Homi Bhabha National Institute

Ph. D. PROGRAMME

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4. Title of the Thesis:	**"**Role of RAP80-ABRAXAS-BRCA1-complex in the DNA Damage Repair: Structural Investigations to Reveal the Molecular Complexity"
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SYNOPSIS

Cancer is a complex disease which arises due to abnormal cell division. Instead of undergoing death, cancer cells continue to grow and form new abnormal cells which may leads to metastasis. Breast cancer can be classified into two categories; Ductal carcinoma in situ (non-invasive breast cancer) and Lobular carcinoma in situ (pre-cancer). About 5 - 10% of breast cancer cases are reported to be hereditary in nature, and the most common cause is an inherited defects in the genes, *BRCA1* and *BRCA2* [1] [2]. *BRCA1* is a well established tumor suppressor, and plays an essential roles in various cellular events like apoptosis, cell cycle checkpoint control, transcription and DNA damage repair [3]. BRCA1 protein performs these functions by forming molecular complexes with different cellular binding partners. BRCA1-complex comprises BRCA1, RAP80, ABRAXAS and MERIT40, and is crucial for DNA damage repair mechanism. Several mutations have been identified in BRCA1 associated proteins that disrupt interactions with binding partners, thereby causing various pathogenic implications [4] [5]. The present study aims to structurally characterise the BRCA1 binding proteins to gain functional insights of pathogenic mutation(s) reported in RAP80 and ABRAXAS. This work would also highlights the importance of BRCA1-complex recruitment to the DNA damage site.

The thesis comprises of seven chapters and a summary chapter.

Chapter 1 provides an introduction about the role of BRCA1-complex in the DNA damage repair pathway.

DNA damage repair is a vital process for the survival of living organisms, and mainly occurs through two pathways, homologous recombination (HR) and non homologous end joining (NHEJ). The BRCA1-complex is involved in the homologous recombination

pathway, while DNA-PK and KU70/80 complexes are involved in NHEJ pathway. In response to DNA damage, a series of phosphorylations are triggered leading to the formation of polyubiquitin chains on the histone protein, H2AX. These polyubiquitin chains are recognized by the Ubiquitin Interacting Motif (UIM) present at the C-terminus of RAP80, thereby helping in the recruitment of BRCA1-complex to the DNA damage site. RAP80 forms a stable complex with BRCA1 through the binding partner, ABRAXAS. At the C-terminus, ABRAXAS contains a consensus sequence motif pS-X-X-F (pS – Phosphoserine, X-any amino acids, F-Phenylalanine), through which it recognizes and binds to phospho-specific binding domain BRCA1-BRCT (BRCA1 C-terminal repeats) [6, 7] [2, 8]. MERIT40 (MEdiator of RAP80 Interaction and Targeting 40) protein is also a key component of the BRCA1-complex, and is essential for maintaining the complex integrity. However, the exact nature of interactions among components of the BRCA1-complex is not yet established. The two tandem BRCTs are the hotspots for clinical mis-sense mutations, which invariably disrupt phosphorylation dependent interactions with the binding partners [9, 10]. The in-frame deletion mutation of the conserved glutamic acid residue at position 81, c.241-243delGAA (ΔE81), found in a patient diagnosed with breast cancer, is located in the UIM1 motif of RAP80 [5]. Similarly, the familial mutation (R361Q) at the C-terminus of ABRAXAS in Finnish population is associated with cancer. It is suspected that these mutations disassemble the BRCA1-complex [11]. In view of the critical role played by BRCA1-complex in DNA damage repair, we have undertaken structural & biophysical studies on the component proteins of the BRCA1-complex.

Chapter 2 is a brief description of the various methodologies and techniques used to carry out research work reported in the thesis.

The gene of interest was sub-cloned from mammalian c-DNA construct to bacterial expression vector and site- directed - mutagenesis was performed on wild- type template to incorporate the mutation. The protein was purified using different chromatography techniques (affinity and size exclusion chromatography), and highly purified protein was characterized using biophysical tools, such as Circular Dichroism, Fluorescence, Differential Scanning Calorimetry, Dynamic Light Scattering and Mass Spectrometry, etc. Protein-protein interactions were analyzed using Isothermal Titration Calorimetry, Surface Plasmon Resonance and pull down assays. Structural insight was obtained with crystallographic methods, and in-silico tools were used for molecular modeling and docking.

