effect of these single amino acid changes in APE1 on its sub-cellular localization in cells. Interestingly, all of the GFP-tagged APE1 population variants exhibited nuclear distribution similar to the WT-APE1-GFP (Section 2.3.2). When they were forced to localize in the cytoplasm the single amino acid changes also did not affect the APE1 distribution (Section 2.3.2). Therefore, this suggests SNPs in APE1 do not play any role in APE1 sub-cellular distribution, but rather result in functional differences of APE1 (Kim et al. 2012).

Of 20 identified human population variants of APE1 (Xi et al. 2004; Wilson et al. 2011), I64V, L104R, E126D, D148E, G306A, and D308A were chosen for our investigation. They were chosen because they have been associated with either disease susceptibility, decreased DNA incision activity, or decreased RNA cleaving activity. The I64V variation is one example which is hypothesized to have no known defect in APE1's functions (Table 9) and decreased lung cancer risk (Zienolddiny et al. 2006). The D148E variation is one example which has high allele frequency in the human population and is associated with various cancer risks (Section 1.4.2). It also has a significantly decreased RNA cleaving activity (Table 9). The G306A variation has no identified association with any disease but has a significantly decreased RNA cleaving activity with a normal abasic DNA incision activity (Table 9). The L104R, E126D, and D308A have both decreased abasic DNA and RNA cleaving activities (Table 9).
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The L104R and E126D variants were of particular interest because they were found to have altered cleavage patterns on c-myc CRD mRNA \textit{in vitro} as compared to that of WT-APE1 (Kim \textit{et al.} 2012). They also were found to be resistant to the effects of a powerful RNase inhibitor called RNasin (Kim \textit{et al.} 2012). There are controversial findings of these two variants and their association with Amyotrophic Lateral Sclerosis (ALS) (Olkowski 1998; Hayward \textit{et al.} 1999; Tomkins \textit{et al.} 2000) implicating their role in the formation and progression of ALS. Further investigations on APE1 polymorphisms in an effort to discover their effects on protein structure, abasic DNA and RNA incision activities, redox regulatory function, and intracellular distribution should be carried out in order to gain a better understanding of the correlation of APE1 polymorphisms and risks of various diseases.

Table 9. Summary of DNA and RNA incision activities of human population variants of APE1 (Kim \textit{et al.} 2012).

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<tr>
<td>WT</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>I64V</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>L104R</td>
<td>56.71 ± 26.80</td>
<td>74</td>
<td>60.0</td>
</tr>
<tr>
<td>E126D</td>
<td>60.43 ± 11.16</td>
<td>102</td>
<td>41.4</td>
</tr>
<tr>
<td>D148E</td>
<td>94.36 ± 6.20</td>
<td>101</td>
<td>23.3</td>
</tr>
<tr>
<td>G306A</td>
<td>107.22 ± 17.13</td>
<td>97</td>
<td>20.4</td>
</tr>
<tr>
<td>D308A</td>
<td>20 ± 3.52</td>
<td>71</td>
<td>11.1</td>
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</table>
4.3 Cellular localization of wild-type, N-terminal deletion, and human population variants of APE1 under cellular stress

The primary objective of this phase of the investigation was to identify the cellular localization of wild-type, N-terminal deletion, and human population variants of APE1 under cellular stress. Co-localization of APE1 with PBs and/or SGs was examined under normal, oxidative and nitrosative stress conditions. Differences in APE1 distribution upon oxidative and nitrosative stresses were also examined along with biochemical analysis.

4.3.1 Co-localization of APE1 with PBs and/or SGs

In an attempt to find a clue on APE1's role in RNA metabolism, we conducted co-localization studies of APE1 with PBs and/or SGs. mRNA decay is known to be carried out at specific sites such as cytoplasmic locales, mitochondria, or ER where mRNAs that need to be regulated are concentrated. Examples are IRE1α which is an endoribonuclease localized to the ER (Han et al. 2009), UPF1 which aids in decapping and deadenylation in PBs (Jin et al. 2009), Pat1β which is involved in decapping in PBs (Ozgur et al. 2010), and QKI-6 which is a RNA binding protein in SGs (Wang et al. 2010). Localization to the specific compartments of a cell helps these enzymes to be placed in a substrate-concentrated environment where they can function more efficiently. Therefore, we hypothesized that if APE1 is localized to PBs and/or SGs under normal and/or cellular stress, we could provide further evidence for its role in RNA metabolism. The results from the co-localization studies of APE1 with PBs and/or SGs indicated that APE1 is unlikely to be involved in mRNA processing that is carried out in PBs and SGs because there was no physical co-localization between these entities (Sections 3.3.1, 3.3.3, and 3.3.5). This raised two questions: (i) the cytoplasmic APE1 and its role in the cytoplasm and (ii) other possible locales where APE1 is involved in RNA degradation.
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4.3.1.1 Cytoplasmic APE1 and its proposed roles

