Chapter 5

Structural & functional analysis of ABRAXAS
5.1 Introduction

ABRAXAS acts as a bridging molecule among the BRCA1-complex family which comprises of RAP80, ABRAXAS, BRCA1, MERIT40, BRCC36 and BRCC45 proteins [6, 15-18]. RAP80 can form DNA damage induced foci of BRCA1 complex in two different manners, one is Ubiquitin Interacting Motif (UIM) dependent while the other is UIM independent but AIR (ABRAXAS Interacting Region) dependent. BRCA1 complex foci formation is essential for execution of homologous recombination repair process after DNA damage. siRNA mediated knockdown of ABRAXAS reduces the BRCA1 complex foci formation in IR induced DNA damage, and the BRCA1 level was found significantly lower compared to the RAP80 UIM truncation [87]. RAP80 double mutants lacking UIM and AIR completely abolish the foci formation, thus indicating potential role of ABRAXAS in BRCA1-complex foci formation [87]. Furthermore, ABRAXAS and RAP80 knockdown cells showed defective homologous recombination repair and increased hypersensitive to IR and UV radiation [20, 24]. Role of ABRAXAS is also suspected in G2/M check point activation since depletion of RAP80 and ABRAXAS display defective G2/M check points [6, 7]. ABRAXAS comprises pS-X-X-F (SPTF) binding motif at its C-terminal which interacts with BRCA1-BRCT phospho-peptide binding domain and brings about foci formation (Figure 5.1). The Ser 404 and Ser406 on the SPTF motif are phosphorylated by ATM/ATR after IR treatment and is required for the phosphodependent interaction between ABRAXAS and BRCA1 BRCT domains [6, 7]. Moreover, S406A mutation abolishes the foci formation between the ABRAXAS and BRCA1, suggesting phosphodependent interaction between them [192]. ABRAXAS acts upstream to BRCA1 and its knockdown significantly reduce accumulation of BRCA1-
complex at DNA damage site. There are possible augmentations that loss of ABRAXAS or BRCA1 could lead to similar phenotype, thereby projecting ABRAXAS as an excellent candidate gene for familial breast cancer [6, 16, 192, 193].

Several reported mutation in BRCA1 associated proteins either disrupt their interaction with BRCA1 or with other binding partner, thereby causing various pathogenic implications [4, 5]. Familial mutation at C-terminus of ABRAXAS in Finnish population (Arg361Gln) is suspected to disassemble the BRCA1 complex [11]. c.1082G>A alteration was observed among 3 of 125 (2.4%) studied breast cancer families but was found to be absent in 868 healthy female volunteers [11]. This variant impairs nuclear localization of BRCA1 complex and its DNA damage repair function, thereby predisposing the individual to repair defects [11]. Immunoprecipitation of epitope tagged ABRAXAS R361Q mutant with BRCA1 and other core complex in cytoplasm displays its failure to form foci in the nucleus after DNA damage, whereas nuclear localization of wild-type is retained [11]. Failure to achieve nuclear retention adversely affects G2/M checkpoint and homology-directed DNA repair, and reduces nuclear retention to DSB site of ABRAXAS interacting partners [11]. Moreover, expression of the ABRAXAS R361Q variant causes hypersensitivity to ionizing radiation and reduced BRCA1 localization at sites of DNA damage in several cell lines[11].

![Figure 5.1](image_url): Domain organization of ABRAXAS.
These reports substantially list ABRAXAS as a new susceptibility gene to cancer predisposition and opened the vast perspective to study the mutant like R361Q discovered in cohort of patient in disease progression.