Chapter 3 describes about the structures of RAP80 and its deletion mutant ΔE81. Binding studies of the wild-type and mutant RAP80 with di-ubiquitin molecules have also been mentioned in this chapter.

The wild- type gene was sub-cloned into the bacterial expression system, pGEX-kT vector, and site- directed -mutagenesis has been carried out to produce the RAP80 ΔE81 variant. The protein was purified in large quantities for structural and interactions studies. The molecular weight of the purified wild-type protein determined using mass spectrometry was 14.95 kDa. CD and fluorescence spectroscopic studies indicate

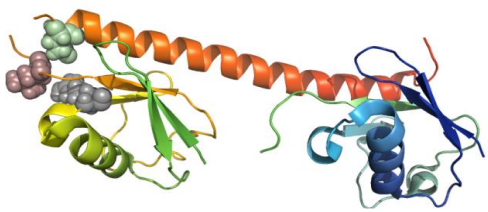

Figure 1: RAP80 wild type and Di-ubiquitin

that the wild -type protein is properly folded and having well defined secondary structure. Crystallization trials were carried out using commercially available Hamptom Research screen solutions, in hanging and sitting drop vapour diffusion method. A molecular model of the wild-type protein has been constructed using Swiss-Model server [12]. The modeled structure suggests that RAP80 is predominately a helical protein (**Figure 1**), a result consistent with CD measurements. Further, modeling of the mutant protein indicates disruption in the longest helix, leading to distorted structure pattern. Interestingly, the mutant is found to be relatively more susceptible to digestion by proteases like trypsin and chymotrypsin. The mutant also has lesser affinity for ubiquitin when probed using pull down and Surface Plasma Resonance techniques.

In conclusion, helical to random structure transition in the mutant resulted in loss of several weak intermolecular hydrogen bond and hydrophobic interactions between the UIMs and Di-Ub (K-63 linked), thereby making the binding interactions unfavorable for ubiquitin. Unstable nature of mutant/ di-ubiquitin complex may be responsible for defective recruitment of BRCA1-complex to the DNA damage sites.

Chapter 4 focuses on biophysical, biochemical and structural characterization of MERIT40**.**

MERIT40 (MEdiator of RAP80 Interaction and Targeting 40) is one of the recently discovered components of the BRCA1-complex, and its function is not yet clearly established. However, it is shown to significantly influence the stability of BRCA1-complex [13]. The wild-type protein has been sub-cloned into bacterial expression pGEX-kT

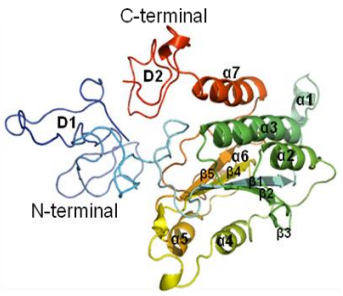

Figure 2: Model structure of MERIT40

vector, and protein was chromatography purified in large quantities for structural and biochemical studies. The full length protein is observed to have a

molecular weight of 36.61 kDa, as established through mass spectrometry. Crystallization attempts have not yet yielded good quality single crystals. Ab-initio molecular modeling was performed using the Robetta server, and the molecular model suggests disordered region at both N and C-terminals (**Figure 2**), a finding consistent with results of in-vitro limited proteolysis using trypsin and chymotrypsin enzymes. Structural similarity of MERIT40 model with vWFA like domain suggests proteosome-like activity and involvement in ubiquitination processes for MERIT40 [14]. Gel filtration, native gel electrophoresis and glutaraldehyde cross-linking studies indicated the presence of a concentration independent dimeric species along with the prominent monomeric species. Thermal and chemical denaturation profiles suggest that MERIT40 undergoes unfolding through an intermediate species. To our conclusion, MERIT40 can be classified as an intrinsically disordered protein due to the presence of N-and C-termini disordered regions. Structural homologous of MERIT40 suggests its plausible role in complement activation pathway. MERIT40 undergoes a three state unfolding transition pathway with a dimeric intermediate.

Chapter 5 describes biophysical characterization of ABRAXAS wild-type and R361Q mutant.

ABRAXAS is the third component of the BRCA1-complex [6, 15-18], and is directly associated with RAP80 and BRCA1-BRCT. The *abraxas* gene was sub-cloned into bacterial expression vector pET28a(+) ,and the protein was expressed in *E.coli Rosetta*

2(DE3). Site- directed-mutagenesis was performed using PCR and the restriction enzyme method. The wild- type and mutant proteins were purified using affinity chromatography (Ni-NTA). The molecular weight of the purified protein was estimated around 41 kDa using size exclusion chromatography.