Researchers have considered the role of cytoplasmic APE1 mainly as a mtDNA repair device in mitochondria upon oxidative stress (Section 1.4.3). Another proposed role of cytoplasmic APE1 was recently highlighted by the existence of the nucleocytoplasmic shuttling of APE1 (Zhang et al. 2011). The nucleocytoplasmic shuttling has emerged as an important regulatory mechanism for the multifunctional proteins involved in DNA repair pathways and maintenance of genomic stability such as BRCA1 and p53 (Zhang et al. 2011). Therefore, they proposed that the nucleocytoplasmic shuttling, spatial and temporal control of APE1 may serve to regulate the nuclear activity of APE1 in cells.

Other possible cytoplasmic roles of APE1 may be identified from the physical association of APE1 with other proteins in cells. Interaction of APE1 with nuclear factor-kappaB (NFκB) and subsequent activation of NFκB induced Cox-2 expression has resulted in tumor progression and metastasis in vitro and in vivo (Wu et al. 2010). Cytoplasmic APE1 caused a poor prognosis and a 3.7-fold increased risk of tumor recurrence and/or metastasis; therefore, cytoplasmic APE1 could enhance lung tumor malignancy (Wu et al. 2010). Overall, this suggests APE1 is a redox factor that can maintain transcription factors in a reduced state so that the downstream activation of cellular mechanisms can occur.

Physical association of APE1 with proteins that are involved in RNA metabolism is well studied (Section 1.3.1) and the discovery of RNA incision activity of APE1 (Sections 1.3.2 and 1.3.3) has been recently highlighted. Altogether, this evidence suggests a direct involvement of APE1 in RNA metabolic pathways. It is still unclear if the role of cytoplasmic APE1 is indeed maintaining and degrading RNA in cells. Further work such as assessing RNA incision activity of
the N-terminus deleted variants of APE1 and looking for the difference in RNA level upon introduction of cytoplasmic APE1 in cells are required to confirm such hypothesis.

4.3.1.2 Possible ways of APE1 involvement in RNA metabolism

APE1 can be involved in RNA metabolism either in the nucleus or in the cytoplasm. First, since APE1 is predominantly present in the nucleus in most cell types, one can assume that it plays a role in RNA metabolism in the nucleus. This has been supported by association and interaction of APE1 to other proteins that are involved in the recognition of damaged RNA, pre-mRNA maturation or splicing, RNA synthesis, rRNA metabolism, and general ribonucleotide metabolism (Section 1.3.1). Participation of APE1 as a RNA cleaving enzyme in the aforementioned pathways of RNA metabolism in the nucleus have, however, not been proven yet. Second, APE1 may play a role in RNA metabolism when it is localized in the cytoplasm. As mentioned earlier in this chapter, scientists have proposed that there is nucleocytoplasmic shuttling as well as spatial and temporal control of APE1 (Zhang et al. 2011). This may serve to regulate both the nuclear and the cytoplasmic activities of APE1 in cells. For instance, when APE1 is shuttled to the cytoplasm for a specific reason it may act as an RNA cleaving enzyme, whereas it acts as DNA repair enzyme in the nucleus. To carry out its cytoplasmic role, APE1 may be localized to any cellular compartments in the cytoplasm such as ER or nonspecifically throughout the cytoplasm, or associated with polysomes where the RNA cleaving activity of the native APE1 was first isolated (Barnes et al. 2009). As the nuclear role of APE1 in RNA metabolism was suggested by its interaction with other proteins that are involved in different RNA metabolism pathways, one can suggest its cytoplasmic role by doing the same sets of experiments. Therefore, a future experiment should look at protein-protein interaction by APE1 co-immuno-precipitation and examine its activity when it is associated with polysomes. If one
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can find a cell line or tissue that is known to have cytoplasmic APE1 predominantly, one can carry out a microarray study to see if there are any differences in particular gene expression due to the cytoplasmic localization of APE1.

