Title
Investigating the Interaction of p53 with Proteins Involved in Nucleotide Excision Repair Pathway

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Publication Date
2017

Peer reviewed|Thesis/dissertation
Investigating the Interaction of p53 with Proteins Involved in Nucleotide Excision Repair Pathway

A dissertation submitted in partial satisfaction of the requirements for the degree of

Doctor of Philosophy

in

Environmental Toxicology

by

Sandeep Sati

September 2017

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University of California, Riverside
Acknowledgements

These last five years have been the most enriching years of my life. This thesis is not only the result of my work at UCR, but also my experience with several remarkable individuals I met here, who I wish to acknowledge.

Firstly, I would like to express my sincere gratitude to my advisor Dr. Li Fan for his continuous support and guidance during my Ph.D. study. I would like to thank him for encouraging my research and for allowing me to grow as a research scientist. I really appreciate his patience, motivation and immense knowledge that has helped me to stay focused and work towards my thesis. I would also like to thank my Ph.D. committee members: Dr. Xuan Liu and Dr. Gregor Blaha for serving in my committee and providing guidance all through these years. I truly want to thank them for their constructive suggestions and discussions in all the committee meetings that have been immense help with all my projects.

I think everyone will agree with me if I say that a good support system is very important to survive the graduate school. Dr. Eduardo Hilario, Dr. Kevin Duprez, Aman Duggal and Feng He have been that support system for me all these years. I would like to thank them for being my friends and great colleagues. They have been always available for advice on lab related issues. I would like to specially thank Dr. Eduardo Hilario for being very patient with me and teaching me all the techniques and fruitful discussions. Along with being a great mentor, he has also been an awesome friend making my time enjoyable here. We have shared several meals and laughs together and I will always cherish these memories forever.
I would also like to thank our collaborators Prof. Hedi Mattoussi and his student Anshika Kapur at the Florida State university (FSU) for contributing to my research. Anshika has also been a great friend and support all these years. I will also like to give a special mention to Bob Manidhar and his family including his wife and beautiful daughters. They have been a family away from home. I deeply appreciate all their care and hospitality. Bob has always been there to support me in every need. I will cherish all the time we have spent together and all the memories made over these years.

Above all, I owe all this to my family- my father Gyana Nand Sati, mother (Late) Shakuntla Sati and my brother Pradeep Sati. All this would never have been possible without their support. I am so thankful to my parents, who have always believed in me and allowed me to pursue all my dreams. I would have never even thought of coming this far without the encouragement from my dad. I feel so lucky to have a father like him who has worked tremendously hard all these years to support me and my brother in our endeavors. I also appreciate my brother, for taking care of my dad while I have been away. I couldn’t have asked for a more loving and supportive family.
ABSTRACT OF THE DISSERTATION

Investigating the Interaction of p53 with Proteins Involved in Nucleotide Excision Repair Pathway

by

Sandeep Sati

Doctor of Philosophy, Graduate Program in Environmental Toxicology
University of California, Riverside, September 2017
Dr. Li Fan, Chairperson

DNA is exposed to relentless challenges by a variety of chemical, enzymatic and environmental agents. Maintaining the integrity of the genome by immediate and precise repair mechanisms is one of the most efficient processes inside cells. The nucleotide excision repair pathway (NER) is one of the most important repair mechanisms used by the cells to remove bulky DNA lesions specifically generated by exposure to UV, ionizing radiations and chemotherapeutic agents like cisplatin. Several proteins work simultaneously to operate this pathway including TFIH multiprotein complex containing ten subunits and ERCC1-XPF. Several studies in the past have proposed that p53 protein, product of a tumor suppressor gene, plays a key role in regulating the process of DNA repair. It has been shown to modulate the activity of multiple proteins involved in NER either by regulating their transcription or directly binding to them. However, as this process is very complex and well regulated, deciphering the mechanism of action has been a challenge.
In this thesis, I have attempted to gain some more insight into the role of p53 in NER by identifying proteins whose activity might be directly influenced by it. The two proteins that have primarily been concentrated on include XPB protein and ERCC1-XPF complex. XPB, the largest subunit of TFIIH complex, uses its ATPase and unidirectional 3’-5’ helicase activities in accordance with the 5’-3’ helicase activity of XPD (another subunit of TFIIH) in NER to unwind the DNA helix near lesions. ERCC1-XPF is a structure specific endonuclease that cleaves the damaged DNA at the 5’ end of DNA lesion for subsequent NER factors to fill in the gap with correct nucleotide sequence complementary to the template. Several in vitro and in vivo techniques were employed to explore the interaction of XPB and ERCC1-XPF with p53 protein and map down the specific modules of each protein important for this interaction.

In Chapter 1, the fundamental reasons behind DNA damage and different DNA repair pathways responsible for maintaining the genome have been introduced. An overview of the process of NER along with detailed description about XPB DNA helicase, ERCC1-XPF endonuclease complex and p53 protein have been provided. This is followed by a discussion about the role of p53 in DNA repair. Finally, the principle of all the techniques used in this study to explore protein-protein interactions have been outlined.

In Chapter 2, I provide evidence confirming that XPB could be one of the factors responsible for the interaction between p53 and TFIIH complex, regulating the DNA repair system. Firstly, this interaction was established using affinity column chromatography for pull-down assay and Fluorescence Resonance Energy Transfer (FRET). Further yeast two hybrid analysis was utilized to illustrate that smaller modules
of XPB including 105-129 a.a at the N-terminus and 730-782 a.a at the extreme C-terminus bind to DNA binding domain (DBD) and C-terminal domain (CTD) of p53. The analysis also demonstrated that XPB-p53 protein-protein interaction is weakened by the T119P mutation in XPB, and is completely abolished by the F99S and XPB11BE mutations. All three mutations are associated with human diseases.

In Chapter 3, the domains of interaction between ERCC1-XPF complex and p53 were mapped down. The results from yeast two hybrid analysis indicated that XPF subunit of ERCC1-XPF complex does not interact with p53. In contrast, ERCC1 binds to DNA binding domain (DBD) and C-terminal domain (CTD) of p53 primarily with its central domain. However, the helix-hairpin-helix (HhH2) domain of ERCC1 might also play a critical role in this interaction.

Finally, a summary of the major developments made in this thesis along with a discussion about the future direction is presented in Chapter 4.
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CHAPTER 1

INTRODUCTION

1.1 DNA DAMAGE AND REPAIR

The eukaryotic genome is under constant threat from exogenous agents like ultraviolet (UV) radiation, ionizing radiation, and numerous genotoxic chemicals. The by-products of endogenous metabolic activities like reactive oxygen species (ROS; i.e., superoxide anions, hydroxyl radicals, and hydrogen peroxide) and alkylating agents also affect the physical constitution of DNA. The damage induced might result in damaged bases, mismatches, single strand breaks, double strand breaks (DSBs) and larger lesions or adducts \(^1\). The impaired DNA may induce activation of cell cycle checkpoint or completely cease cell cycle resulting in apoptosis. The resulting mutations and chromosomal aberrations might activate oncogenes and suppress tumor suppressor genes leading to cancer (see schematic in Figure 1.1). Given the central role of DNA, it is crucial to promptly remove any DNA lesions or adducts to maintain the genomic integrity of cells. If DNA lesion left unrepaired, it leads to stoppage of different cellular processes like transcription and replication, which are important for cell survival, which later lead to mutagenesis and ultimate lead to cellular toxicity. DNA damage can lead to several inherited diseases, aging, and carcinogenesis. There is an intricate system of DNA repair pathways that work together to remove any lesions present in DNA to maintain the integrity of the genome. These include Base excision repair (BER), Mismatch repair...
(MMR), Double strand break repair (DSB) and Nucleotide excision repair (NER) pathway. Each pathway works exclusively for removal of a specific type of DNA lesion 2-9.

**Figure 1.1**- Schematic representation of different agents leading to DNA damage, the DNA repair mechanisms involved in the removal of the lesions and the long-term consequences if DNA remains unrepaired. Image from Hoeijmakers *et al* 4.

**1.1.1 Base excision repair pathway:** The pathway is normally used for the removal of oxidative damages to the bases. Enzymes in this pathway recognize relatively few, but frequent DNA lesions such as abasic sites, deaminated C and A, and individual bases damaged by oxidative intermediates or alkylating agents. Most of these lesions do not distort the DNA helix structure to a great extent. In both prokaryotes and eukaryotes
BER is initiated by DNA glycosylases, a class of enzymes that recognizes a specific set of modified bases. DNA glycosylases cleave the N-glycosylic bond between the target base and the deoxyribose, releasing a free base and leaving an apurinic/apyrimidinic (AP) site. In mammalian cells, two pathways for the processing of AP sites have been described: (i) a single nucleotide insertion (or "short patch") pathway catalyzed by DNA polymerase β and (ii) a proliferating cell nuclear antigen (PCNA) dependent ("long patch") pathway, involving a re-synthesis patch of 2 to <13 nucleotides. Additional variations on each of these BER pathways have also been reported 10.

1.1.2 Mismatch repair pathway: This pathway is used by cells to remove erroneous bases normally incorporated during DNA replication and recombination. Basically, this type of damage occurs because of slippage of DNA polymerase due to repetitive sequences present on DNA during replication. The mismatched bases normally disturb the hydrogen bonding between the DNA strands. This pathway includes four steps: i) recognition of mismatch bases, ii) recruitment of MMR protein factors, iii) verification of the strand containing the mismatch followed by degradation of the strand lying past the mismatch, and iv) re-synthesis of the strand. Eukaryotic system contains mutS related protein complex MSH2-MSH3 to perform MMR 11.

1.1.3 Nucleotide excision repair pathway: This pathway is one of the more flexible DNA repair pathways that can repair a variety of DNA lesions by sensing any types of helix distortions in DNA. In particular, it is responsible for removal of bulky DNA
lesions like cyclobutane pyrimidine dimers (CPD) and 6-4 photoproducts produced mainly by exposure to UV radiations. Once the lesion has been detected, it uses two different structure specific endonucleases to cleave DNA on both the sides of the lesion; this usually removes approximately 28-30 base pairs (including the lesion) from the strand. The resulting gap is then filled by addition of bases complementary to the template strand by DNA polymerase.

1.1.4 Double strand break pathway: Double strand breaks are (DSB) the most serious form of DNA damage mainly caused due to exposure to ionization radiation and is repaired by double strand break (DSB) repair pathway. This type of damage is the worst because it affects both strands of DNA preventing the use of complementary strand as a template. This pathway can follow two different mechanisms each dealing with a specific kind of damage. These include- Homologous recombination (HR) and Non-homologous end-joining (NHEJ) pathways. Homologous recombination is an error free repair pathway that uses identical or nearly identical sequence as a template to retrieve the genetic information. Whereas in NHEJ, the two ends of DNA molecules are directly joined end to end resulting in some deletions; hence NHEJ mechanism is more prone to errors. Proteins used in NHEJ pathway is a heterodimeric Ku70/Ku80 complex.
1.2 NUCLEOTIDE EXCISION REPAIR PATHWAY

Nucleotide excision repair pathway is one of the most flexible DNA repair pathways in mammals responsible to remove a variety of bulky DNA adducts including, cyclobutane pyrimidine dimers (CPD) and 6-4 pyrimidine-pyrimidone photoproducts (6-4 PPs) that are majorly produced by exposure to UV radiation, environmental mutagens, and chemotherapeutic agents like cisplatin. This pathway is also usually able to detect and remove a vast variety of DNA lesions that are bulky and can thermodynamically destabilize DNA.\textsuperscript{16–19}

NER has two sub-pathways: 1) GG-NER (Global genome repair) pathway, 2) TC-NER (Transcriptional coupled) \textsuperscript{20}. Both the sub-pathways are different only in the way they detect the DNA damage.

1.2.1 Global genome repair pathway: GG-NER pathway as the name suggests is effective in repairing the DNA lesions present anywhere in the genome. Here, the damages are first recognized by XPC-Rad23B or UV-DDB protein followed by the process of repair. XPC-Rad23B protein is a heterodimer that along with centrin (CENT) protein can specifically detect the lesions that produce distortion in the helical structure of DNA like 6-4 PPs lesions \textsuperscript{16,21}. Later, it recruits the NER machinery to the damaged site for the repair procedure. XPC, a 125kDa protein, preferentially binds to damaged DNA. It is always found as a part of a complex containing two human homologues of yeast Rad23 proteins i.e. Rad23A and Rad23B. The exact function of the latter two
proteins are still unknown, but have been proposed to enhance the activity of XPC protein. Additionally, Rad 23 protein contains a ubiquitin like moiety at its N-terminus suggesting that it might be involved in maintain the stability of XPC protein via ubiquitin-degradation pathway.

A few DNA lesions, like CPD lesions, are not capable of distorting the DNA structure, making it difficult for XPC-Rad23B complex to detect them. In such situations, UV-induced DNA damage binding protein (UV-DDB) recognizes the lesions and then recruits the XPC to the damage site (schematic summarizing this in Figure 1.2). UV-DDB is a hetero-dimer composed of two subunits including p127/DDB1 (127kDa) and p48/DDB2 (48kDa). Mutation in DDB2 subunit has been shown to cause mild xeroderma pigmentosum and skin cancer also known as XPE disease. This validates the importance of UV-DDB protein complex in efficient functioning of GG-NER.

After the damage recognition, various NER factors (detailed in Table 1.1) are recruited at the damage site to cleave the DNA lesion. For this, a multi-protein complex known Transcription Factor II H (TFIIH) is recruited by XPC-Rad23B at the damage site. This complex is conserved in organisms from yeast to human and has been proven crucial for cell survival. TFIIH is mainly made up of two sub complexes including the core complex and the Cdk- activation kinase (CAK) complex. The core of the TFIIH consist of seven protein subunits i.e. XPB, XPD, p52, p62, p44, p34, and p8 that together form a ring like structure. The CAK complex is heterotrimeric composed of three proteins including Cdk 7, Cyclin H, and Menage a trois 1 (MAT1) that are involved in cell cycle control. It has been shown that the subunits of TFIIH complex interact with each other and
Figure 1.2- Mode of detection of different types of DNA lesions during NER pathway. CPD lesions produce small helix distortion in DNA that can be recognized directly by UV-DDB complex followed by binding of XPC-RAD23B to the DNA strand opposite to the lesion. The 6-4PP lesions produce large helix distortion in DNA which can easily be detected by XPC-RAD23B. Image from Marteijn et al.¹².

This is important for proper functioning of the complex during NER. For instance, p52 subunit of TFIIH interacts with XPB and control its ATPase activity.³⁵ Similarly, p44 subunit has been reported to interact with XPD and control its helicase activity.³⁶ XPB has also been described to interact with many subunits of TFIIH including p62, p52, p44, and p8.³⁷
Table 1.1- All the core protein factors involved in DNA repair process by NER pathway.