To understand the deleterious effects of mutations and possible consequences, biophysicochemical characterization and interaction analysis of ABRAXAS wild-type and R361Q mutant with BRCA1 were performed. This includes cloning, expression and purification of functional domains of ABRAXAS wild-type and mutant and their biophysical analysis

5.2 Material and Methods

A comparative structural, stability and binding analysis of ABRAXAS (6-373) wild-type and ABRAXAS (6-373) R361Q mutant was carried out. In addition, different regions of ABRAXAS (6-268 a.a), ABRAXAS (6-301 a.a), ABRAXAS (6-373 a.a), ABRAXAS (6-409 a.a) were also sub-cloned to perform binding analysis with MERIT40. Nucleotide sequence of primers used in PCR amplification for ABRAXAS (6-268), ABRAXAS (6-301), ABRAXAS (6-373), ABRAXAS (6-409) regions are shown in Figure 5.2, respectively. PCR condition is as follow; 95 °C denaturation (5 minutes), 95 °C denaturation (45 seconds), annealing 68°C for 35 seconds, Extension 72°C at 0.5kb/min, final extension 72°C for 10 minutes, 25 cycles. The PCR amplified product was digested with Nco1 and Xho1 and ligated into pET28(a)+ using one-step cloning protocol. The ligation mixture was transformed into E.coli DH5α cells. Selected potential clones were screened for the presence of insert by digesting with enzymes Nco1 and Xho1 and loading on agarose gel. Final confirmation was done by DNA sequencing.
Sequentially confirmed clones were used for protein expression and purification. The methods and materials have been described in detail in chapter 3 (material and methods). Functional domains of ABRAXAS were expressed in bacterial system *E.coli* BL21 (DE3) cells. For protein expression, 100 ng/µl plasmid construct was transformed into *E.coli* BL21 (DE3) cells and grown on LB agar plate containing antibiotic kanamycin (100 µg/ml). Further protocol is described below.

**Figure 5.2:** (A) Basic scheme for sub-cloning of different functional domain of ABRAXAS in pET28. (B) ABRAXAS primers used for PCR amplification; 1-(Abraxas_6 amino acids_F), 2-(Abraxas_268 amino acids_R'), 3-(Abraxas_301 amino acids_R'), 4-(Abraxas_373 amino acids_R'), 5-(Abraxas_409 amino acids_R). F stand for forward and R for reverse primer.

**Protocol for protein expression:**

1. **Inoculation:** Pick-up a single transformed colony from antibiotic resistant LB agar plate and inoculate it in 100 ml LB broth containing 100 µg/ml of kanamycin. Incubate on shaker incubator at 37 °C over night.
2. **Dilution:** Inoculate 10 ml of starting culture to 1000 ml (1: 100 ratios) of autoclaved LB broth containing the 100 µg/ml of kanamycin. Incubate the flasks on shaker incubator at 37 °C until it has reached mid-log phase i.e. A<sub>600</sub> between 0.6-0.8.

3. **Induction:** Cool down the flasks and add 400 µl IPTG (stock 1M) and incubate on shaker incubator at 24°C for 16 hours.

4. **Harvesting:** Transfer the culture to centrifuge bottles and centrifuge for 10 minutes at 6000 rpm at 4°C. Resuspend the pellet in small amount of supernatant and centrifuged for 15 minutes at 5000 rpm, 4 °C.

5. **Storage:** The pellet obtained was stored at -80 °C for further use.

Further, proteins were purified with affinity chromatography followed by FPLC. ABRAXAS (6-373) R361Q mutant was expressed in *E.coli* (BL21) and purified as per wild- type protocol.

**Protocol for purification of ABRAXAS (6-373) wild-type and R361Q mutant:**

*Purification buffer composition:* 300 mM NaCl, 10 mM HEPES, 0.1mM EDTA, 2mM BME pH 7.5

*FPLC buffer composition:* 300 mM NaCl, 10 mM HEPES, 0.1mM EDTA, 2mM BME pH 7.5

1. **Re-suspension:** Re-suspend the pellet of ABRAXAS in 40 ml of purification buffer; containing 200 µl of 200 mM PMSF and 20ul of protease inhibitor were added.

2. **Ultra sonication:** Transfer the suspension into the centrifuge tube and sonicate at 50 pulse rate and 50 power with 1.45 minutes of duty cycle.
3. **Centrifugation**: After sonication the suspension is subjected to centrifugation at 18000 rpm for 45 minutes at 4°C. The aim here is to obtain cell free extract. Collect the supernatant and discard the pellet as it contains cell debris.