4.3.2 Stress responsive degradation of N-terminal deletion variant of APE1

When the cells were treated with both oxidative and nitrosative stresses we did not observe any changes in the WT-APE1-GFP and ND41-GFP distributions based on live cell imaging (Sections 3.3.1 and 3.3.5). The only change in APE1 distribution upon cellular stress was the loss of the cytoplasmic ND20-GFP (Sections 3.3.3, 3.3.7 and 3.3.8). Interestingly, when we confirmed our live cell imaging data with the western blot analysis we noted a discrepancy of the WT-APE1-GFP distribution. This is discussed in detail in section 3.3.8. The observed changes in the ND20-GFP distributions upon cellular stress using western blot analysis are consistent with the data from live cell imaging (Section 3.3.8).

There are four possibilities which could explain the ND20 re-distribution upon cellular stress. The first two hypotheses were inhibition of nuclear export of the ND20 and increased nuclear import of the ND20 upon cellular stress that resulted in decreased cytoplasmic ND20. This suggests that it might have been due to the cell’s need of ND20 in the nucleus upon cellular stress. The third hypothesis was to confirm whether the re-distribution of the ND20 was due to the changes in the existing ND20 or newly synthesized ND20. Using chemical inhibitors, we found that none of the above was the cause of the ND20 re-distribution (Section 3.3.7). Lastly, we tested the possibility of ND20 degradation upon cellular stress. After treatment with proteasome inhibitor to stall all protein degradation in cells, we found that the re-distribution of ND20 did not occur upon cellular stress (Section 3.3.7). Therefore, we were able to conclude that
there is an important 21-40 amino acid region of APE1 which is responsible for the APE1
degradation upon cellular stress.

This finding is in good agreement with previous studies on critical lysine residues within
the 21-40 amino acid region of APE1 and its effect on APE1 functions and localization (Busso et
al. 2009; Fantini et al. 2010; Busso et al. 2011). There are five critical lysine residues including
K24, K25, K27, K31, and K32 which are important for APE1 interaction with NPM1 upon
acetylation (Fantini et al. 2010). These residues also serve important roles as ubiquitin acceptor
sites (Busso et al. 2009). Among these five residues, K24, K25, and K27 are known as the main
sites for ubiquitination (Busso et al. 2009); however, eliminating K24/25/27 by substituting with
Arg did not completely abolish APE1 ubiquitination, suggesting the supporting role of K31/32
and C-terminus region of APE1 in its ubiquitination (Busso et al. 2011). Depending on cell
types, cytoplasmic localization of mono-ubiquitinated APE1 in the mouse NIH3T3 cell line
(Busso et al. 2009) and nuclear localization in the HCT116 cell line (Busso et al. 2011) have
been observed. These mono-ubiquitinated APE1 may get poly-ubiquitinated and degraded in the
cell upon cellular stress (Section 3.3.7).

These five lysine residues may also play an important role in controlling APE1's
functional activation of its role in RNA metabolism which is supported by two different findings.
Researchers have found that ubiquitinated APE1 enhanced the global gene suppression as
demonstrated by an increased DNA affinity of ubiquitin-APE1 fusion protein (ub-APE1) (Busso
et al. 2011). They suggested that ub-APE1 may increase its existence on genomic DNA and its
role as a gene repressor. They also noted many genes involved in stress responses were down-
regulated in the cells expressing ub-APE1 as compared to that of the WT-APE1, including
ferritin and TUBA1 which was confirmed using qPCR assays (Busso et al. 2011). Since it was
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not reported whether the down-regulation of these genes were due to APE1 acting as gene repressor by tightly binding to DNA, we can speculate that its down-regulation may be due to the activation of RNA incision activity of APE1. This speculation is supported by another finding regarding the role of these critical lysine residues. Acetylation of the five critical lysine residues were found to inhibit APE1 action in RNA metabolism by preventing its RNA-binding activity (Fantini et al. 2010). Therefore, we propose that these post-translational modifications of these lysine residues may act as a switch for APE1’s involvement in RNA metabolism. Upon acetylation, these lysine residues may stall APE1’s involvement in RNA metabolism. On the other hand, ubiquitinated APE1 may be an active form for carrying out APE1’s role in RNA metabolism. Further investigation of ub-APE1 activity on RNA incision activity will be needed to identify such role of the lysine residues.