<table>
<thead>
<tr>
<th>NER factor</th>
<th>subcomplex</th>
<th>lesion and facilitates repair complex assembly</th>
<th>basal RNA Pol II transcription and cell cycle regulation</th>
<th>additional non-NER-specific role in transcription-coupled repair</th>
<th>Subunits</th>
</tr>
</thead>
<tbody>
<tr>
<td>XPC-hHR23B</td>
<td>damage sensor and repair recruitment factor</td>
<td>only in GC-NER; not involved in TC-NER</td>
<td>XPC</td>
<td>protein size (aa): 940</td>
<td>Rad4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>hHR23A</td>
<td>rodent mutant: Rad23</td>
<td>S. cerevisiae homolog: affininty for damaged DNA</td>
</tr>
<tr>
<td>TFIH</td>
<td>catalyzes open complex formation around the lesion and facilitates repair complex assembly</td>
<td>basal RNA Pol II transcription and cell cycle regulation</td>
<td>XPF</td>
<td>protein size (aa): 782</td>
<td>ERCC3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>XPD</td>
<td>rodent mutant: Rad25/SSL2</td>
<td>ERCC2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>p53</td>
<td>5' → 3' helicase</td>
<td>Rad3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>p44</td>
<td>DNA binding?</td>
<td>TFH4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>p62</td>
<td>DNA binding?</td>
<td>SSL1</td>
</tr>
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<td></td>
<td></td>
<td>p52</td>
<td>CAK subcomplex</td>
<td>Cdk7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mat1</td>
<td>CAK subcomplex</td>
<td>Cyclin H</td>
</tr>
<tr>
<td>XPA</td>
<td>binds damaged DNA and facilitates repair complex assembly</td>
<td>replication recombination</td>
<td>XPA</td>
<td>protein size (aa): 273</td>
<td>Rad14</td>
</tr>
<tr>
<td>RPA</td>
<td>stabilizes opened DNA complex and positions nucleases</td>
<td>replication recombination</td>
<td>RPA70</td>
<td>protein size (aa): 616</td>
<td>Rif1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>RPA32</td>
<td>ssDNA binding</td>
<td>Rif2</td>
</tr>
<tr>
<td>XPG</td>
<td>catalyzes 3' incision and stabilizes full open complex</td>
<td>additional non-Ner-specific role in transcription-coupled repair</td>
<td>XPG</td>
<td>protein size (aa): 1186</td>
<td>ERCC5</td>
</tr>
<tr>
<td>ERCC1-XPF</td>
<td>catalyzes 5' incision</td>
<td>interstrand cross-link repair recombination via single-strand annealing</td>
<td>ERCC1</td>
<td>protein size (aa): 297</td>
<td>Rad10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>XPF</td>
<td>rodent mutant: Rad9</td>
<td>ERCC4</td>
</tr>
</tbody>
</table>

The CAK sub-complex of TFIH complex has been demonstrated to control cell cycle as well as gene transcription. When the CAK sub-complex is not bound to the TFIH core, it phosphorylates the Cdk kinases and hence plays a role in the cell cycle check point. However, when it is bound to the TFIH complex, it phosphorylates the C-terminus of RNA-polymerase II and other transcription factors that helps in the transcription of the genes.
The core of TFIIH complex harbors two DNA helicases including XPB (ERCC-3) and XPD (ERCC-2). Both the helicases have opposite polarities (3’-5’ for XPB and 5’-3’ for XPD) and work together to open the double stranded DNA around the lesion to allow other NER factors to bind to the pre-incision complex \(^{43}\). XPB and XPD possess ATPase and helicase activity such that only ATP-ase activity of XPB and helicase activity of XPD are required for proper functioning of NER \(^{44}\).

Once the DNA lesion has been encountered and verified by XPD, it gets blocked recruiting other factors like XPA and RPA at the damaged site \(^{45,46}\). XPA is a 32 kDa protein that preferentially binds to damaged

**Figure 1.3**- A schematic summary of different steps involved in GG-NER and TC-NER. Image from Marteijn *et al* \(^{12}\).
DNA with a stronger binding to double stranded DNA than single stranded DNA. It plays a structural role in NER by maintaining the pre-incision complex intact and interacting with other NER factors to orient them in proper position. Replication Protein A (RPA) also referred as single strand binding protein (SSB) binds to single stranded DNA around the lesion. It primarily binds to the strand opposite to the DNA strand containing the lesion to mask and protect it from getting attacked by the nucleases. It also allows to properly orient the endonucleases of TFIIH complex including ERCC1-XPF and XPG by protein-protein interactions. XPG binds to 3’-end of the damaged DNA completing the pre-incision complex. This forces the XPC-Rad23B protein to leave the complex. ERCC1-XPF then binds to the 5’-end of the damaged site by interacting with XPA. This is followed by the first incision by ERCC1-XPF at 5’end of damaged site and the second incision by XPG at 3’-end removing around 24-32 nucleotide containing the lesion. Then, the gap is filled by DNA polymerase by using the undamaged strand as template. The newly synthesized strand is finally ligated to the rest of the DNA segments by DNA ligase III (ERCC1).

1.2.2. Transcriptional coupled pathway: TC-NER is particularly responsible for repair of the lesions in transcriptionally active strands. This pathway is initiated when the RNA polymerase II get stalled in the lesion site during the transcription process. The repair process starts by recruiting the TC-NER specific cockayne syndrome protein A (CSA) and cockayne syndrome protein B (CSB) at the damage site. The CSA and CSB proteins are essential to recruit other NER related protein factors like TFIIH complex to
bind to the damage site. After the damage has been detected, the repair process involves the same sequential steps as explained previously in GG-NER. The steps in GG-NER and TC-NER are summarized in Figure 1.3.

1.2.3. Diseases associated with NER: Several diseases have been associated with non-functional NER pathway including:

a) Xeroderma pigmentosum (XP): XP was the first human disease identified that is caused due to ill functioning NER pathway. This is an inherited rare autosomal recessive disease which is mainly characterized by extreme sensitivity to sunlight leading to sunburns and skin pigmentation ultimately increasing the rate of skin cancer. Mutations in any of the eight XP (XP-A through G) genes that renders the protein non-functional can lead to this disease. The frequency of occurrence for XP is approximately 2.3 individuals per million of the general population in western Europe. Individuals who are heterozygous for this disease are mainly unaffected however, homozygous mutation leads to severely effects. Incidence of cancer in an individual with this mutation is 2000 times higher than normal individuals. Patients suffering with XP-related diseases show many neurodegenerative defect, dwarfism, deafness, impaired sexual development.

b) Cockayne syndrome (CS): This also an inherited, rare autosomal recessive disease that is characterized by short stature, and neurodegenerative and developmental disorders. This disease is a result of mutation in the CSA and CSB genes. There are three types of CS including: (i) Cockayne syndrome type-1 which is moderate in nature; (ii)
Cockayne syndrome type-2 which is the most severe form of disease; and (iii) Cockayne syndrome type-3 which is also considered as the milder form of this disease. Patients with these diseases normally have a life span between 1 year to 20 years.  

c) Trichothiodystrophy (TTD): This disease is also an inherited autosomal rare disease characterized by brittle, sulfur deficient hairs. Other symptoms include cutaneous, neurologic and growth abnormalities. There is also report of abnormal characteristic at birth, decrease fertility, which showed the role of the gene mutated in this disease in DNA repair and short stature in individual affected with this disease.  

1.3 XERODERMA PIGMENTOSUM COMPLEMENTING GROUP B (XPB)  

1.3.1 Structure and role of XPB in NER: A very important component of the TFIIH complex that is important for its activity in NER and as a transcription factor is Xeroderma Pigmentosum complementing group B (XPB) protein. XPB is a 90 kDa, 3’-5’ DNA helicase that belongs to the SF-2 helicase superfamily. It is also known as ERCC3 in eukaryotes and Rad25 in archaea. It helps in the promoter melting during transcription and unwinding DNA along with placement of TFIIH factors onto the damaged DNA during NER pathway. The crystallographic structure of the full length *Archaeoglobus fulgidus* XPB (Af-XPB) and the human C-terminal XPB have been reported. Structure of Af-XPB shares 42% similarity with the central region of human XPB indicating that the central core structure is conserved from archaea to human (see
Figure 1.4). The crystal structure of Af-XPB shows two Rec-A like helicase domains (HD-1, HD-2) connected via a flexible linker region. XPB contains seven conserved helicase motifs i.e. four helicase motifs are present on HD-1 (I, Ia, II, III) while other three on the HD-2 domain (IV, V, VI) \(^{69}\). Helicase motif I (GxGKT,) also known as walker A motif, is involved in phosphate binding \(^{70}\). Substitution of K with R in this motif abolishes the DNA repair activity of XPB. Helicase motif II is important to bind to ATP \(^{70}\). The important helicase motif III is involved in ATP hydrolysis which provides energy for DNA unwinding \(^{71}\). Another motif also known as RED motif is present near motif III and consists of charged acidic residues i.e. R210, E211 and D212. This motif is important for helicase activity of XPB that has been confirmed by the reduced helicase activity due to mutations in RED motif. The other three motifs (IV-VI) present on HD-2 domain are also involved in DNA repair; any alterations to these motifs also impair the DNA repair activity \(^{73}\).

Human XPB contains two additional domains beside HD-1 and HD-2 including: (i) damage recognition domain (DRD) present on the N-terminal XPB and is linked to HD-1. DRD domain shares similar structure as the mismatch recognition domain (MRS) of
MutS protein from MMR pathway. DRD domain is proposed to bind to the DNA lesions or kinks. (ii) Thumb domain (ThM) present on the top of HD-2 domain is structurally similar to the thumb domain of T7 DNA polymerase. It contains several positively charged residues that might be involved in protein-DNA interaction.

XPB is an unconventional SF-2 DNA helicase. This suggests that HD-1 and HD-2 domains of XPB do not form an inter-domain groove for ATP binding and hydrolysis as present in other conventional DNA helicases. To make an inter-domain groove for ATP binding and hydrolysis, instead, it has been proposed that HD-2 domain rotates approximately by 170° around an inter-domain hinge region (see Figure 1.5). This rotation of XPB helicase domain-2 is hypothesized to be crucial for XPB to perform its function.

**Figure 1.5**- Proposed conformational change in Af-XPB upon binding to ATP. Image procured from Fan et al.


1.4 ERCC1-XPF

1.4.1 Structure and role of ERCC1-XPF in NER: ERCC1-XPF is a structure specific endonuclease which has roles in multiple DNA repair pathways including NER and DSB to remove the DNA lesion specifically generated by UV light exposure [77]. This endonuclease belongs to the XPF/MUS81 superfamily and is highly conserved throughout evolution as all eukaryotes studied so far have homologs of ERCC1-XPF. Most of the members of XPF/MUS81 family are present as homodimers in archaea and heterodimers in eukaryotes where one of the subunit is catalytically active while the other is not [50,78–82]. Following this pattern, ERCC1-XPF is also a heterodimer where non-catalytic ERCC1 subunit is responsible for efficient protein-protein and protein-DNA interactions while the catalytic XPF subunit can cut the single/double stranded-DNA. Presence of ERCC1 is indispensable for the catalytic activity of XPF [83]. Moreover, the formation of ERCC1-XPF complex is also important as the individual subunits are insoluble and tend to aggregate due to improper folding [84,85].

The eukaryotic XPF protein (916 amino acids) consists of three domains including: N-terminal SF-2 inactive DEAD/DExH helicase domain [18], active nuclease domain containing GDX₉ERKX₃D motif and helix-hairpin–helix domain (HhH₂) at the C-terminal end [86–88]. ERCC1 (297 amino acid) has two domains: central domain which has inactive nuclease and helix-hairpin-helix domain (HhH₂) at the C-terminal end that binds to XPF [89]. *Saccharomyces Cerevisiae* also has the orthologues for ERCC1-XPF known as Rad1-Rad10 where Rad1 has the catalytic domain and Rad10 has the non-catalytic
domain \(^{90,91}\). Similarly, \textit{S. pombe} has Rad16-Swi10 where Rad16 has the catalytic domain while Swi10 has the non-catalytic domain \(^{92}\). Structural and biochemical studies of ERCC1-XPF have shown that only HhH\(_2\) of ERCC1 is required for binding with DNA; this indicates that ERCC1 targets the ERCC1-XPF complex at the correct position on DNA which is later cleaved by the nuclease domain of XPF. Functioning of these structure specific endonuclease must be tightly regulated as non-specific excision of DNA might lead to genome instability \(^{93}\). These structure specific endonucleases can cut the damaged as well as undamaged DNA with the same efficiency, hence their activity is tightly regulated. \(^{93}\).

1.4.2 ERCC1-XPF role in cancer chemotherapy: The activity of ERCC1-XPF complex to remove DNA lesions has recently been exploited to assess the efficiency of platinum based chemotherapy. The exposure to cisplatin usually results in bulky DNA adducts that are removed by ERCC1-XPF endonuclease in NER pathway. So, the efficiency of these platinum based chemotherapy depends on the activity of ERCC1-XPF. Therefore, ERCC1-XPF can be used as a biomarker to predict the outcome of the chemotherapy \(^{85}\).

Several studies have reported that low level of ERCC1-XPF can be correlated to successful treatment of cancer cells during chemotherapy as the cells are unable to repair the damage induced by the drugs leading to apoptosis. Whereas high levels of ERCC1-XPF correspond to poor response to chemotherapy as any cisplatin induced the DNA lesions are removed by the ERCC1-XPF complex \(^{94}\). Hence, reduction of ERCC1-XPF level in the cells can be beneficial to improve the probability of successful chemotherapy.
Several strategies have been developed to decrease the level and/or functional activity of the ERCC1-XP complex. For instance, 1) modifying the dimerization domains of ERCC1 and XPF subunits would interrupt with the complex formation. 2) disturbing the interaction of ERCC1 with XPA will have an effect as this interaction helps to recruit the complex at the right site for cleavage. 3) any alteration to the nuclease domain of XPF rendering it inactive would also impair the complex from repairing any damaged DNA.

1.5 p53 PROTEIN

p53 protein is one of the tumor suppressor proteins that play key roles in controlling many cellular processes like cell cycle control, apoptosis, differentiation, senescence and DNA repair. Any mutations or alterations in the gene has been shown to affect the regulation of cellular functions and can eventually result in tumor growth. Its importance in controlling cancer can be judged by the fact that this protein is found to be mutated in more than 50% of cancers.

It is a 393 amino acids long phosphoprotein that is usually present in almost undetectable amounts in normal cells due to continual degradation by the proteasomal pathway. However, during stress conditions the level of p53 increases dramatically, achieved by post-translational modifications that increase the half-life of the protein. There are many factors that can elevate p53 protein levels in cells including several genotoxic and non-genotoxic factors like mutagens, heat shock, oxidative stress, and exposure to UV-light or ionizing radiation. Once accumulated p53 can act as a transcription factor and
initiate transcription of its downstream effector genes including p21, GADD45 and cyclin G. The products of these genes may induce cell cycle arrest which allows the cells to repair the damaged DNA. However, if the cell fails to repair the DNA damage, p53 can also induce apoptosis to permanently remove the damaged mutated cells. Several studies have also demonstrated that p53 can also maintain genomic integrity by directly interacting with the DNA damage or the proteins involved in repair mechanisms thereby regulating their functions. As p53 is important for maintaining the genomic integrity, it is also known as the guardian of the genome.