4. **Calibration of Ni-NTA beads**: Give two column volume washes with distilled water to remove the traces of ethanol as the beads are stored in 20% ethanol and then three column washes with purification buffer.

5. **Binding**: The soluble fraction obtained is brought to room temperature and then carefully mixed with affinity resin, and incubated at room temperature for an hour.

6. **Washing**: After binding, the column is given 4 column washes with wash buffer so as to remove non specific protein. Take 40µl of beads to load on gel to observe the bound protein.

7. **Cleavage**: Add 400µl of TEV protease enzyme, 40µl of protease inhibitor cocktail and 100µl of PMSF in 20 ml of purification buffer and proceed the cleavage step for 3 hours by passing the solution through column at interval of 1 hour. This step is performed to cleave the His-tagged from fusion protein to get the purified native protein. Take out 40µl of beads to observe the cleavage of protein.

8. **Elution**: After TEV cleavage the protein is eluted in 30 ml of purification buffer.

9. **Calibration of Ni-NTA resin**: Give 2 column washes with double distilled water and then 5 to 6 column wash with washing Buffer.

10. **Metal Ion Chelate Affinity Chromatography**: After equilibration of Ni-NTA resin, pass the eluted fractions through it.
11. **Equilibration of centricon:** Wash the centricon (Millipore 10 kDa Molecular weight cut off) with water to remove traces of alcohol, rinse it with washing buffer by centrifuging it at 4500 rpm for 10 minutes at 4 °C.

12. **Concentrating the protein:** Transfer the eluted protein in the 10 KDa centricon and concentrate the protein upto 2 ml by centrifuging it at 4500 rpm for 10 minutes at 4 °C. Check the concentration on Nanodrop spectrophotometer (280 nm). Centrifuge for 10 minutes at 5000 rpm at 4 °C for removal of any traces of impurities.

13. **Gel filtration:** Inject 2 ml of concentrated protein on AKTA- FPLC against FPLC buffer.

14. **Fraction collection:** Collect the purified protein obtained through FPLC in 1.7 ml eppendorf at its elution volume according to gel filtration spectra profile of sample.

15. **Loading on SDS-PAGE 12% gel:** Load 20µl of FPLC fractions on SDS-PAGE, stain it with commassie dye, and then destain to visualize the protein of interest.

16. **Concentrate the protein:** The fractions which showed purified protein bands of interest were further concentrated as per requirement.

The purified proteins were used in various bio-physicochemical experiments. The complete details of protocol have been discussed in chapter 3 (material and methods).

**5.3 Results and discussion**

**5.3.1 Sequence alignments:** Multiple sequence alignment of ABRAXAS protein sequences among various species showed a highly conserved nature of Arg 361 which indicates that it could be a critical residue for maintaining the protein structure and function (**Figure 5.3**). Hence, *in vitro* study was performed to analyze the effect of substitution mutation in ABRAXAS protein.
Figure 5.3: Multiple sequence alignment of ABRAXAS showing highly conserved nature of Arg361 [194].

5.3.2 Cloning, expression and purification of ABRAXAS functional domain: All deletion construct of ABRAXAS showed the insert release at proper size on Agarose gel after digestion with Nco1 and Xho1 (Figure 5.4). ABRAXAS wild-type eluted from Superdex-200 column as monomer, with some proportion of aggregated protein (Figure 5.5). ABRAXAS(6-373) wild-type and R361Q mutant eluted at the same elution volume corresponding to monomer.

![Figure 5.4](image-url)

**Figure 5.4:** Agarose gel showing the PCR amplified product (A) and insert release from potential clones’ plasmids (B) of pET 28(a)-ABRAXAS

![Figure 5.5](image-url)

**Figure 5.5:** (A) Purification of ABRAXAS. SDS-PAGE showing purified protein of ABRAXAS (6-268) lane 2-6, ABRAXAS (6-301) lane 7-11 and ABRAXAS (6-409) lane 12-14. (B) ABRAXAS (6-373) wild-type (lane 1), mutant (lane 2). (C) Overlay of gel filtration spectra of ABRAXAS (6-373) wild-type and R361Q (Superdex 200) [194].
5.3.3 Oligomeric characterization of ABRAXAS (6-373) wild-type and R361Q mutant: Substitution mutation results into distortion of secondary or tertiary structure which may lead change in the oligomeric behavior of the protein [195, 196]. ABRAXAS R361Q mutation involves substitution of a basic residue with non polar charged residue, which might change the oligomeric property of the protein.