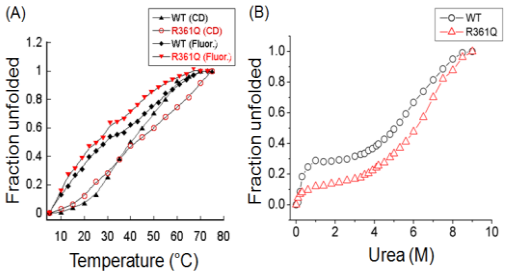

Figure 3: Thermal and urea denaturation of ABRAXAS

DLS experiment has shown that the mutant had a larger hydrodynamic diameter (wild type 9.0±0.3nm and mutant 10±0.5nm). Comparable susceptibility of ABRAXAS mutant towards protease digestion suggests that substitution of R361Q is not destabilizing the domain integrity of ABRAXAS. CD and fluorescence analyses also reveal that the secondary structures of wild -type and mutant proteins are similar, while changes in Trp and Tyr microenvironment suggest a mild alteration in tertiary structure. Thermal stability and folding pathway of wild- type and mutant were found to be similar when probed by varying the temperature (**Figure 3**). However, chemical denaturation using urea as the unfolding agent showed a prominent intermediate in case of wild-type compared to mutant.

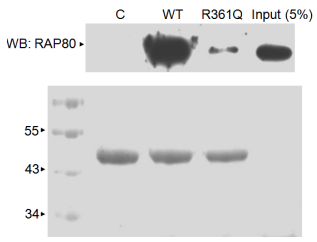

Figure 4: Pull down of ABRAXAS

In conclusion, in-vitro and in-silico studies indicate that R361Q mutation is causing several localized changes which adversely affect RAP80 binding (**Figure 4**) and recruitment of BRCA1-complex to the DNA damage site.

Chapter 6 describes the molecular assembly of BRCA1-complex**.**

Though the BRCA1-complex consists of six proteins, RAP80, ABRAXAS, BRCA1, MERIT40, BRCC36 and BRCC45, it is not known how these are organized in the complex [13, 19, 20]. In other words, it is not completely known which components interact directly or indirectly. A direct association between RAP80 and ABRAXAS has been reported earlier[7]. Similarly, pS-X-X-F motif of ABRAXAS directly binds to BRCA1-BRCT[7] in a phosphorylation dependent manner. This study focused on identification of direct binding partners of MERIT40. The ABRAXAS, RAP80 and MERIT40 were sub-cloned into bacterial vector, protein was expressed and purified to carry out interaction analysis using pull down and calorimetry

A quantitative pull down interaction analysis established direct interaction of MERIT40 with BRCA1-BRCT and ABRAXAS. The observed ITC isotherm of MERIT40 and BRCA1-BRCT interaction depict a low binding affinity (K=1.02 ± 0.15 E^5). Interactions profile of MERIT40 and ABRAXAS suggests that ABRAXAS C-terminal region is involved in interaction with MERIT40 with a binding affinity (K= 5.8 ± 0.35 E^5M^{-1}). However, MERIT40 doesn't show any interaction with RAP80 (**Figure 5**).In conclusion, interactive relationship of MERIT40 with ABRAXAS as well as BRCA1-BRCT mediates the formation of stable BRCA1-complex. Thus, MERIT40 is the core mediator molecule, and is obligatory for organization of stable BRCA1-complex.

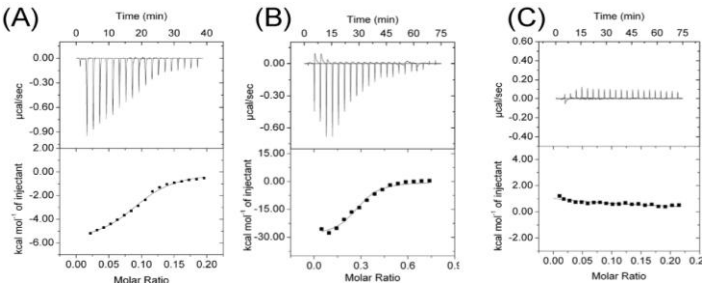

Figure 5: ITC of MERIT40 with (A) BRCA1 BRCT (B) ABRAXAS and (C)RAP80

Chapter 7 of the thesis describes the crystallographic studies of BRCA1 interacting partners.