1.5.1 Structure of p53: p53 protein is a modulator protein like other transcription factors and contains five functionally conserved domains (see Figure 1.6) including:

1) Transactivation domain (TAD): The N-terminal p53 contains an acidic transcriptional activation domain that is involved in activation of transcription factor.

2) Proline Rich Region (PRR): This region of the protein contains proline rich residues PXXP, and MDM2 protein binds to this domain as well with transcriptional activation domain.

3) DNA Binding Domain (DBD): This domain is involved in sequence specific binding of DNA. This domain is found to be mutated in more than 90 % of the cancer.

4) Oligomerization Domain (OD): This domain allows oligomerization of the protein to form a tetramer, which is the active form of the protein. This domain also contains nuclear localization sequences (NLS).
5) **C-terminal Regulatory Domain (CTRD):** This domain is involved in the negative regulation of DNA binding by the central domain of p53.

![Diagram showing structural domains of p53 protein]

**Figure 1.6**—Schematic diagram showing all the structural domains of p53 protein.

### 1.5.2 Role of p53 in NER:
Over the past few years, many articles have demonstrated that p53 is directly or indirectly responsible to maintain the genomic integrity by contributing to the DNA repair pathways, specifically NER \(^{110,111}\). The role of p53 protein in regulating both global genomic repair (GGR) and transcription-coupled repair (TCR) has already been demonstrated in several studies. The cells lacking functional p53 displayed more sensitivity to UV induced DNA damage and a slower repair \(^{112–117}\).

In a study, human colon carcinoma RKO cells with human papillomavirus E6 oncoprotein or a dominant-negative mutant p53 transgene (disrupts p53 function) demonstrated reduced repair of UV induced DNA damage in host cell reactivation experiments and \textit{in vitro} DNA repair assays \(^{112}\). In another study, the cells with mutated p53 gene show reduced removal of UV induced CPDs from overall genomic DNA in comparison to the normal cells or fibroblasts with heterozygous mutations in p53 gene \(^{114}\). Further, several studies reported that p53 directly binds to TFIIH subunits including XPD and XPB confirming its direct role in GGR \(^{118,119}\). The p53 protein has also been
reported to transcriptionally regulate the expression of DDB2 gene by directly binding to it and XPC gene by binding to the p53 response element present in XPC promoter region \(^1\). All these results combined signify the role of p53 protein in regulating GGR pathway in NER.

A multitude of investigations also confirm the role of p53 protein in TCR. For instance, Yamaizumi and Sugano \(^2\) found that by using a-amanitin, an inhibitor of transcription in normal and XP cells, resulted in increased p53 expression in both cells. This indicated that UV-induced DNA damage in active genes can lead to accumulation of p53. Further, Wang et al observed that p53 interacts with CSB helicase protein that is a part of transcription-coupled preferential-strand NER (TC-NER) \(^3\). Further, in another study the UV-enhanced reactivation (UVER) and heat shock-enhanced reactivation (HSER), both dependent on TCR, triggered the host cell reactivation (HCR) of UV damaged reporter gene in normal fibroblasts. However, this was not observed in case of p53 mutant Li-Fraumeni cells suggesting that p53 is necessary to induce TCR \(^4\).

All these evidences confirming the role of p53 in NER clearly suggest that any alterations in the p53 gene or protein may negatively affect the genomic stability of the cells by lowering the DNA repair efficiency. However, the mechanism governing the role of p53 in NER is still not entirely understood. Hence, in this study to get further insights, the interaction of p53 with TFIIH subunit, XPB DNA helicase and structure specific endonuclease ERCC1-XPF was tested by using \textit{in vivo} and \textit{in vitro} methods.
1.6 TECHNIQUES USED TO STUDY PROTEIN-PROTEIN INTERACTION

1.6.1 Förster resonance energy transfer (FRET): FRET is a photo-physical process that involves non-radiative transfer of energy from a photo-excited donor fluorophore (D) to a ground state acceptor molecule (A) through dipole-dipole interactions. The pair of molecules that interacts resulting in FRET is often referred to as the donor-acceptor pair. Briefly, the donor fluorophore emits at shorter wavelengths that overlap with the absorption spectrum of the acceptor molecule (see Jablonski diagram in figure 1.7A). This results in a reduction of the donor fluorescence (quenching) and excited state lifetime along with an increase in acceptor fluorescence intensity (if it is a fluorophore) \(^{124-127}\). There are a few factors that are critical for efficient FRET interactions including: (a) proximity between donor and acceptor (\(\leq 10\) nm); (b) a finite spectral overlap between the donor emission and acceptor absorption profiles (see Figure 1.7B). Additionally, the relative orientation of the donor and acceptor dipoles also strongly governs the efficiency such that the perfectly parallel dipoles produce highest FRET efficiency while the perpendicular dipoles show no interaction \(^{127,128}\).
Figure 1.7- Principle behind Fluorescence Resonance Energy Transfer (FRET) (A) Jablonski diagram representing the energy transfer between the donor and acceptor electron vibrational energy states resulting in FRET. (B) Absorption and emission spectra for an ideal donor-acceptor pair depicting the spectral overlap between the fluorescence spectrum of donor and absorption spectrum of acceptor.

The rate of energy transfer ($k$) for a donor-acceptor pair strongly depends on their center to center distance (inverse sixth power) and is expressed as,

$$k = \frac{1}{\tau_D} \left( \frac{R_0}{r} \right)^6$$

(Eq. 1)

where $\tau_D$ designates the excitation lifetime for the donor, $r$ is the distance between the donor and the acceptor, and $R_0$ also referred to as Förster radius, is the distance at which energy transfer is 50% efficient. The magnitude of the $R_0$ is dependent on the spectral properties of the donor and the acceptor and is given by:

$$R_0 = \left( \frac{9000 \times \ln(10) \times k_D^2 \times Q_D I}{128 \pi^5 n_D^4 N_A} \right)$$

(Eq. 2)

Here, $I$ is the integral of the spectral overlap function, $Q_D$ is the donor PL quantum yield, $n_D$ is refractive index of the medium, $N_A$ is Avogadro’s number, and $\alpha_D^2$ is the dipole
orientation factor that is usually equal to 2/3 for a randomly oriented donor-acceptor pair \[126,127\].

The FRET can be easily detected and quantified with respect to the decrease in fluorescence of donor molecule or as an increase in fluorescence of the acceptor. The FRET efficiency \((E)\) can hence be extracted by using \[127\]:

\[
E = 1 - \frac{F_{DA}}{F_D} \quad \text{(Eq. 3)}
\]

In the above expression, \(F_D\) and \(F_{DA}\) designate the experimentally measured average PL intensities for the donor molecule in absence and presence of the acceptor molecule respectively. The strong distance dependence of FRET efficiency allows to probe any slight changes in the distance between the donor and the acceptor molecules. This technique is best suited for the separation distances in the range of 10-100 Å which is comparable to the diameters of many proteins, thickness of biological membranes, and distances between sites on multi-subunit proteins. Hence, this technique is ideal tool for several biological applications such as probing time-dependent protein conformational change, monitoring protein dynamics over an extended period, and exploring ligand-receptor binding and protein-protein interactions \[126,129-137\].

Most of these biological investigations using FRET have relied on using conventional fluorophores including molecular (or organic) dyes and genetically encoded fluorescent proteins. The emissive fluorophores can act as both donors and/or acceptors, while the dark dyes can operate only as acceptors that lead to quenching. However, these fluorophores have several inherent limitations, including low quantum yields and less resistance to chemical and/or photo-degradation/bleaching. They have narrow excitation
and broad emission spectra which makes it difficult to pair donors and acceptors that have optimal spectral overlaps while avoiding direct excitation of the acceptor. However, recent advances in growth of colloidally stable luminescent quantum dots have attracted great interest in employing them as potential fluorophores in sensing applications using FRET. They offer unique optical and physical properties including size- and composition dependent broad absorption, narrow and symmetric emission spectra along with high quantum yield and remarkable photo- and chemical-stability. The progress made in surface functionalization of these nanomaterials has been utilized in several FRET investigations to design sensor platforms and investigate protein-protein/protein-DNA interactions.

1.6.2 Yeast two hybrid: Yeast two hybrid system was first developed by Field and Song in the year 1989. It is a very commonly used technique to evaluate protein-protein interaction which is based on the reconstitution of a transcription factor as a result of interaction between the two proteins. Soon after, this technique gained popularity for investigating protein interactions due to several advantages. For instance, the use of eukaryotic yeast as the host system offers greater chance for proteins to be properly expressed with post-translational modifications and folding in comparison to prokaryotic (bacterial) system or using in vitro conditions. The use of transcription of reporter gene as an interaction indicator allows the signal to be amplified making it possible to detect even weak and transient interactions. However, if the protein of interest by itself can initiate the transcription of reporter gene (also known as auto-activation) it might show some
false positives. Therefore, every protein construct should to be tested for auto-activation before use in the interaction studies. All the interaction in this system occurs in the yeast nucleus, so any protein with strong targeting signal might not be suitable for this system.

Yeast two hybrid exploits the property of Gal4 transcription factor that regulates the galactose metabolism in *Saccharomyces Cerevisiae*. Gal 4 has two separable and functionally active domains: the N-terminal domain which binds to specific DNA (UAS) and the C-terminal acidic domain which is important for the activation of transcription (see Figure 1.8A). This system can be used to detect relatively weak as well as transient protein-protein interactions that might be difficult to examine by other biochemical approaches. This method can also afford to provide information about specific domains or even residues of the proteins involved in the interaction.

For interaction studies, protein X gene is fused to DNA binding domain (DBD) vector and protein Y gene is fused to activation domain (AD) vector. The yeast is transformed with both the vectors and allowed to grow. The interaction of Protein X and Y will bring AD and DBD domains close enough to complete transcription factor making it functionally active. This will initiate the transcription of the reporter gene which is under the control of that UAS sequence. LacZ and MEL-1 are commonly used reporter genes that transcribe to β-galactosidase and α-galactosidase respectively (see Figure 1.8B). X-
Figure 1.8- Schematic representation of yeast two hybrid system (A) and the constructs for yeast AH109 strain (B). Images adapted from Clontech user manuals.

alpha Gal (5-Bromo-4-Chloro-3-indolyl a-D-galactopyranoside) is a chromogenic substrate for these enzymes. The yeast colonies showing positive protein interaction turn blue when grown on the medium supplemented with X-alpha gal.
CHAPTER 2

INTERACTION OF p53 PROTEIN WITH XPB HELICASE

2.1 INTRODUCTION

Nucleotide excision repair pathway (NER) is a DNA repair mechanism which eliminates a vast variety of bulky DNA adducts formed by exposure to several genotoxic agents and UV rays. Several studies have shown that TFIIH, a multi protein transcription factor complex, along with its basal transcriptional functions plays an important role in NER. The xeroderma pigmentosum complementation group B (XPB) protein is a component of the TFIIH complex and is responsible for ATPase driven DNA helicase activity that is crucial to unwind the DNA. Mutations in the xeroderma pigmentosum (XP) genes encoding for XPB and XPD cause incurable genetic disorders with the loss of DNA repair activity leading to skin cancer.

Several articles have reported that the tumor suppressor protein, p53, that modulates several processes like cell cycle, apoptosis, senescence, DNA repair, and metabolism can also affect the activity of the TFIIH complex. Though the exact mechanism behind this activity is not clear, some studies emphasize the importance of interactions between p53 and several other cellular factors as a significant contributor. Alternatively, some evidences also point towards the direct interaction of p53 with various TFIIH complex components. For instance, Wang et al demonstrated that both XPB and XPD helicases
interact with p53 protein under *in vitro* and *in vivo* conditions\textsuperscript{118,119}. However, the specific residues that are involved in this interaction are still unknown.

In this study, the interaction between p53 and XPB using different *in vivo* and *in vitro* methods was investigated to get further structural insight. For this three different approaches including pull down assay, fluorescence resonance energy transfer (FRET), and yeast two hybrid analysis were utilized. The first two methods confirm the *in vitro* interaction of p53 with XPB. The yeast two hybrid analysis afforded to map down the interaction to specific domains of p53 and regions of XPB. The results demonstrated that XPB contains two interacting regions along its structure that allow it to bind with p53 i.e. 105-129 a.a region present on the N-terminus of XPB and 730-782 a.a region present at the extreme C-terminus of XPB. Further, it was observed that DBD and CTD domains of p53 are primarily involved in this interaction where only CTD domains bind to the 691-782 a.a. region of XPB. Expanding this approach to different mutant forms of XPB, elucidated that F99S, T119P, and XPB11BE mutations negatively affect the binding of XPB to p53 while S751D and S751E mutations do not show any effect.

### 2.2 MATERIALS AND METHOD

#### 2.2.1 Protein expression and purification

a) GST-tagged N-terminal XPB protein (30-494 a.a): A small aliquot of frozen Rosetta bacterial cells transformed with pGEX-plasmid vector was added to 10 mL of antibiotic (50 μg/mL of ampicillin and 35 μg/mL of chloramphenicol) supplemented LB media.
This starter culture was allowed to grow overnight at 37 °C. The grown bacteria were transferred to 1 L of LB media (with antibiotics) and allowed to grow until the optical density (O.D.) at 600 nm was 0.6. Then, the protein expression was induced by adding at 0.2 mM IPTG and the culture was incubated at 20 °C for 16 hours. The cells were then collected by centrifuging the culture at 4000 RPM for 20 mins followed by pellet resuspension in lysis buffer (50 mM Tris-Cl pH 8.0, 150 mM NaCl, 10% glycerol, 5 mM DTT, 1% NP-40). The cells were lysed by sonicating for 20 mins (70% power with 10 second on & 20 second off pulse rate) on ice bath. The lysate was centrifuged at 40,000 g for 20 mins at 4 °C and the supernatant was used for further protein purifications.