4.4 Immuno-histochemistry of Cancer tissues

Biomarkers are the molecules that are used as indicators of a biological state. It is a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention (Sahab et al. 2011). In cancer, biomarkers have the ability to assess the presence, stage, and malignancy of cancerous tissue and they also aid in cancer diagnosis, prognosis, and therapy assessment (Sahab et al. 2011).

There have been many studies looking at APE1 distribution or expression in various cancers and the complex localizations of APE1 in them have been well-reviewed (Evan et al. 2000; Tell et al. 2005). The nuclear/cytoplasmic and cytoplasmic distributions of APE1 are found to be correlated with more aggressive carcinomas and poorer prognoses for the patients.
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This is why APE1 has been proposed as a good biomarker for ovarian cancer (Sheng et al. 2011), cervical cancer (Qung et al. 2009), prostate cancer (Kelley et al. 2001), non-small cell lung cancer (Yoo et al. 2008), lip squamous cell carcinoma (Souza et al. 2011), and hepatocellular carcinoma (Avellini et al. 2010; Di Maso et al. 2007). There is currently no immuno-histochemical data available for APE1 in breast, kidney (renal cell), bladder, and thyroid cancer tissues. Therefore, to initiate the study, we aimed to examine the cytoplasmic expression of APE1 in breast FFPE carcinoma tissues specifically looking for any co-localization within PBs and SGs (Section 3.3.9). With very little histology background, we encountered some difficulties to distinguish normal breast cells from cells at different stages of cancer and difficulties staining the tissues with PBs and SGs (Section 3.3.9). Future experiments to improve these are suggested in detail in section 3.3.9.

This initial and preliminary study has provided an analysis of the benign and highly differentiated breast cancers from an immunohistochemical perspective. In future, APE1 and its expression may be used as a prognostic biomarker for aggressive or different stages of breast cancers. We suggest to also include the studies of renal cell carcinoma, transitional cell carcinoma, and thyroid follicular carcinoma tissues.

4.5 Concluding remarks

Several studies have identified the correlation between the cytoplasmic distribution of APE1 with aggressive tumor progression and poor disease prognoses. Recently, there have been a number of studies investigating the role of cytoplasmic APE1 but no consensus has been reached. The main goals of this thesis were to confirm the cellular localization of wild-type and N-terminal deletion variants of APE1 and to identify the cellular localization of APE1 human
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population variants. We also investigated the cellular localization of APE1 under cellular stress and the co-localization of APE1 with PBs and/or SGs as an attempt to identify APE1’s role in RNA metabolism. The role and importance of NLS in APE1 sub-cellular distribution was confirmed from the results of the expression of WT-APE1-, ND20-, and ND41-GFP in both HeLa and HepG2 cells; however, attempts to find out the exact mechanisms regulating APE1 distribution were not achieved in this thesis. A lack of changes in the distribution of APE1 human population variants implies that the SNPs of APE1 do not play any role in the sub-cellular distribution of APE1. This thesis is the first study to report the localization of APE1 human population variants. The discrepancy in the ND20 distribution upon cellular stress from the live cell imaging and western blot analysis was due to the loss of the cytoplasmic ND20-GFP through ubiquitination and degradation. This highlights the important five lysine residues that are responsible for ubiquitination and APE1 degradation upon cellular stress. These five lysine residues may also play an important role in modulating the activation APE1 in its role in RNA metabolism. Future studies on ub-APE1 RNA incision activity are expected to provide more insight into the role of lysine residues and their modification in APE1 function. Lastly, the initial and preliminary APE1 immuno-histchemical analysis of the FFPE breast cancer tissue provide the first insight into APE1 distribution in breast cancer. Future studies should include comparison of normal breast tissue and different stages of breast cancer tissues, and other cancer tissues that have not been examined. This is expected to provide extensive analyses of the cancers from an immuno-histochemical perspective and insight into the adequacy of using APE1 as a prognostic biomarker for aggressiveness or staging of cancers.
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