![Image](A)

**Figure 5.6:** Dynamic Light Scattering profile of ABRAXAS (6-373) wild-type and R361Q. Inset showing the magnified region of the peak corresponds to monomeric population (A). Correlation coefficient of wild-type (B) and mutant (C) [194].

The observed hydrodynamic diameter of wild-type was 9.0±0.3 nm while mutant showed a hydrodynamic diameter of 10.0±0.5 nm (Figure 5.6). Relative increase in the effective diameter of mutant protein may be due to expansion of molecule dimension, nevertheless, the increment was not due to oligomerization. Moreover, the aggregates detected in case of mutant showed a broader variety as compared to wild-type and spread over a long molecular weight range. This indicates that there may be some structural changes or disordered region present in mutant which are different from wild-type. These findings supported that ABRAXAS R361Q mutation probably incorporates short or long range structural changes in the protein without changing its oligomeric behaviour.

5.3.4 Structural Organization of ABRAXAS (6-373) wild-type and R361Q: Limited proteolysis is one of the widely used approaches for determining any structural alteration in protein due to acquired mutation [177]. In general, a compact globular domain of
protein considerably resists the protease digestion, while disordered region undergoes rapid digestion [177]. To understand domain integrity and determine the resistivity of ABRAXAS wild-type and mutant against the protease digestion, limited digestion with trypsin and chymotrypsin proteases was performed. Wild-type and mutant proteins were treated with same concentration of proteases for limited time and results were analyzed on SDS-PAGE (Figure 5.7). ABRAXAS wild-type resistance towards protease digestion was comparable to that of mutant protein which indicates existence of similar structural domain(s). Parallel susceptibility of ABRAXAS mutant towards protease digestion suggests that substitution of R361Q is not destabilizing the domain integrity of ABRAXAS. Since the observed domain’s organization in limited proteolysis is similar it indicates that wild-type and mutant proteins may have similar or slightly changed secondary and tertiary structures. To

Figure 5.7: Resistivity profile of ABRAXAS wild-type and mutant towards Protease digestion. Limited proteolysis of ABRAXAS (6-373) wild-type (A, C) and mutant (B, D) using trypsin (A, B) and Chymotrypsin (C, D) as proteases. Ctl- control as untreated with trypsin and chymotrypsin, respectively [194].
evaluate this, secondary structure of ABRAXAS wild-type and mutant was compared using far-UV Circular Dichroism (Figure 5.8). It has been observed that ABRAXAS wild-type and mutant have well-defined α/β characteristics, however, β-sheets characters were more prominent. Data analysis using Dichroweb server suggested that wild-type and mutant are having α-helix (wild-type 15%, mutant 15.5%) and β-sheets (wild-type 24%, mutant 23%) [186]. Secondary structural comparison of wild-type and

![Figure 5.8](image-url): Secondary and tertiary structure evaluation of ABRAXAS wild-type and mutant. (A) Overlay of Far-UV Circular Dichroism spectrum of wild-type and mutant. (B) Overlay of fluorescence emission spectrum of wild-type and mutant [194].

mutant deciphered the similar α-helical and β-sheets characteristics which illustrates less effect of R361Q on the ABRAXAS structure. ABRAXAS R361Q mutation may be inducing short range 2° structural changes which might be collectively responsible for tertiary structural modification.

To study the three dimensional structure of ABRAXAS wild-type and mutant, Trp and Tyr microenvironment were monitored using fluorescence spectroscopy. An emission maxima at λ 332 nm obtained for wild-type and 334 nm for mutant indicated slight changes in Trp and Tyr microenvironment (Figure 5.8). Thus, it can be concluded that
tertiary structural components become moderately altered without causing drastic conformational changes.