To purify the protein, the supernatant was loaded onto the glutathione agarose resin (GST column) which was pre-equilibrated in the equilibration buffer (50 mM Tris pH 8.0, 150 mM NaCl, 10% Glycerol). This was followed by washing the column with wash buffer (50 mM Tris pH 8.0, 150 mM NaCl, 10% Glycerol) and finally protein elution with elution buffer (50 mM Tris pH 8.0, 150 mM NaCl, 10% Glycerol, 10 mM reduced glutathione). All the elution fractions were combined and concentrated using 50 kDa amicon tube filter and further purified using size exclusion chromatography (S-200) using S-200 buffer (20 mM Tris pH 8.0, 200 mM NaCl, 10% Glycerol) at 4 °C. The elution fractions of S-200 containing GST-tagged N-terminal XPB protein (XPB-F5) were combined and concentrated using 50kDa amicon filter and the resulting concentrated sample was stored at -80 °C for further experiments.
b) Full length His-Tagged-p53 (1-393 a.a): A small aliquot of frozen Rosetta bacterial cells transformed with pET-plasmid vector was added to 10 mL of antibiotic (50 μg/mL of kanamycin and 35 μg/mL of chloramphenicol) supplemented LB media. This starter culture was allowed to grow overnight at 37 ºC. The grown bacteria were transferred to 1 L of LB media (with antibiotics) and allowed to grow until the optical density (O.D.) at 600 nm was 0.6. Then, the protein expression was induced by adding at 0.2 mM IPTG and the culture was incubated at 25 ºC for 5 hours. The cells were collected by centrifugation and lysed in lysis buffer (50 mM Tris pH 8.0, 300 mM NaCl, 10% Glycerol, 1% NP-40, 30 mM IMZ) by sonicating for 20 mins (70% power with 10 second on & 20 second off pulse rate) on ice bath. Lysed cells were centrifuged at 40,000 g at 4 ºC for 20 mins and the supernatant was used for loading onto the Ni-NTA resin column, pre-equilibrated in the equilibration buffer (50 mM Tris pH 8.0, 300 mM NaCl, 10% Glycerol, 30mM IMZ). The column was washed with wash buffer (50 mM Tris pH 8.0, 300 mM NaCl, 10% Glycerol, 40 mM IMZ) and the protein was eluted using elution buffer (50 mM Tris pH 8.0, 300 mM NaCl, 10% Glycerol, 500 mM IMZ). The elution fractions were dialyzed overnight in dialysis buffer (20 mM Tris pH 8.0, 50 mM NaCl, 10% glycerol, 2 mM DTT) in cold room (4 ºC). The dialyzed protein was further purified using Ion-exchange chromatography (SPFF column and QFF column) at 10 ºC using NaCl gradient. The p53 protein binds to SPFF column while the contaminants bind to QFF column. The buffers used include: Buffer A (20 mM Tris pH 8.0, 50 mM NaCl, 10% Glycerol, 2 mM DTT), Buffer B (50 mM Tris pH 8.0, 1M NaCl, 10% Glycerol, 2 mM DTT). The SPFF p53 elution fractions were combined and concentrated using 50
kDa amicon tube at 4 °C. The concentrated sample of p53 protein was stored at -80 °C until further use.

2.2.2 Pull-down assay

a) Interaction of XPB-F5 with His-tagged p53 protein: For *in-vitro* protein binding studies, pull down assay was performed. Briefly, 80 μL of Ni-NTA agarose resin column was equilibrated in the equilibration buffer (50 mM Tris-Cl pH 8.0, 300 mM NaCl, 10% Glycerol, 1 mM DTT, 30 mM IMZ). 50 μg of His-tagged p53 (full length) protein was mixed with 90 ug of XPB-F5 and incubated overnight at 10 °C. After incubation, the protein mixture was mixed with Ni-NTA agarose resin and incubated for 1 hour at RT with gentle mixing in between. The resin was washed extensively with equilibration buffer 6 times (200 μL each time) with equilibration buffer and then the bound protein was eluted with equilibration buffer containing 500 mM IMZ. The eluted fractions were analyzed for proteins present using SDS-PAGE followed by coomassive brilliant R-250 dye staining. For control experiments, same protocol was utilized except replacing His-tagged p53 protein with equilibration buffer for pull down assay.

b) Interaction of Mutant GST-tagged XPB (30-328 a.a, T119P mutation) with His-tagged p53 protein: The interaction of p53 protein with a mutant XPB protein was also investigated. This XPB mutant has threonine residue at 119th position replaced with a Proline resulting in TTD, a rare inherited disease. To study the effect of mutation on its interaction with p53 protein similar pull-down assay was performed with slight
variations. Briefly, 30 μL of Ni-NTA agarose resin was equilibrated in the equilibration buffer (100 mM Bis-Tris pH 7.0, 300 mM NaCl, 10% Glycerol, 1mM DTT, 50 mM IMZ). 30 μg of His-p53 (full length) protein was mixed with 30 μg of GST-XPB 30-328a.a T119P (N-Terminal XPB with T119P disease mutation) and incubated for 4 hours at RT. After incubation, protein mixture was mixed with Ni-NTA agarose resin and incubated the resin- protein mixture for 1 hour at RT with gentle mixing in between. The resin was washed extensively 6 times (1 mL each time) with equilibration buffer and then the bound protein was eluted with equilibration buffer containing 500 mM IMZ. The eluted fractions were analyzed for proteins present using SDS-PAGE followed by coomassive brilliant R-250 dye staining. For control experiments, same protocol was utilized except replacing His-tagged p53 protein with equilibration buffer for pull down assay.

2.2.3 Förster resonance energy transfer (FRET)

a) Synthesis and ligand exchange of QDs: The CdSe-ZnS core-shell QDs with emission peak centered at 538 nm were grown following protocols described previous literature. Briefly, the organometallic precursors were reduced at high temperature in the coordinating solvent mixtures made of alkylphosphines, alklyphosphine-carboxyl and alkylamines. This was followed by ZnS-overcoating to grow a few monolayers of ZnS over the native QD core. The native hydrophobic capping ligands were further exchanged with His-PIMA-ZW ligand with subsequent purification as described in previous reports.
b) Covalent conjugation of XPB protein subdomains to Cy3 dye: The thiol groups available on N-terminal/C-terminal GST-tagged XPB proteins and GST tag were covalently coupled to maleimide functionalized sulfo-cy3 dye to provide protein dye conjugates for FRET studies. The conventional protocol with a few modifications was employed to prepare N-terminal-XPB-Cy3 (F5-Cy3), C-terminal XPB (F6-Cy3) and GST-Cy3 conjugates. Here the preparation of F5-Cy3 conjugates is detailed. Briefly, 4.5 μL of 0.7 mM TCEP solution prepared in PB (10 mM, pH 7.5) was mixed with 300 μL of 21 μM N-terminal XPB protein in a scintillation vial and left stirring for 30 min at RT. This step allows reduction of any thiol moieties available on the proteins. Then, 4.8 μL of 10.6 mM maleimide functionalized sulfo-Cy3 dye solution, prepared in DMSO, (8 times molar excess dye with respect to the protein) was added to the above mixture. The solution was diluted to 500 μL with PB (10 mM, pH 7.5) and allowed to react in dark for approximately 3 hours at RT with continuous stirring. The conjugates were separated from unbound dye by size exclusion chromatography (using PD 10 column). The first eluted fraction containing protein-dye conjugates was characterized by UV-vis spectroscopy to determine the dye and protein concentrations using their extinction coefficients (XPB-F5- 107565 M⁻¹ cm⁻¹, XPB-F6- 68105 M⁻¹ cm⁻¹, GST tag- 43110 M⁻¹ cm⁻¹ and Cy3- 150000 M⁻¹ cm⁻¹).

c) p53 interaction with protein dye conjugates: The histidine-tagged p53 protein was allowed to interact with protein dye conjugates by mixing them together in same molar equivalents. For instance, 80 μL of 11.5μM his-tagged p53 protein was mixed with 146
μL of 6.32 μM of F5-Cy3 conjugates in a scintillation vial. The two proteins were allowed to interact for 3 hours at 4 °C.

d) Assembly of QD-Protein-dye Conjugates: The histidine tagged-p53-protein-dye conjugates including (p53-F5-Cy3, p53-F6-Cy3 and p53-GST-Cy3) were conjugated to the QDs via metal-histidine mediated self-assembly. The conjugation was carried out using the same steps for all three protein dye conjugates. Here the assembly of QD-p53-F5-Cy3 conjugates with varying valence is detailed. Different QD-p53-F5-Cy3 conjugates were prepared by varying the QD:p53-F5-Cy3 valence from 1:2 to 1:12 corresponding to QD:dye ratio of 1:0.7 to 1:4. The desired amounts of p53-F5-Cy3 solutions were loaded into separate Eppendorf tubes, followed by the addition of PB (pH 8.0, 20 mM) to bring the total volume to 495.6 μL. Approximately, 4.6 μL of stock QD dispersion (5.4 μM) was added to each tube and the solution was mixed well with pipette followed by incubation at 4 °C for 1 hour to allow for self-assembly. The samples were then characterized by collecting the emission spectra.

2.3.4 Yeast Two Hybrid Analysis

a) Polymerase Chain Reaction (PCR): Amplification of desired gene fragments of p53 & XPB were carried out using PCR method. Closed circular plasmid containing the gene of interest (template) and DNA primers including, forward and reverse primers (details in Appendix 1) with restriction endonuclease site at both the ends were used in this PCR. 50 μL of PCR mix was prepared by mixing following ingredients: 100 ng of template DNA,
0.5 μM of forward & reverse primers, 200 μM dNTPs, 1X Taq Polymerase buffer, and 1.25 U Taq DNA polymerase. The annealing temperature and extension time required for all the PCR reactions was determined depending on the length of the primers used and the gene of interest. The DNA was denatured at 90 °C for 3 minutes followed by 30 cycles of amplifications whose conditions are mentioned in Table 2.1.

Table 2.1- Conditions followed for different steps in PCR amplification

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>95 °C</td>
<td>30 sec</td>
</tr>
<tr>
<td>Annealing</td>
<td>Depends on the primers</td>
<td>30 sec</td>
</tr>
<tr>
<td>Extension</td>
<td>68 °C</td>
<td>Depends on the gene</td>
</tr>
<tr>
<td>Final Extension</td>
<td>68 °C</td>
<td>10 min</td>
</tr>
<tr>
<td>Hold</td>
<td>4 °C</td>
<td>Not applicable</td>
</tr>
</tbody>
</table>

b) Agarose gel purification: All PCR products were confirmed for their size and purified using agarose gel electrophoresis. 1% - 2% (w/v) of agarose gel in 1X-TAE buffer supplemented with ethidium bromide (for visualization) was used depending on the expected size of the PCR products. Gels were run at 100V in an electrophoretic container. The DNA bands were observed under UV-light using codec imager. For size comparison of the PCR products, 1 Kb plus DNA marker was used. Further, each DNA band was purified by using Ultra-Sep gel extraction kit (Omega) according to manufacturer guidelines.
c) **Restriction endonuclease digestion:** The purified PCR products were double digested using EcoRI & BamHI restriction endonucleases (details in Appendix 1). For this digestion reaction 1 µg of DNA was mixed with 5 µL of 10X cut-smart buffer and 1U of restriction endonuclease enzyme. The final volume of the reaction mixture was adjusted to 50 µL using double distilled autoclaved water and was incubated at 37 ºC for digestion. The digested PCR products were further purified using the agarose gel electrophoresis followed by using Ultra-Sep gel extraction kit as described before.

The Activation Domain and DNA-Binding Domain vectors containing multiple cloning sites (MCS) were also digested in similar way using the same endonucleases as used in PCR product digestion and further purified using the agarose gel electrophoresis. All the vectors used in this study were purchased from Clonetech. pGBK7 vector that encodes for the DNA binding domain (BD) of the GAL4 transcriptional factor was used. This vector expresses proteins fused to 1-147 a.a of the GAL4 DNA binding domain (DNA-BD). It carries a yeast nutritional marker tryptophan gene and kanamycin gene for bacterial selection. The gene sequence of the protein to be used as bait was cloned in frame in this vector. Another vector used for the study was pGADT7 that encodes for the activation domain (AD) of the GAL4 transcriptional factor. This vector expresses proteins fused to 768-881 a.a of the GAL4 activation domain (DNA-AD). It carries a yeast nutritional marker leucine gene and ampicillin gene for bacterial selection. Gene sequence of the protein to be used as prey was cloned within the frame of BD vector. Vectors which were used as a positive control in this experiment were: (1) pGBK7-p53 which encodes for p53 protein fused with DNA binding domain (BD) and; (2) pGADT7-
T antigen, which encodes for T-antigen protein fused with activation domain (AD) of the Gal4 transcription factor. Both these proteins i.e. BD-p53 and AD-T antigen are already known to interact strongly in yeast two hybrid system.

d) Ligations: The digested PCR products and vectors were ligated to prepare the plasmid containing the gene of interest. For this, the amplified DNA and vectors were mixed in the molar ratio of 3:1 (Insert: Vector) along with 1 μg of DNA, 2 μL of 10X T4 buffer (50 mM Tris-HCl, 10 mM MgCl₂, 10 mM DTT, 1 mM ATP, pH 7.5), and 1 U of T4 ligase enzyme (NEB). The final volume was adjusted to 20 μL using double distilled autoclaved water. The ligation reaction was incubated overnight at 16 °C temperature.

e) Bacterial Transformations: Competent DH10B cells (50 μL), thawed on ice, were mixed with 5 μL of ligated plasmid and the mixture was incubated on ice for 20 minutes. Control experiment was also done in the same way in which DH10B cells were mixed with water instead of plasmid. After 20 minutes, the cells were heat shocked for 2 minutes at 42 °C followed by addition of 400 μL of LB broth. This mixture was incubated at 37 °C for 1 hour followed by plating on LB-agar plates supplemented with appropriate antibiotic. The plates were incubated overnight at 37 °C allow bacterial colonies to grow. Single bacterial colonies were picked from transformation plate and grew in 6 ml LB broth containing 50 μg/μL of required antibiotic at 37 °C while shaking at 225 RPM overnight. Plasmids from these cultures were isolated by using alkaline lysing method using literature protocol followed by double digestion using restriction endonuclease
enzymes (EcoRI & BamH1) as discussed above. The plasmids containing the gene of interest (positive clones) were identified by running the digested samples on 1% - 2% (w/v) agarose gels.

f) **Yeast transformation:** Yeast (*Saccharomyces Cerevisiae*) AH109 strain was utilized for this study. Yeast AH109 strain (MATa, trp 1-901, leu 2-3, 112, ura 3-52, his 3-200, gal4Δ, gal80Δ, LYS2 : : GAL1UAS-GAL1TATA-HIS3, GAL2UAS-GAL2TATA-ADE2, URA3 : : MEL1UAS-MEL1 TATA-lacZ) had three reporter genes *His*, *Ade2* and *Mel-1* that were under the control of distinct GAL4 upstream activating sequences and TATA boxes. The yeast transformation was performed by using the lithium acetate method. The recipe for all the constituents used for this method are detailed in Table 2.2. For this, yeast colony was taken from YPD plate and grown in 100 mL of YPD medium overnight at 30 °C. The growth of yeast was monitored by measuring the optical density (O.D.) of the culture at 600 nm. At the O.D. of 1-1.5, the culture was centrifuged at 4300 rpm for 10 min at RT to collect the cells. The pellet was washed twice with autoclaved ddH₂O, followed by three washes with 0.1M lithium acetate. Then the pellet was resuspended in required amount of 0.1 M lithium acetate for transformation. In a separate Eppendorf, the two plasmids (AD vector and DBD vector), containing the gene of interest, were mixed carefully with 20 μL of salmon sperm DNA using pipette. To this 100 μL of yeast cells (prepared above) and 500 μL of PEG/TEL solution (8 mL of 50% PEG 3350, 1 mL of 1M LiAc, and 1 mL of 10X TAE buffer) was added and the mixture were incubated at 30 °C overnight. The cells were heat shocked at 42 °C for 10 minutes followed by a quick
spin to pellet the cells. The pellet was resuspended in 100 - 200 μL of ddH₂O (autoclaved) and then plated onto double drop out plate (SD/-Trp/-Leu) and then incubated at 30 °C until yeast colonies were observed.