One of the basic requirements for three-dimensional structure determination of macromolecules using X-ray crystallography is the highly purified protein in large amount and well-ordered crystals. Protein crystallization experiments were carried out by the vapour diffusion method separately in hanging and sitting drops. The concentration of protein used to prepare the drop, which equilibrated with a precipitating solution, ranged from 15 – 25 mg/ml. Commercially available solutions, Hampton Crystal Screen 1, 2 , SaltX, Sigma crystal screen and Qiagen screens were used as the precipitating solutions. The protein and precipitating solutions were mixed in 1:1 volume ratio, and the mixture was allowed to equilibrate at 22°C or at 4°C with 500-1000 µl mother liquor in a closed system. We observed a clear drop or precipitation in most of the drops in cases of MERIT40, RAP80 and ABRAXAS. However, the crystallization trial of BRCA1-BRCT and KIF1 peptide complex resulted in diffraction quality crystals. These crystals were used for x-ray diffraction data collection, and the structure of the complex has been determined to low resolution.

kif1b gene encodes a motor protein that transports mitochondria and synaptic vesicle precursors, and mutations in this gene cause the formation of Charcot-Marie-Tooth disease. Kinesis-like KIF1B can be a potential interacting partner of the BRCA1-BRCT domain [21] and is suspected to be phosphorylated in case of cancer. In order to understand the molecular associations with BRCA1-BRCT and KIF1B, the synthetic construct of KIF1B (NH2- DRTP(**pS**)PTFST-COOH) (pS- phosphoserine) peptide was analyzed for its binding with BRCA1-BRCT. The binding isotherm suggests a moderate binding with affinity constant, $K= 3.53\ E^5\ M^{-1}$. Crystals of the complex were obtained by co-crystallization under following conditions: 0.2 M ammonium sulphate, 30% PEG

5000 MME, 0.1 M MES pH 7.5. Diffraction data were collected at 100 K by the oscillation method using the MAR DTB mounted on a Microstar rotating anode X-ray generator (Bruker). The diffraction data processed using Mosflm software, indicates that the crystals belong to the space group $P2_12_12_1$ with unit cell parameters: a= 84.01 Å , b=180.47 Å , c=194.22 Å. The useful data extended to 3.5 Å with a completeness of 86%. The structure was solved by molecular replacement method as implemented in the software package *Phaser*. There are six molecules in the asymmetric unit leading to a solvent content of 60%. The structure was further refined using *Refmac-5*. In the crystal structure, there is clear electron density for the KIF1 oligopeptides bound to all the six subunits. The major interactions between the KIF1 peptide and the BRCA1 BRCT domain is through hydrogen bonds between phosphoserine 909 from the peptide and residues Ser1655 and Gly1656 from the BRCT domain. Efforts are being made to improve the quality of crystals for better diffraction.

Chapter 8 describes about important findings and conclusions of the work

RAP80, ABRAXAS, MERIT40 and BRCA1 play crucial role in DNA damage repair pathway, and mutations such as ΔE81 in RAP80 and R361Q in ABRAXAS cause significant impairment of BRCA1-complex function. Limited proteolysis of purified RAP80 and its ΔE81 mutant shows that the mutant is less compact and more susceptible to proteolysis. Molecular modeling studies indicate that RAP80 ΔE81 mutation leads to destabilization of an alpha-helix causing loss of binding with ubiquitin. This, in turn, leads to defective recruitment of BRCA1 to the DNA repair site. Similarly, biophysical and in-silico experiments indicate that R361Q mutation brings some localized changes in the structure of ABRAXAS that is likely to affect transport of BRCA1-complex from the

cytoplasm to the nucleus. MERIT40 is a recently identified protein that acts as a stabilizer of the BRCA1-complex. It is found to bridge RAP80 and BRCA1 through direct interactions with adapter protein ABRAXAS, and BRCA1, thus maintaining the integrity of the whole complex. The sequence of MERIT40 is not similar to any proteins contained in the PDB to enable homology modeling. Ab-initio modeling using Robetta server revealed that MERIT40 is structurally similar to another protein involved in complement activation. These studies presented in this thesis unravel the interactions involved among different BRCA1-complex members which are essential for DNA repair function of complex. It will further explore the possibility of structure based inhibitor design for therapeutic application that can compensate the effect of mutations.

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List of publications:

(a) Published

1) ***Vikrant***, *Ulka Sawant, Ashok K Varma, (2014).* Role of MERIT40 in stabilization of BRCA1-complex: A protein-protein interaction study. Biochem Biophys Research Communication. (http://dx.doi.org/10.1016/j.bbrc.2014.03.073)

2) *Dilip C. Badgujar, Ulka Sawant,* ***Vikrant****, Lumbini Yadav, M. V. Hosur and Ashok K.* Varma, *(2013).* Preliminary Crystallographic Studies of BRCA1 BRCT- ABRAXAS Complex. Acta Crystallography. F69. 1401-1404

3) ***Vikrant****, Pallavi Nakhwa, Dilip C. Badgujar, Rajan Kumar, Khushboo K.S. Rathore, Ashok K. Varma, (2013).* Structural and functional characterization of the MERIT40 to understand its role in DNA repair. Journal of Biomolecular Structure and Dynamics. (http://dx.doi.org/10.1080/07391102.2013.843473)

4) ***Vikrant****, Rajan Kumar, Lumbini R. Yadav, Pallavi Nakhwa, Sanjeev K. Waghmare, Peyush Goyal, Ashok K. Varma, (2013).* Structural and Functional Implication of RAP80 Δ81Glu Mutation. PloS One. 8. e72707

5) ***Vikrant****, Ashok K Varma, (2011).* Structural investigation of RAP80; A novel BRCA1 interacting protein involved in the mediation of DNA damage repair function. Acta Crystallography. A67. 346

6) ***Vikrant****, Rajan Kumar, Quadir, S. Waghmare, Ashok K Varma.* Mechanism of BRCA1-complex due to ABRAXAS Arg361Gln mutation. JBSD, 10.1080/07391102.2014.945484

(C) Other Publications:

1) *Lumbini, Mahamaya,* ***Vikrant****, M V Hosur, Ashok Varma.* Tetrameric ZBRK1 DNA binding domain has affinity towards cognate DNA in absence of Zinc ions- **BBRC** | Volume 450 | Pages 283–288

2) *Rakesh C. Chandarana,* ***Vikrant****, Ashok K. Varma, Anil Saran, Evans C. Coutinho, Jacinta S. D'Souza (2013).* Over-expression, purification and isotopic labeling of a tag-less human glucose dependent insulinotropic polypeptide. 3 Biotech. 13205-013-0181

3) *Bhanu P Jagilinki, Nikhil Gadewal, Harshal Mehta, Hafiza Mahadik,* ***Vikrant****, Anamika Pandey, Ulka Sawant, Prasad A Wadegaonkar, Peyush Goyal, Satish Kumar, Ashok K Varma (2014)* Conserved residues at the MAPKs binding interfaces that regulate transcriptional machinery. **JBSD** | DOI: http://dx.doi.org/10.1016/j.bbrc.2014.05.104

Date: 16/4/2014 Signature of Student

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List of Abbreviations

BRCA1	: BReast CAncer susceptibility gene1
CtIP	: CtBP Interacting Protein
RING	: Really Interesting New Gene
BARD1	: BRCA1 Associated Ring Domain-1
HR	: Homologous Recombination
NHEJ	: Non-Homologous End Joining
ATM	: Ataxia Talangiectasia Mutated
ATR	: ATM and RAD3 related
MDC1	: Mediator of DNA Damage Checkpoint -1
RNF	: RING finger containing protein
UBC	: Ubiquitin Conjugating Enzyme
MRE11	: Meiotic Recombination 11 homolog
MRN	: Mre11-Rad50-Nbs1 complex
RAP80	: Receptor Associated Protein-80
Chk1	: Checkpoint kinase 1
BACH1	: BTB and CNC homology 1
ACC1	: Acetyl Coenzyme A Carboxylase
CD	: Circular Dichroism
MALDI-TOF	: Matrix-Assisted Laser Desorption and Ionisation-Time Of Flight
ITC	: Isothermal Titration Calorimetry
MR	: Molecular Replacement

COOT	: Crystallographic Object-Oriented Toolkit
NCoA2	: Nuclear receptor CoActivator 2
TEV	: Tobacco Etch Virus protease
CCP4	: Collaborative Computational Project No.4
FPLC	: Fast Protein Liquid Chromatography
NMR	: Nuclear Magnetic Resonance
RMSD	: Root Mean Square Deviation
Top BP1	: Topoisomerase (DNA) II Binding Protein 1
53BP1	: p53 Binding Protein
PARP1	: Poly (ADP-ribose) Polymerase 1
RFC	: Replication Factor C
ARD	: Ankyrin Repeat Domain
SPR	: Surface Plasmon Resonance

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