**Table 2.2** - The recipe for all the constituents used in lithium acetate assay for yeast transformation.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Recipe</th>
</tr>
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<tbody>
<tr>
<td>0.1M Lithium Acetate</td>
<td>1.02g of LiAc in 100ml of ddH₂O, Autoclave it.</td>
</tr>
<tr>
<td>1M Lithium Acetate</td>
<td>10.2g of LiAc in 100ml of ddH₂O, Autoclave it.</td>
</tr>
<tr>
<td>50 % PEG 3350 (W/V)</td>
<td>50g of PEG 3350 and make the final volume to 100ml H₂O using ddH₂O, Autoclave it.</td>
</tr>
<tr>
<td>Salmon sperm DNA (Carrier DNA)</td>
<td>Make stock of 2mg/ml, boil for 10 minutes, put on ice to make single stranded DNA and uses 20ul (40ug) for transformation.</td>
</tr>
</tbody>
</table>

**2.3 RESULTS AND DISCUSSION**

XPB, also known as ERCC3, is a DNA helicase that unwinds the double helix of DNA to single stranded DNA using ATP as energy source and is involved in many cellular processes like transcription and especially DNA repair by NER pathway. Mutations or any defects in this protein affecting its structure and/or function might lead to rare human inherited genetic disorders. Another crucial mammalian protein, p53, also known as the guardian of the genome, controls many important cellular functions like cell cycle control, apoptosis, and DNA repair. Previous studies have shown that both these proteins (p53 and XPB) interact with each other that might be crucial in governing the process of...
DNA repair by NER pathway. Studies from other labs have shown that C-terminal domain (CTD) of p53 is required for its interaction with XPB\textsuperscript{119}. However, there is no evidence indicating the specific residues of XPB that contribute to this interaction.

In this study, the interaction of p53 and XPB protein was assessed by using several different techniques including pull-down assay, FRET and yeast two hybrid system. Several different constructs of p53 and XPB protein were prepared to explore their interaction and map it down to specific residues involved.

\textbf{2.3.1 Pull-down assay:} This is one of the \textit{in vitro} techniques employed to detect protein-protein interactions based on the principle of affinity chromatography. This method is commonly used to demonstrate the physical interaction between proteins that form stable protein complexes. This approach was employed to investigate the interaction of XPB helicase with p53 protein. For this, two domains of XPB protein including GST-tagged-N-terminal XPB (residues 30-494, referred as XPB-F5) and C-terminal XPB (residues 494-782, referred as XPB-F6) were expressed in bacteria and partially purified using affinity and size exclusion chromatography. Similarly, the full-length histidine-tagged-p53 protein (residues 1-393, referred to as p53) was also expressed in bacteria and purified by affinity and ion-exchange chromatography.

The interaction of p53 with XPB-F5 and XPB-F6 was probed using Ni-NTA column as the means for affinity chromatography. For this, the p53 protein was mixed with XPB-F5 and XPB-F6 separately to allow them to interact with each other and form any complexes. The mixed protein samples were then run through the Ni-NTA column
individually, following similar approach as applied to purify any histidine-tagged protein. Since p53 protein was expressed with a histidine-tag, it was expected to bind to the Ni-NTA column, immobilizing any proteins interacting with it onto the column as well. After washing the column with excess buffer, the bound proteins were eluted and examined using SDS-PAGE. Figure 2.1 shows the representative gel obtained from the assay for p53-XPB-F5 interaction.

**Figure 2.1** - The SDS-PAGE gels (12%) from pull-down assay for interaction of the full length His-tagged p53 protein with XPB-F5. **(A)** The gel obtained for p53-XPB-F5 mixture passed through the Ni-NTA column. The p53 and XPB lanes show the band position for both the proteins. The presence of XPB-F5 and p53 protein band in E1 and E2 lanes is an indication of their interaction. **(B, C, D)** The gels obtained for the control experiments where XPB-F5 (B), GST-tag (C) and GST-tag with p53 (D) was run through the Ni-NTA column. Here, M is marker, L is loading sample, FT is flow through, W1-W6 are washes and E1-E2 are elutions.
study. The absence of p53 and XPB-F5 bands in last wash suggests that any unbound proteins were removed from the column prior to elution. Interestingly, both bands reappear in the elution lanes indicating that XPB-F5 was also immobilized onto the Ni-column along with p53 protein. This result confirms that p53 protein interacts with N-terminal domain of XPB protein under in-vitro conditions. Alternatively, the XPB-F6 protein band was not observed in the elution lane (data not shown) suggesting that the C-terminal XPB domain might not be involved in the interaction with p53. To confirm that XPB-F5 and GST tag present on it do not non-specifically bind to the Ni column or the GST tag does not interact with p53 protein, some control experiments were also performed. The gel pictures shown in Figure 2.1 (B-D) confirm that none of these non-specific interactions are happening during the experiment further complementing the conclusion.

2.3.2 **FRET:** Fluorescence resonance energy transfer is a photo-physical process that involves non-radiative transfer of excitation energy from an excited donor molecule (D) to a proximal ground state acceptor molecule (A) via dipole-dipole interactions. This procedure due to its sensitivity to distance between the donor and the acceptor has been utilized as a potential spectroscopic tool to probe the protein-protein interactions over the past few years. This technique was utilized to supplement the previous results. For this XPB-F5 and XPB-F6 proteins were coupled to dyes (acceptor), and were allowed to interact with p53 protein that can be directly self-assembled onto the QDs (donor) using metal-histidine coordination. The prepared conjugates (different valence of QD:protein
dye conjugates) were characterized by fluorescence spectroscopy to inspect the interaction of p53 with these proteins.

a) **Covalent conjugation of XPB subdomains to Cy3 dye:** XPB-F5-Cy3 (F5-Cy3), XPB-F6-Cy3 (F6-Cy3) and GST-Cy3 conjugates were prepared by coupling the available thiol moieties to maleimide functionalized Cy3 dye by following conventional protocol. After separation from the unreacted dye, the conjugated proteins were analyzed by absorption spectroscopy. By deconvolution of the conjugate absorption spectra, the average number of Cy3 dyes coupled per XPB-F5, XPB-F6 and GST-tag protein was estimated to be 0.34, 0.27 and 0.41 respectively. This shows that the coupling efficiency of dye to each protein was rather low, however, the fluorescence still lied within the detection limit.

b) **Conjugation of proteins to QDs and FRET analysis:** The F5-Cy3, F6-Cy3 and GST-Cy3 conjugates were allowed to interact with p53 protein. These conjugates (p53-F5-Cy3, p53-F6-Cy3 and p53-GST-Cy3) were then allowed to self-assemble onto the His-PLMA-ZW functionalized QDs via metal histidine coordination owing to the his-tag present on p53 protein. Using F5 and F6 dye conjugates allowed to identify the specific domains of XPB helicase that interact with p53 protein. In addition, the GST-Cy3 conjugate was utilized as a control protein to confirm the non-specific interaction of p53 protein with GST tag.
Figure 2.2 shows the PL spectra obtained by excitation of different QD-protein-dye conjugates including, QD-p53-F5-Cy3, QD-p53-F6-Cy3, and QD-p53-GST-Cy3 at varied valence ratios. These spectra correspond to the PL collected by excitation at 400 nm which does not excite Cy3 dye directly. The progressive decrease in the fluorescence of the QDs was observed as the molar ratio of p53-F5-Cy3 per QD increased (shown in Figure 2.2A). This illustrates that the Cy3 dye was successfully involved in fluorescence resonance energy interaction with QDs. This would only be possible when F5-Cy3 conjugate was interacting with his-tagged p53 protein that was able to bind to QDs due to the terminal histidine tag. In contrast, no significant PL reduction was observed in case of p53-F6-Cy3 (Figure 2.2B) and p53-GST-Cy3 (Figure 2.2C) coupling to the QDs at similar valences. This suggests that F6 and GST tag do not show any interaction with p53 protein resulting in no dye interaction with the QDs.

Figure 2.2- PL spectra obtained from excitation of different QD-protein-dye conjugates including QD-p53-F5-Cy3 (A), QD-p53-F6-Cy3 (B), and QD-p53-GST-Cy3 (C).

Figure 2.3 A-C shows the composite quenching efficiency plots extracted from the PL spectra analysis for all the protein-dye conjugates under study. The QD-p53-F5-Cy3
resulted in a maximum FRET efficiency (E) of ~40% when 4 dyes were coupled onto each QD. However, only ~10% and ~7% E was observed in case of QD-p53-F6-Cy3, and QD-p53-GST-Cy3 respectively with similar dye equivalents per QD.

![Figure 2.3- Plots showing the FRET efficiency for different QD-protein conjugate pairs including QD-p53-F5-Cy3 (A), QD-p53-F6-Cy3 (B), and QD-p53-GST-Cy3 (C).](image)

All these results combined support the previous results obtained from pull down assay, suggesting that the XPB helicase interacts directly with the p53 protein. And more importantly, the N-terminal XPB domain (XPB-F5) is responsible for this interaction.

2.3.3 Yeast Two Hybrid Analysis

a) Modules of XPB and p53 involved in the interaction: Furthermore, to identify the specific residues of the XPB and p53 protein responsible for their interaction yeast two hybrid analysis was used. This technique is one of the best approaches that is employed to study protein-protein under \textit{in-vivo} conditions. Moreover, this technique allowed to breakdown both XPB and p53 protein into smaller fragments to further narrow-down the precise residues that control the interaction. For this the interaction of smaller fragments
of XPB was investigated with different functional domains of p53 protein including, trans-activation domain (TAD), proline rich region (PRR), DNA binding domain (DBD), oligomerization domain (OD), and C-terminal regulatory domain (CTRD); the latter two domains together are often referred to as C-terminal domain (CTD). Briefly, different fragments of XPB were fused into the BD-vector while different constructs of p53 were ligated into the AD-vector. Then, the yeast was transformed with different combinations for p53 & XPB to test the interaction by comparing the growth of yeast on different drop out plates that is a good indicator for the strength of interaction. The different drop-out plates tested were triple drop out (SD/-Leu/-Trp/-His), and four drop out (SD/-Leu/-Trp/-His/-Ade) plates, where yeast growth in the latter plate suggests stronger interaction. It is worth mentioning here, that the first 60 residues of p53, corresponding to TAD, were not included in this study as it might initiate transcription of reporter gene (auto-activation) without any interaction, resulting in pseudo positives. Hence, in latter sections, full length p53 protein refers to the p53 without any TAD.

Firstly, the interaction of full length p53 (p53_{PRR+DBD+CTD}) was evaluated with the full-length XPB (19-782 a.a) and fragmented XPB modules including, XPB_{43-494}, XPB_{243-692}, and XPB_{691-782}. No significant yeast growth was observed for any of these constructs (see Figure 2.4) indicating that the proteins did not interact.
Figure 2.4 - Yeast two hybrid analysis showing that various XPB modules do not interact with p53 (PRR+DBD+CTD). (A) Diagram showing the representative p53 and XPB constructs with specified domains and modules respectively. (B) The images showing the growth of yeast transformed with different XPB and p53 constructs after 72 hours. Interaction was monitored with respect to the growth of yeast on SD/-Leu/-Trp/-His and SD/-Leu/-Trp/-His/-Ade drop out plates. (C) Summary of the yeast two hybrid analysis. No growth is indicated as (-) while growth in triple drop out and four drop out plate is indicated as (+) and (++) respectively.

It is important to specify here that, the slight growth observed in triple drop-out plate for XPB<sub>691-782</sub> with p53 is the result of auto-activation as similar growth was observed in the control experiment for XPB<sub>691-782</sub> with empty AD-vector. Similar results were obtained
for study of full length XPB (19-782 a.a) with separate domains of p53 including p53PRR, p53DBD, p53OD, p53CTRD, and p53CTD indicating no interaction for these constructs as well (see Figure 2.5).

**Figure 2.5**-Yeast two hybrid analysis showing that various individual p53 domains do not interact with full length XPB (19-782 a.a). (A) Diagram showing the representative p53 and XPB constructs with specified domains and modules respectively. (B) The images showing the growth of yeast transformed with different XPB and p53 construct after 72 hours. Interaction was monitored with respect to the growth of yeast on SD/-Leu/-Trp/-His and SD/-Leu/-Trp/-His/-Ade drop out plates. (C) Summary of the yeast two hybrid analysis. No growth is indicated as (-) while growth in triple drop out and four drop out plate is indicated as (+) and (++) respectively.
Interestingly, full length XPB (19-782 a.a) showed strong interaction with p53<sub>DBD+CTD</sub> by activating both histidine and adenine reporter genes as shown by growth of yeast in SD/-Leu/-Trp/-His and SD/-Leu/-Trp/-His/-Ade plates. However, no growth was observed for with BD-vector with p53<sub>PRR+DBD</sub> (see Figure 2.6).

**Figure 2.6** - Yeast two hybrid analysis showing that the full length XPB (19-782 a.a) interacts with p53 (DBD+CTD) but not with p53 (PRR+DBD). (A) Diagram showing the representative XPB and p53 constructs with specified modules and domains respectively. (B) The images showing the growth of yeast transformed with various XPB and p53 constructs after 72 hours. Interaction was monitored with respect to the growth of yeast on SD/-Leu/-Trp/-His and SD/-Leu/-Trp/-His/-Ade drop out plates. (C) Summary of the yeast two hybrid analysis. No growth is indicated as (-) while growth in triple drop out and four drop out plate is indicated as (+) and (++) respectively. The blue colonies in four drop out plates containing X-α-Gal (XAG) confirm positive interaction.
This result demonstrates that the N-terminus PRR domain of p53 is not important for interaction with XPB while both DBD and CTD domains are required. BD-p53 & AD-T antigen was used as positive control for this experiment as both show strong interaction in yeast two hybrid assay \(^{159}\). As the structure of the full length XPB and p53 are unknown, it can be proposed that the interacting domains for them might not be readily exposed making it difficult for them to interact: hence no interaction was observed in the previous Y2H experiments. Nevertheless, when smaller domains of p53 are utilized the interacting domains are sterically less hindered explaining the positive interaction obtained in latter experiments.

Once, the p53 domains necessary for interaction with XPB were identified, the regions of XPB involved in the interaction were mapped down. For this, many BD vector fused constructs of XPB were prepared, i.e., XPB\(_{43-494}\), XPB\(_{243-692}\), XPB\(_{494-782}\), XPB\(_{691-782}\) and XPB\(_{730-782}\) covering almost the full length of XPB except the first 43 residues. These XPB constructs were co-transformed with AD vector fused with p53\(_{PRR+DBD}\) and p53\(_{DBD+CTD}\). XPB\(_{43-494}\), XPB\(_{691-782}\), (see Figure 2.7) XPB\(_{494-782}\), and XPB\(_{730-782}\) (see Figure 2.8) showed interaction with p53\(_{DBD+CTD}\), but not with p53\(_{PRR+DBD}\). The XPB\(_{243-692}\) does not show any interaction with p53 constructs (see Figure 2.7). These results indicate that the p53\(_{DBD+CTD}\) interacts primarily with the N-terminus region (43-494 a.a) and extreme C-terminus region (730-782 a.a) of XPB. Next, the N-terminal region of XPB was subdivided to further narrow-down the crucial residues. The BD-constructs were
assembled with XPB_{1-160}, XPB_{105-494}, XPB_{130-494}, and XPB_{70-494}. The results (shown in Figure 2.9) indicate that p53 strongly interacts with all the constructs of XBP except XBP_{130-494}.

**Figure 2.7**- Yeast two hybrid analysis showing that XBP (43-494 a.a), and XBP (691-782 a.a) but not XBP (243-692 a.a) interacts with p53 (DBD+CTD). (A) Diagram showing the representative XBP and p53 constructs with specified modules and domains respectively. (B) The images showing the growth of yeast transformed with various XBP and p53 constructs after 72 hours. Interaction was monitored with respect to the growth of yeast on SD/-Leu/-Trp/-His and SD/-Leu/-Trp/-His/-Ade drop out plates. (C) Summary of the yeast two hybrid analysis. No growth is indicated as (-) while growth in triple drop out and four drop out plate is indicated as (+) and (++) respectively. The blue colonies in four drop out plates containing X-α-Gal (XAG) confirm positive interaction.
These results combined, indicate that 105.a.a-129.a.a. and 730.a.a.-782 a.a. of XPB are critical for interaction with DBD and CTD domains of p53.

Another experiment, confirmed that XPB 691-782 a.a region does not require DBD domain of p53 to interact with the protein (shown in Figure 2.10). Hence, only CTD (including OD and CTRD) is responsible for interaction with extreme C-terminal residues of XPB.

**Figure 2.8**- Yeast two hybrid analysis showing that XPB (494-782 a.a), and XPB (730-782 a.a) interact individually with p53 (DBD+CTD). (A) Diagram showing the representative XPB and p53 constructs with specified modules and domains respectively. (B) The images showing the growth of yeast transformed with various XPB and p53 constructs after 72 hours. Interaction was monitored with respect to the growth of yeast on SD/-Leu/-Trp/-His and SD/-Leu/-Trp/-His/-Ade drop out plates. (C) Summary of the yeast two hybrid analysis. No growth is indicated as (-) while growth in triple drop out and four drop out plate is indicated as (+) and (++) respectively. The blue colonies in four drop out plates containing X-α-Gal (XAG) confirm positive interaction.
Figure 2.9- Yeast two hybrid analysis showing that the 106-129 a.a region of XPB protein is critical for interaction with p53 (DBD+CTD). (A) Diagram showing the representative XPB and p53 constructs with specified modules and domains respectively. (B) The images showing the growth of yeast transformed with different XPB and p53 constructs after 72 hours. Interaction was monitored with respect to the growth of yeast on SD/-Leu/-Trp/-His and SD/-Leu/-Trp/-His/-Ade drop out plates. (C) Summary of the yeast two hybrid analysis. No growth is indicated as (-) while growth in triple drop out and four drop out plate is indicated as (+) and (++) respectively. The blue colonies in four drop out plates containing X-β-Gal (XAG) confirm positive interaction.
Figure 2.10- Yeast two hybrid analysis showing that XPB (691-782 a.a) primarily interacts with the CTD domain of p53. (A) Diagram showing the representative XPB and p53 constructs with specified modules and domains respectively. (B) The images showing the growth of yeast transformed with different XPB and p53 constructs after 72 hours. Interaction was monitored with respect to the growth of yeast on SD/-Leu/-Trp/-His and SD/-Leu/-Trp/-His/-Ade drop out plates. (C) Summary of the yeast two hybrid analysis. No growth is indicated as (-) while growth in triple drop out and four drop out plate is indicated as (+) and (++) respectively. The blue colonies in four drop out plates containing X-α-Gal (XAG) confirm positive interaction.

b) Effect of disease causing mutations on p53 and XPB interaction: Several mutations like, F99S, T119P, XPB11BE, S751D, and S751E in the XPB protein have been shown to affect its DNA repair activity leading to several diseases. For instance, F99S and T119P point mutations have been shown to decrease the DNA repair activity of XPB. F99S disease normally occurs due to the presence of heterozygous mutation at phenylalanine 99 (Phe99) which changes the residue to serine (S99). F99 is a conserved, hydrophobic residue that according to the N-terminal XPB (NTD-XPB) modelling by
Saha et al is exposed on the protein surface; hence, has been proposed to be important for interaction with p52 subunit in XPB-p52 interaction. The XPB-p52 interaction very important for its role in transcription as well as NER pathway. F99S mutation interrupts the XPB-p52 interaction resulting in reduced levels of XPB and decreased rates of DNA repair and basal transcription. Another disease is caused by homozygous T119 mutation, where threonine 119 is mutated to proline. The same model by Sasha et al suggests that threonine 119 residue is present in the small loop that join the two motifs of NTD-XPB. So, T119P mutation restricts the rotation of the two motifs, altering the 3-D structure of motif 2 with respect to motif 1 that might disturb the structure of NTD-XPB. This all might disturb the proper functioning of NTD-XPB during transcription and DNA repair, which might be the cause behind the disease.

As the yeast two hybrid results discussed above showed that p53DBD+CTD interacts with the N-terminus region of XPB (105a.a -129a.a), the effect of these mutations on the interaction between XPB and p53 was explored. So, BD-fused XPB70-494 with F99S and XPB70-494 with T119P constructs were designed and transformed into yeast along with AD-fused p53DBD+CTD construct. Interaction studies (Figure 2.11) showed that F99S mutation in XPB almost ceases the interaction of XPB with p53 protein while the T119P mutation weakens it in comparison to the interaction between wild type proteins. It should also be mentioned here that the GST tagged mutated XPB protein (with T119P mutation) was also expressed in bacteria (the purified protein was graciously provided by
Figure 2.11- Yeast two hybrid analysis showing the effects of disease causing mutations in XPB (F99S and T119P) on its interaction with p53. (A) Diagram showing the representative mutated XPB and p53 constructs with specified modules and domains respectively. (B) The images showing the growth of yeast transformed with different XPB and p53 constructs after 72 hours. Interaction was monitored with respect to the growth of yeast on SD/-Leu/-Trp/-His and SD/-Leu/-Trp/-His/-Ade drop out plates. (C) Summary of the yeast two hybrid analysis. No growth is indicated as (-) while growth in triple drop out and four drop out plate is indicated as (+) and (++) respectively. The blue colonies in four drop out plates containing X-α-Gal (XAG) confirm positive interaction.

Dr. Eduardo Hilario). The binding of mutated XPB to His-tagged p53 protein was tested using pull-down assay. Figure 2.12 shows images obtained from the SDS gels that also supplement the yeast two hybrid conclusion suggesting that T119P mutation weakens the interaction of XPB with p53.
Figure 2.12- The SDS-PAGE gels (12%) from pull-down assay for interaction of the full length His-tagged p53 protein with mutated XPB (T119P). (A) The gel obtained for p53-XPB (T119P) mixture passed through the Ni-NTA column. The p53 and XPB (T119P) lanes show the band position for both the proteins. The presence of XPB (T119P) band in FT lane and its absence in E1 lane indicates that p53 and XPB (T119P) proteins do not interact with each other. (B) The gel obtained for the control experiments where XPB (T119P) was run through the Ni-NTA column. Here, M is marker, L is loading sample, FT is flow through, W1-W5 are washes and E is elution.

A frame shift mutation, XPB11BE, occurs due to trans-version of C to A which introduces 4 base pair insertions in the mRNA. This changes the last 41 residues at the C-terminal end of the XPB with only 781 residues instead of 782 residues. The mutant XPB also shows less solubility in comparison to the wild type protein that might affect the activity of TFIIF complex including NER. Based on yeast two hybrid results, 730-782 residues of XPB are important for interaction with p53; hence it would be interesting to test if this mutation will also influence the interaction of XPB with p53. So, XPB with XPB11BE frame-shift mutation was cloned and then transformed in yeast AH109 cells.
along with p53\text{DBD+CTD}. Yeast two hybrid result (see Figure 2.13) indicates a decrease in the strength of the interaction as growth was only observed in case of triple (SD/-Trp/-Leu/-His) drop-out plate, in contrast to four (SD/-Trp/-Leu/-His/-Ade) plate for wild type XPB. This result further suggests that the motif required for interaction with p53 might be present in the last 41 residues of XPB.

Further expanding this analysis, the effects of S751D and S751E mutations in XPB (also present in the last 40 residues of XPB) were also explored for its interaction with p53. Previous studies have demonstrated that these mutations affect the interaction of XPB with ERCC1-XPF, a structure specific endonuclease, leading to inefficient NER. However, their effect on XPB-p53 mutation has not yet been explored. Hence, the residue Ser751 in XPB (691-782 a.a) was mutated to aspartic acid (S751D) and glutamic acid (S751E) separately and then tested for their interactions with p53\text{DBD+CTD} using yeast two hybrid system. Similar growth pattern of yeast was observed in case of mutated and the wild type XPB 691-782 (Figure 2.14). These results indicate that S751D and S751E mutations do not affect the interaction between XPB and p53 and hence Ser residue at 751\text{th} position is not the most crucial part of this interaction as it is for interaction of XPB with ERCC1-XPF.
Figure 2.13- Yeast two hybrid analysis showing the effect of XPB11BE mutation on XPB interaction with p53. (A) Diagram showing the representative mutated XPB and p53 constructs with specified modules and domains respectively. (B) The images showing the growth of yeast transformed with XPB and p53 constructs after 72 hours. Interaction was monitored with respect to the growth of yeast on SD/-Leu/-Trp/-His and SD/-Leu/-Trp/-His/-Ade drop out plates. (C) Summary of the yeast two hybrid analysis. No growth is indicated as (-) while growth in triple drop out and four drop out plate is indicated as (+) and (++) respectively. The blue colonies in four drop out plates containing X-α-Gal (XAG) confirm positive interaction.
Figure 2.14- Yeast two hybrid analysis showing the effects of point mutations (S751D and S751E) in XPB on its interaction with p53. (A) Diagram showing the representative mutated XPB and p53 constructs with specified modules and domains respectively. (B) The images showing the growth of yeast transformed with XPB and p53 constructs after 72 hours. Interaction was monitored with respect to the growth of yeast on SD/-Leu/-Trp/-His and SD/-Leu/-Trp/-His/-Ade drop out plates. (C) Summary of the yeast two hybrid analysis. No growth is indicated as (-) while growth in triple drop out and four drop out plate is indicated as (+) and (++) respectively. The blue colonies in four drop out plates containing X-α-Gal (XAG) confirm positive interaction.

All the results combined provide enough evidence to propose that 105a.a-129a.a and 730a.a-782a.a of XPB are critical for interaction with DBD and CTD domains of p53, except the last 90 residues of XPB (691-782a.a) that only binds to the CTD domain. It was interesting to observe that p53 interacts only with specific residues of XPB and with
not with full length protein. This could be attributed to the fact that full length XPB protein is a big protein composed of 782 amino acids. Henceforth, the folding of protein might hide the interaction sites making them inaccessible for binding of p53. Moreover, no interaction was observed between the C-terminal domain (494-782a.a) of XPB with CTD of p53, pointing toward the same reason.
CHAPTER 3

INTERACTION OF p53 PROTEIN WITH ERCC1-XPF ENDONUCLEASE

3.1 INTRODUCTION

ERCC1-XPF, a heterodimer protein complex, is a structure specific endonuclease that is involved in several different DNA repair pathways including nucleotide excision repair, inter-strand cross link (ICL) and double strand break repair pathway \(^{77}\). This complex excises the DNA lesions, especially the ones created by UV exposure. The ERCC1 subunit in this complex is catalytically inactive and is primarily responsible for interaction with DNA and other NER proteins. It is also important for anchoring XPF to the damaged DNA at correct position. The XPF subunit is catalytically active and has a nuclease domain that cleaves the damaged DNA at the 5’end. The interaction of both the subunits is critical for solubility and functionality of the complex \(^{161}\).

p53, a tumor suppressor protein, also plays a very important role in maintaining the genomic stability of cells by controlling various processes like cell cycle, DNA repair, apoptosis and senescence \(^{110}\). Several studies have demonstrated that p53 contributes to different DNA repair pathways through transcription dependent as well as transcription independent ways \(^{162}\). For instance, studies have reported that p53 modulates the transactivation of the p48-XPE and XPC genes whose protein products are involved in NER. Another study discovered a p53 DNA binding region in the promoter segment of
the DDB2 gene that codes for XPE also involved in NER. Separately, many articles have demonstrated that p53 can bind to various proteins like XPB, XPD, CSA and CSB that are important components of NER pathway. It will be interesting to know if p53 also interacts with any other elements of NER\footnote{120,163,164}.

In this chapter, the interaction of p53 with ERCC1-XPF complex was investigated. The results from yeast two hybrid analysis demonstrated that the central domain of ERCC1 is the main site of interaction with p53 whereas the helix-hairpin-helix domain (HhH\textsubscript{2}) shows relatively weaker interaction. The p53 protein on the other hand needed two domains i.e. DNA binding domain (DBD) and oligomerization domain (O.D) to interact with the ERCC1 central domain. Additionally, the p53 C-terminal regulatory domain (CTRD) is necessary for stronger interaction with HhH\textsubscript{2} domain of ERCC1. Furthermore, the results from pull down assay also support the above findings suggesting that p53 interacts with the central domain of ERCC1 subunit. This study hence provides further evidence that p53 directly interacts with proteins involved in several DNA repair pathways.

3.2 MATERIALS AND METHOD

3.2.1 Protein expression and purification

a) GST-tagged ERCC1 (Central domain) protein (96-214 a.a): A small aliquot of frozen Rosetta bacterial cells transformed with pGEX-plasmid vector was added to 10 mL of antibiotic (50 μg/mL of ampicillin and 35 μg/mL of chloramphenicol)
supplemented LB media. This starter culture was allowed to grow overnight at 37 °C. The grown bacteria were transferred to 1 L of LB media (with antibiotics) and allowed to grow until the optical density (O.D.) at 600 nm was 0.6. Then, the protein expression was induced by adding at 0.2 mM IPTG and the culture was incubated at 25 °C for overnight. The cells were then collected by centrifuging the culture at 4000 rpm for 20 mins followed by pellet resuspension in lysis buffer (50 mM Tris-Cl pH 7.5, 300 mM NaCl, 10% glycerol, 1% NP-40). The cells were lysed by sonicating for 20 mins (70% power with 10 second on and 20 second off pulse rate) on ice bath. The lysate was centrifuged at 40,000 g for 20 mins at 4 °C and the supernatant was used for further protein purifications.

To purify the protein, the supernatant was loaded onto the glutathione agarose resin (GST column) which was pre-equilibrated in the equilibration buffer (50 mM Tris pH 7.5, 300 mM NaCl, 10% Glycerol). This was followed by washing the column with wash buffer (50 mM Tris pH 7.5, 300 mM NaCl, 10% Glycerol) and finally protein elution with elution buffer (50 mM Tris pH 7.5, 300 mM NaCl, 10% Glycerol, 10 mM reduced glutathione). All the elution fractions were combined dialyzed for overnight in the dialyzing buffer (50 mM Tris-Cl pH 7.5, 10% glycerol) in cold room (4 °C). Overnight dialyzed protein was used for Ion exchange chromatography (SPFF column and QFF column) at 10 °C and protein was eluted with salt gradient. Buffer used were: Buffer A (50 mM Tris pH 7.5, 50 mM NaCl, 10% Glycerol), Buffer B (50 mM Tris-Cl pH 7.5, 1M NaCl, 10% Glycerol). Elution’s containing protein of interest were concentrated using 50 kDa amicon tube filter and further purified using size exclusion chromatography (S-200).
using S-200 buffer (20 mM Tris pH 7.5, 300 mM NaCl, 10% Glycerol) at 4 °C. The elution fractions of S-200 containing GST-Tagged-ERCC1 (central domain) were combined and concentrated using 50kDa amicon filter and the resulting concentrated sample was stored at -80 °C for further experiments.

b) p53 (1-393 a.a) expression and purification: Plasmid transformed BL-21 (DE3) E. coli colonies were grown in 1L LB media at 37 °C until O.D reached 0.4, then temperature was lowered to 22 °C until O.D reached to 0.6, when IPTG (0.2 mM) was added and the cells were incubated for another 5 hrs. The cells were then harvested by centrifugation and lysed in lysis buffer (25 mM HEPES/KOH pH 8.0, 250 mM KCl, 10% Glycerol, 2mM BME, 1 mM BZ, 0.1% Tween-20, 2 mM EDTA) by sonication. The cell debris was separated by centrifugation and supernatant was diluted 5 times in the dilution buffer (50 mM NaH$_2$PO$_4$, pH 8.0, 10mM Imidazole, 1mM BME, 1 mM BZ, 0.1 mM PMSF), followed by loading to Ni-NTA agarose resin. The column was extensively washed with wash buffer (50 mM NaH$_2$PO$_4$ pH 8.0, 300 mM KCl, 10 mM Imidazole, 1mM BME, 1mM BZ, 0.1mM PMSF) to remove any impurities. p53 protein was then eluted in the elution buffer (50 mM NaH$_2$PO$_4$ pH 8.0, 300 mM KCl, 1mM BME, 1mM BZ, 0.1mM PMSF, 500 mM IMZ).

3.2.2 Yeast two hybrid: Yeast two hybrid technique was used to confirm the p53 interaction with ERCC1-XPF protein. This approach required preparation of AD and BD vectors (listed in Appendix II and III), that were prepared following the same protocol as
described in Chapter 2. The primers and restriction enzymes that were used in this process have been listed in Appendix I These vectors were then utilized for yeast transformation that has also been described in detail in previous chapter.

3.2.3 Pull-down assay

Interaction of GST-tagged ERCC1 (96-214 a.a) with His-tagged p53 protein (1-393 a.a): For in vitro protein binding study, 100 μL of Ni-NTA agarose resin was equilibrated in the equilibration buffer (50 mM Tris-Cl pH 7.5, 50 mM NaCl, 10% Glycerol). 30 μg of His-tagged p53 (full length) protein was mixed with 15 μg of GST-ERCC1 (Central Domain) and incubated on ice for 3 hours. After incubation, the protein mixture was mixed with Ni-NTA agarose resin and incubated the resin-protein mixture on ice for more 20 minutes. The resin was washed extensively with equilibration buffer 6 times with different concentration of Imidazole (20 mM, 40 mM, 60 mM IMZ) and then the bound protein was eluted with equilibration buffer containing 500 mM IMZ. Proteins were analyzed by SDS-PAGE followed by coomassive brilliant R-250 dye staining. For control experiments, same protocol was utilized except replacing His-tagged p53 protein with equilibration buffer for pull down assay.

3.3 RESULTS AND DISCUSSION

3.3.1 Yeast Two Hybrid study:

a) Interaction of XPF subunit with p53: To get a better understanding about the interaction of p53 with ERCC1-XPF complex, firstly the binding of p53 to XPF subunit
was examined. For this, BD vector constructs containing N-terminal of XPF (XPF\textsubscript{N}, residues 11-640 a.a) and C-terminal of XPF (XPF\textsubscript{C}, residue 650-905 a.a) were designed. These were co-transformed into yeast cells with AD vector containing \textit{p53\textsubscript{PRR+DBD+CTD}}.

Figure 3.1 shows that no significant growth was seen in any of the cases that is an evidence for no binding between the two proteins.

To address the question that the interacting domains of the two proteins might not be available, the interaction was also tested in presence of smaller p53 fragments. For this, AD vector fused XPF\textsubscript{N} and XPF\textsubscript{C} were co-transformed into yeast cells with BD vector containing \textit{p53\textsubscript{PRR+DBD}} and \textit{p53\textsubscript{DBD+CTD}}. Here also no yeast growth was observed (see Figure 3.2) indicating that XPF subunit of the endonuclease complex does not interact with p53.

\textbf{b) Interaction of ERCC1 subunit with p53:} To investigate the interaction of p53 with ERCC1 subunit, AD vector was fused with different constructs of ERCC1\textsubscript{1-297} (Full Length) while BD vector was fused with various p53 constructs respectively. The yeast AH109 cells were co-transformed with AD and BD constructs. The interaction was evaluated by the growth of yeast on synthetic drop out medium lacking Tryptophan, Leucine, Histidine (SD/-Trp/-Leu/-His) and Tryptophan, Leucine, Histidine and Adenine (SD/-Trp/-Leu/-His/-Ade). BD-p53 and AD-T antigen was used as positive control for
Figure 3.1- Yeast two hybrid analysis showing that XPF (XPF\textsubscript{N} and XPF\textsubscript{C}) does not interact with p53 (PRR+DBD+CTD). (A) Diagram showing the representative XPF and p53 constructs with specified modules and domain respectively. (B) The images showing the growth of yeast transformed with XPF and p53 constructs after 72 hours. Interaction was monitored with respect to the growth of yeast on SD/-Leu/-Trp/-His and SD/-Leu/-Trp/-His/-Ade drop out plates. (C) Summary of the yeast two hybrid analysis. No interaction was indicated as (-), growth in triple drop out media indicated as (+), and in four drop out media indicated as (++).
Figure 3.2- Yeast two hybrid analysis showing that XPF (XPF\textsubscript{N} and XPF\textsubscript{C}) does not interact with p53 (PRR+DBD) nor p53 (DBD+CTD). (A) Diagram showing the representative XPF and p53 constructs with specified modules and domain respectively. (B) The images showing the growth of yeast transformed with XPF and p53 constructs after 72 hours. Interaction was monitored with respect to the growth of yeast on SD/-Leu/-Trp/-His and SD/-Leu/-Trp/-His/-Ade drop out plates. (C) Summary of the yeast two hybrid analysis. No interaction was indicated as (-), growth in triple drop out media indicated as (+), and in four drop out media indicated as (++).
this experiment as it was shown that both shows strong interaction in yeast two hybrid assay.

The investigation was begun by assessing the interaction of full length ERCC1 (1-297a.a) with p53_{DBD+CTD} and p53_{PRR+DBD}. Figure 3.3 shows that yeast growth was observed in both SD/-Trp/-Leu/-His and SD/-Trp/-Leu/-His/-Ade drop out plates for both p53 constructs. This is an evidence suggesting that ERCC1 subunit of ERCC1-XPF complex interacts with p53 protein.

![Diagram showing the representative ERCC1 and p53 constructs with specified modules and domain respectively.](image)

Figure 3.3- Yeast two hybrid analysis showing that full length ERCC1 interacts with p53 (PRR+DBD) and p53 (DBD+CTD). (A) Diagram showing the representative ERCC1 and p53 constructs with specified modules and domain respectively. (B) The images showing the growth of yeast transformed with ERCC1 and p53 constructs after 72 hours. Interaction was monitored with respect to the growth of yeast on SD/-Leu/-Trp/-His and SD/-Leu/-Trp/-His/-Ade drop out plates. (C) Summary of the yeast two hybrid analysis. No interaction was indicated as (-), growth in triple drop out media indicated as (+), and in four drop out media indicated as (++).
To further narrow down the specific domains of p53 involved in interaction with ERCC1, five different AD vectors constructs were prepared, each corresponding to different domains of including PRR (residues 61-94.a.a), DBD (residues 102-292.a.a), O.D (residues 324-355.a.a), CTRD (residues 363-393.a.a), and CTD (residues 324-393.a.a). No significant yeast growth was observed for any of these constructs (Figure 3.4) indicating that individual domains of p53 are not enough to interact with ERCC1; different domains (DBD+PRR and DBD+CTD) of p53 come together to form a structure that binds to ERCC1.

The interaction of ERCC1 and p53 was further examined to determine the interacting regions of ERCC1. Four BD vectors with overlapping fragments of ERCC1 were constructed: ERCC1NLS (residues 1-90.a.a), ERCC1CD+HhH2 (residues 90-297.a.a), ERCC1CD (residues 90-214.a.a), and ERCC1HhH2 (residues 220-297.a.a). These were co-transformed with AD vectors containing different p53 constructs i.e. p53PRR+DBD, p53DBD+CTD in yeast AH109. The results are shown in Figure 3.5. The PRR+DBD domain of p53 did not show any interaction with any of the ERCC1 constructs. Similarly, NLS domain of ERCC1 did not show any interaction with p53. Whereas, p53DBD+CTD showed strong binding to ERCC1CD+HhH2 and ERCC1CD. A weaker interaction of p53 DBD+CTD domains was observed with HhH2. These results suggest that even though both CD and HhH2 domains of ERCC1 interact with p53, the central domain of ERCC1 is primarily responsible for this interaction. Furthermore, PRR+DBD domains of p53 is not involved in this interaction; rather DBD+CTD domains are important.
Figure 3.4 - Yeast two hybrid analysis showing that the full length ERCC1 does not interact with individual domains of p53. (A) Diagram showing the representative ERCC1 and p53 constructs with specified modules and domain respectively. (B) The images showing the growth of yeast transformed with ERCC1 and p53 constructs after 72 hours. Interaction was monitored with respect to the growth of yeast on SD/-Leu/-Trp/-His and SD/-Leu/-Trp/-His/-Ade drop out plates. (C) Summary of the yeast two hybrid analysis. No interaction was indicated as (-), growth in triple drop out media indicated as (+), and in four drop out media indicated as (++).
**Figure 3.5** - Yeast two hybrid analysis showing that the central domain of ERCC1 is the main site of interaction with p53 (DBD+CTD). (A) Diagram showing the representative ERCC1 and p53 constructs with specified modules and domain respectively. (B) The images showing the growth of yeast transformed with ERCC1 and p53 constructs after 72 hours. Interaction was monitored with respect to the growth of yeast on SD/-Leu/-Trp/-His and SD/-Leu/-Trp/-His/-Ade drop out plates. (C) Summary of the yeast two hybrid analysis. No interaction was indicated as (−), growth in triple drop out media indicated as (+), and in four drop out media indicated as (++). 

Further the distinct interaction regions in central domain of ERCC1 and the precise domains of p53 protein were also identified. For this, AD vector construct of p53 containing the DBD+CTD and DBD+O.D domains were built. DBD+O.D domain was achieved by deleting the CTRD domain (30 residues) from C-terminal end of p53. This was co-transformed into yeast with different BD vector fused ERCC1 constructs. These included ERCC1_{full-length}, ERCC1_{CD+HhH2}, ERCC1_{CD}, ERCC1_{HhH2}, along with smaller regions of CD (96-150 a.a. and 151-214 a.a.) and HhH2 (220-258 a.a. and 259-297 a.a.)
domains. Figure 3.6 shows yeast growth for all ERCC1 constructs including ERCC1_{full-length}, ERCC1_{CD+HhH2}, ERCC1_{CD}, and ERCC1_{HhH2} with p53_{DBD+O.D} domain in the SD/-Trp/-Leu/-His drop out plates, with an exception for ERCC1_{CD} that also showed growth.

**Figure 3.6** - Yeast two hybrid analysis showing that p53 (DBD+OD) is critical for interaction with ERCC1 (CD). (A) Diagram showing the representative ERCC1 and p53 constructs with specified modules and domain respectively. (B) The images showing the growth of yeast transformed with ERCC1 and p53 constructs after 72 hours. Interaction was monitored with respect to the growth of yeast on SD/-Leu/-Trp/-His and SD/-Leu/-Trp/-His/-Ade drop out plates. (C) Summary of the yeast two hybrid analysis. No interaction was indicated as (-), growth in triple drop out media indicated as (+), and in four drop out media indicated as (++).
in the SD/-Trp/-Leu/-His/-Ade drop out plates. This suggests that removal of CTRD domain from p53 protein weakens its interaction with full length ERCC1 protein or CD+HhH\_2 and HhH\_2 domains. Nevertheless, it has no effect on p53 interaction with the central domain of ERCC1. This can be explained with a hypothesis that the CTRD domain of p53 interacts with HhH\_2 domain of ERCC1 resulting in a confirmation change in ERCC1 protein that exposes its central domain to bind with DBD+OD domains of p53. Additionally, Figure 3.7 shows yeast growth in the SD/-Trp/-Leu/-His drop out plates in

Figure 3.7- Yeast two hybrid analysis showing that residues 96-150 of ERCC1 (CD) are critical for interaction with p53(DBD+O.D). (A) Diagram showing the representative ERCC1 and p53 constructs with specified modules and domain respectively. (B) The images showing the growth of yeast transformed with ERCC1 and p53 constructs after 72 hours. Interaction was monitored with respect to the growth of yeast on SD/-Leu/-Trp/-His and SD/-Leu/-Trp/-His/-Ade drop out plates. (C) Summary of the yeast two hybrid analysis. No interaction was indicated as (-), growth in triple drop out media indicated as (+), and in four drop out media indicated as (++).
case of ERCC1\textsubscript{151-214} BD vector co-transformed with p53\textsubscript{DBD+O,D} AD vector demonstrating that the first 60 residues of ERCC1 central domain are involved in interaction with p53. However, when this is compared to the growth seen in case of full central domain with p53\textsubscript{DBD+O,D} it can be stated that this interaction is comparatively weaker. Hence, it can be advocated that although the first half of the ERCC1 central domain interacts with p53, the second half also plays a role in strengthening this interaction; this makes the full central domain of ERCC1 very critical for strong binding to p53. Moreover, Figure 3.8 compares the interaction of p53 DBD+CTD with the entire p53

**Figure 3.8**- Yeast two hybrid analysis showing that residues 258-297 of ERCC1 (HhH2) are critical for interaction with p53(DBD+CTD). (A) Diagram showing the representative ERCC1 and p53 constructs with specified modules and domain respectively. (B) The images showing the growth of yeast transformed with ERCC1 and p53 constructs after 72 hours. Interaction was monitored with respect to the growth of yeast on SD/-Leu/-Trp/-His and SD/-Leu/-Trp/-His/-Ade
drop out plates. (C) Summary of the yeast two hybrid analysis. No interaction was indicated as (-), growth in triple drop out media indicated as (+), and in four drop out media indicated as (++)

HhH$_2$ domain and its fragments including ERCC1$_{220-258}$a.a and ERCC1$_{259-297}$a.a. These results indicate that the first 38 residues of HhH$_2$ domain is responsible for interaction with p53. All these interaction outcomes were also confirmed positive by adding X-α-Gal into the media in which yeast was grown. All the compiled results are shown Figure 3.9.

Figure 3.9- The images for growth of yeast transformed with different ERCC1 and p53 constructs indicating positive interaction between different residues of ERCC1 and p53 with X-α-Gal treatment.
3.3.2 Pull-down assay: This in vitro technique was utilized to further confirm the results obtained above. For this, the central domain of ERCC1 with GST-tag (GST-ERCC1<sub>CD</sub>, residues 96-214) and histidine-tagged-p53 protein (residues 1-393) were expressed in bacteria and partially purified using various chromatography procedures. As explained in the Chapter 2, the interaction of p53 with ERCC1 was explored using Ni-NTA column as the means for affinity chromatography. The p53 protein was mixed with ERCC1 and allowed to interact for 3 hours followed by passing the mixture through the Ni column. The histidine-tagged p53 protein was expected to bind to the Ni-NTA column along with any bound protein. Figure 3.10 shows the representative SDS-PAGE gels obtained from the assay. The absence of p53 and GST-ERCC1<sub>CD</sub> bands in last wash (see Figure 3.10 A) suggests that any unbound proteins were removed from the column prior to elution. However, both the proteins can be seen in the elution lanes indicating that GST-ERCC1<sub>CD</sub> was also

![Figure 3.10](image)

**Figure 3.10**- The SDS-PAGE gels (12%) from pull-down assay for interaction of the full length His-tagged p53 protein with ERCC1<sub>CD</sub>. (A) The gel obtained for p53- ERCC1<sub>CD</sub> mixture passed through the Ni-NTA column. The p53 and ER lanes show the band position for both the proteins. The presence of ERCC1<sub>CD</sub> and p53 protein band in E1, E2 and E3 lanes is an indication of their interaction. (B) The gels obtained for the control experiments where ERCC1<sub>CD</sub> was run through the Ni column. Here, M is marker, L is loading sample, FT is flow through, W1-W3 are washes and E1-E3 are elution lanes.
immobilized onto the Ni-column along with p53 protein. To confirm that GST-ERCC1\textsubscript{CD} protein does not interact non-specifically with Ni-NTA column, a control experiment was also performed where the protein was completely removed from the column during wash steps; no bands were seen in the elution lanes (see Figure 3.10 B). These results complement the yeast two hybrid analysis confirming that p53 protein interacts with the central domain of ERCC1 protein under in-vitro conditions as well.

Using yeast two hybrid and pull-down assay, it has been established that the p53 protein might be a modulator in different DNA repair pathways by interacting with the structure specific endonuclease, ERCC1-XPF, and controlling its activity. It can be reported that the ERCC1 subunit of this complex is responsible for this interaction, while the XPF protein does not bind to p53. The specific domains of p53 and ERCC1 that are involved in this interaction were also narrowed-down. The evidence suggests that the DNA binding and C-terminal domain of p53 bind mainly to the central domain of ERCC1 protein. However, relatively weaker interaction was also observed with helix hairpin helix domain of ERCC1. Based on these results, it can be proposed that the later domain of ERCC1 interacts with p53 leading to a confirmation change that makes the central domain more accessible for binding with p53. These results provide very useful information that can be applied to further understand the mechanism by which p53 regulates the DNA repair pathways.
p53 is considered as the guardian of the genome. It controls various cellular processes like cell cycle control, DNA repair, senescence, and apoptosis\(^\text{165}\). The importance of this protein in maintaining genomic stability can be validated from the fact that p53 is the most commonly mutated gene in more than 50% of human cancers\(^\text{166}\). p53 has been shown to regulate the DNA repair by modulating transcription of the genes involved in the repair pathways\(^\text{167}\). Additionally, several recent studies have shown that p53 can also regulate the repair mechanism by directly interacting with and modulating the activity of the participating proteins\(^\text{118,168}\). NER is one of the pathways that has been reported to be regulated by p53 as it can interact directly with XPB, XPD, RPA, CSA, and CSB that play significant roles in repair\(^\text{119,155,169,170}\). Henceforth, any mutation in the p53 gene that can disturb its interaction with NER components can lead to genomic instability due to reduced DNA repair; this can eventually impair the cell cycle checkpoint and result in apoptosis, which are also controlled by p53. Hence, it is of prime importance to gather more information about the role of p53 in DNA repair.

This dissertation is concentrated on identifying the proteins involved in NER pathway that might be regulated by direct binding to the p53 protein. Transcription Factor II H (TFIIH) is a multi-protein complex that participates in transcription of various protein-coding genes and DNA repair specifically, NER. XPB, a 3’-5’ DNA helicase, is a component protein of the TFIIH complex that is responsible to unwind the double
stranded DNA around the lesion to allow other NER factors to bind and continue with DNA repair. Additionally, another important protein complex, ERCC1-XPF, is required in concert with the XPG endonuclease to cleave the DNA lesion out of the damaged DNA. It is a structure specific endonuclease that distinctly removes the lesions generated by exposure to UV light. Hence, both XPB and ERCC1-XPF play major roles in NER. Hence, in this study the use of in vivo & in vitro biochemical techniques to investigate the interaction of p53 with XPB and ERCC1-XPF has been reported. The results established that both XPB and ERCC1-XPF (only the ERCC1 subunit) interact physically with p53 protein. Moreover, the precise regions in these proteins that are involved in this interaction were also mapped down.

4.1 Interaction of XPB with p53 protein: Starting with XPB, in chapter 2 the evidence for its interaction with p53 has been provided. Three different approaches were employed including affinity pull-down assay, fluorescence resonance energy transfer (FRET) and yeast two hybrid assay (Y2H). For pull-down assay and FRET studies, histidine tagged p53 protein (1-393 a.a), GST tagged N-terminal XPB (XPB-F5, 30-494 a.a), GST tagged C-terminal XPB (XPB-F6, 494-782 a.a) and GST tag protein as a control were efficiently expressed in bacterial system and purified using various chromatography techniques. In pull-down assay, p53 protein was separately mixed with XPB-F5, XPB-F6 and GST tag and then loaded onto the Ni-NTA column that should immobilize any histidine tagged proteins. The results showed that along with histidine tagged p53 protein, XPB-F5 was also bound to the Ni-NTA column until eluted with imidazole rich buffer. Alternatively,
XPB-F6 and GST tag protein (used as a control for non-specific interaction with p53) were not sticking to the column and instead flowed through it. This suggests that p53 interacts with the N-terminal portion of the XPB protein keeping it onto the Ni column. These results were also supported by the FRET studies where the QDs were used as energy donors and p53-Y-Cy3 dye conjugates were used as energy acceptors (here, Y represent any one of the following proteins: XPB-F5, XPB-F6 and GST tag). The quenching efficiency obtained for interaction of XPB-F5, XPB-F6 and GST tag with p53 protein was 40%, 10% and 7% respectively. These results clearly corroborate with the previous results suggesting that p53 interacts primarily with XPB-F5 portion of the protein.

Furthermore, this interaction was also tested under in vivo conditions with yeast two hybrid analysis. The benefit of using this technique is that it will allowed to assess the interaction in conditions more like a eukaryotic cell where proteins should be relatively more comparable to in situ conditions (with post-translational modifications). In addition, this is a very sensitive technique that can amplify and hence detect any weaker interactions that might not be measurable by other assays used above. As expected, the results indicated positively towards the interaction of p53 protein with XPB. Taking advantage of this approach, the specific regions of interaction between the two proteins were also identified. The compiled results presented that there are smaller regions at both the N-terminus of XPB (105-129 a.a) and extreme C-terminus of XPB (730-782 a.a) that bind to p53. p53 also requires two specific domains including DNA binding domain (DBD) and C-terminal domain (CTD) together to interact with XPB.
The results were also narrowed down to some specific residues of XPB by extending this study to different XPB related rare inherited-disease-causing mutations including F99S, T119P and XPB11BE. Based on the above results, the mutated regions for all these diseases lie close to or within the XPB residues that are involved in interaction with p53. Hence, the effect of these mutations on XPB-p53 interaction was identified, providing more insight into the role of p53 in these diseases. F99S is a rare heterozygous point-mutation in which the phenylalanine present at the 99th residue in XPB is changed to serine (F99S); T119P is another homozygous point mutation in which threonine residue at 119th position is mutated to proline. Both these residues i.e. phenylalanine and threonine are conserved residues and have functional and structural role in proper functioning of XPB. The yeast two hybrid analysis results demonstrated that the XPB-p53 interaction is weakened in the case of T119P while is completely abolished in the case of F99S mutations respectively. These results together indicated that F99 and T119 residues play critical role in the interaction of p53 with XPB.

XPB11BE is a frame shift mutation that changes the last 42 residues at the C-terminus of XPB that has also been shown to be involved in its interaction with p53 by the yeast two hybrid studies. This mutation is a result of trans-version of C to A introducing 4 base pair insertions in the mRNA of XPB. Patients suffering from this mutation have only 781 a.a long XPB instead of 782 a.a in the wild type XPB. This mutation decreases the solubility of XPB making it limited for its activity in the TFIIH complex ultimately affecting NER pathway. The results illustrated that the XPB11BE mutation ceases the binding of XPB with p53 completely, supporting the previous results that C-terminal residues of
XPB are critical for interaction with p53 protein. This interaction was probably not observed in the case of pull-down assay and FRET due to the amplification ability of yeast two hybrid analysis that makes it easier to detect even weaker interactions. Alternatively, the XPB protein might attain a specific confirmation in *in vivo* conditions that increases the accessibility of the C-terminal interaction region to bind with p53.

**4.2 Interaction of ERCC1-XPF complex with p53 protein:** Moving the attention to the ERCC1-XPF protein complex, in the third chapter, its interaction with p53 protein was investigated using *in vivo* & *in vitro* methods including yeast two hybrid and pull-down assay respectively. The results from yeast two hybrid analysis showed that p53 interacts with the ERCC1 subunit but not with XPF subunit of the ERCC1-XPF complex. In particular, the central domain of ERCC1 subunit is the main site of interaction with p53 whereas the helix-hairpin-helix (HhH2) domain interacts relatively weakly. Additionally, p53 protein needs two domains i.e. DNA binding domain (DBD) and C-terminal domain (CTD) to interact with the ERCC1 subunit. The findings showed that removal of the C-terminal regulatory domain (CTRD) from the CTD p53 (p53<sub>DBD+OD</sub>) weakens its interaction with ERCC1<sub>CD+HhH2</sub>. This can be explained by the hypothesis that the central domain of ERCC1, which is critical to interact with p53, is not easily accessible for binding. When the ERCC1<sub>HhH2</sub> interacts weakly with the p53<sub>CTRD</sub> it might undergo a conformational change, thus making the ERCC1<sub>CD</sub> more accessible to bind to p53 protein.
Furthermore, to support this conclusion some *in vitro* studies confirming the interaction of p53 protein with ERCC1$_{CD}$ (96-214 a.a) were also performed. The full length p53 (1-393 a.a) and ERCC1$_{CD}$ (96-214 a.a) proteins were successfully collected using a bacterial expression and purification system. These proteins were then tested for interaction using the pull-down assay. As expected, positive results further complementing previous investigations were obtained.

4.3 *Future Directions*: This study provides substantial proof for the interaction of p53 protein with two major contributors of NER pathway including XPB and ERCC1-XPF proteins. Following on these results further experimentations can be done to gather more insight.

1) It will be beneficial to have some structural proof supplementing these results. ERCC1, XPB and p53 proteins can be expressed following the protocols described above and then allowed to interact with each other separately. These mixtures containing the complexes can be utilized for crystallization studies. I have been already working on this idea, however the crystallization process has not been completely optimized yet. This idea will be pursued by upcoming Ph.D. students in our laboratory.

2) The second idea will be to find the functional role of these interactions in maintaining the genomic stability of the cell. Different *in vivo* and *in vitro* functional assays could be utilized to gather valuable information. For instance, ERCC1-XPF enzyme activity can be evaluated in the presence and absence of p53 protein. This will investigate if the interaction of ERCC1 with p53 enhances or diminishes the endonuclease activity of the
complex. Similarly, the effect of interaction with p53 on XPB helicase activity can also be assessed. All the gathered information can constructively be applied to find better ways to treat NER related disorders.
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APPENDIX I

List of forward and reverse primers used in yeast two hybrid study

<table>
<thead>
<tr>
<th>S.No</th>
<th>XPB Inserts</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tbody>
<tr>
<td>1</td>
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<td>5’-CCG GAA TTC TAT GAG GAT GAA GAG GAT GAT-3’</td>
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# APPENDIX II

**List of AD vector constructs**

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<th>Constructs</th>
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# APPENDIX III

List of BD vector constructs

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## APPENDIX IV

### List of abbreviations

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<th>Abbreviation</th>
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<td>1U</td>
<td>one unit</td>
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<tr>
<td>6-4PPs</td>
<td>Pyrimidine-(6-4)-pyrimidone photoproducts</td>
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<td>Activation domain</td>
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<td>Adenine</td>
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<td>ATP</td>
<td>Adenosine triphosphate</td>
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<td>Cyclobutane pyrimidine dimer</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DSB</td>
<td>Double strand break</td>
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<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
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<tr>
<td>ERCC1</td>
<td>Excision repair cross complementing 1</td>
</tr>
<tr>
<td>FRET</td>
<td>Förster resonance energy transfer</td>
</tr>
<tr>
<td>GG-NER</td>
<td>Global genome-NER</td>
</tr>
<tr>
<td>GGR</td>
<td>Global genome repair</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
</tr>
<tr>
<td>His</td>
<td>Histidine</td>
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<tr>
<td>HPLC</td>
<td>High pressure liquid chromatography</td>
</tr>
<tr>
<td>HR</td>
<td>Homologous recombination</td>
</tr>
<tr>
<td>IMZ</td>
<td>Imidazole</td>
</tr>
</tbody>
</table>
LB       Luria- Bertani
Leu      Leucine
LiAc     Lithium acetate
MCS      Multiple cloning sites
MgCl₂    Magnesium chloride
MMR      Mismatch repair
NaCl     Sodium chloride
NER      Nucleotide excision repair
NHEJ     Non-homologous end joining
NP-40    Nonidet P-40
O.D      Optical density
PCR      Polymerase chain reaction
PEG      Polyethylene glycol
RNA Pol II RNA polymerase II
RPA      Replication protein A
SD       Synthetic dropout
TC-NER   Transcription coupled-NER
TCR      Transcriptional coupled repair
TFIIH    Transcription factor –II H
Trp      Tryptophan
TTD      Trichothiodystrophy
UAS      Upstream activating sequence
UV       Ultra-Violet
XAG      X-Alpha-Gal (5-Bromo-4-chloro-3-indolyl-a-D galactopyranoside)
XP       Xeroderma Pigmentosum
XPA      Xeroderma pigmentosum complementation group A
XPB      Xeroderma pigmentosum complementation group B
XPC      Xeroderma pigmentosum complementation group C
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tr>
<td>XPD</td>
<td>Xeroderma pigmentosum complementation group D</td>
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<td>XPF</td>
<td>Xeroderma pigmentosum complementation group F</td>
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<tr>
<td>XPG</td>
<td>Xeroderma pigmentosum complementation group G</td>
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<tr>
<td>YPD (YEPD)</td>
<td>Yeast extract peptone dextrose</td>
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