5.3.5 Thermal stability and folding pathway of ABRAXAS (6-373) wild-type and R361Q Mutant: Thermal stability of ABRAXAS wild-type and mutant was compared at secondary (CD) and tertiary structure (fluorescence) levels. The spectrum obtained from CD corresponding to λ222 nm showed the maximum change in ellipticity and high signal to noise ratio. Change in ellipticity was plotted against the different temperatures and fraction unfolded was determined (Figure 5.9). Thermal stability of ABRAXAS wild-type (Tm 35°C, ΔG° H2O 2.1±0.5 Kcal/mol) was found equivalent to mutant (Tm 34.8°C, ΔG° H2O 1.53±0.6 Kcal/mol), and endures similar folding pathway. For stability assessment of tertiary structure, emission maxima of wild-type and mutant were monitored at different temperatures corresponding to λ280 and fraction unfolded was calculated. Fluorescence spectroscopy revealed comparable unfolding pattern but different stability, and derived Tm value 23±3.1°C for mutant (ΔG° H2O 1.25±0.5 Kcal/mol) and 27±2.5°C for wild-type (ΔG° H2O 1.08 Kcal/mol) (Figure 5.9). Furthermore, it revealed that proteins most likely unfold through an intermediate species and undergo three state transition (Figure 5.9). To determine the chemical stability of wild-type and mutant, protein samples (2µM) were incubated in different concentrations of urea (0-8 M) till equilibrium was reached and emission maxima were obtained for the wavelength of λ280. Thermodynamic parameters (wild-type ΔG° H2O 2.53±0.45 kcal/mol, mutant ΔG° H2O 1.91±0.44 kcal/mol) were calculated by plotting fraction unfolded
Figure 5.9: Thermal and chemical stability of ABRAXAS (6-373) wild-type and mutant. (A) Thermal and (B) chemical stability profiles of wild-type and mutant showing overlay of protein fraction unfolded at different temperature/urea concentration. (C) Differential scanning calorimetry of wild-type and mutant protein [194].

against urea concentration. Chemical stability of mutant was found different, nevertheless, both unfold through acquiring an intermediate species which was more predominant in case of wild-type (Figure 5.9). To substantiate these findings and re-evaluate the reversibility of unfolding of wild-type and mutant, Differential Scanning Calorimetry (DSC) was performed. A peak maxima was considered as temperature of melting and area under the curve was used to determine enthalpy contents and folding pathway. DSC data has shown overlapping of two transitions which could be corresponding to an intermediate formed during unfolding process, and displayed three state reversible folding pathway of wild-type and mutant. The observed Tm values for wild-type was significantly higher compared to mutant (wild-type Tm 32±2.5, ΔH 440±11.6 kcal/mol; mutant Tm 27±3.1, ΔH 660±15 kcal/mol) (Figure 5.9). Furthermore, the observed intermediate species was reluctant to exist in case of mutant while it was predominant in the wild-type. These results suggest that three-dimensional structural
stability of ABRAXAS is different in R361Q mutation and undergoes different folding pathways.

5.3.6 Binding interaction of RAP80 with ABRAXAS (6-373) wild-type and R361Q mutant: ABRAXAS is a member of BRCA1 complex and is involved in direct interaction with RAP80 and BRCA1[7]. ABRAXAS binds directly to RAP80 AIR and through (pS)-P-T-F motif located at the C-terminal to BRCA1 BRCT, thus facilitating the recruitment of BRCA1 complex to the DNA damage site. Secondary structural component of wild-type and mutant remained equivalent irrespective of mutation. However, the three dimensionl folding pattern and unfolding pathway indicate lesser stability of mutant. Since, structural components and folding pathway monitored at global structural level has shown differences, we speculate that the binding interaction between ABRAXAS wild-type and mutant with RAP80 would be affected. To test this hypothesis, His pull-down assay of native RAP80 (1-405) with His tagged ABRAXAS wild-type and mutant was performed. We observed that the binding of wild-type and RAP80 is significantly higher as compared to mutant (Figure 5.10). Dissimilar binding profile suggested that due to mutation, structure alteration hinders the association between RAP80 and ABRAXAS.

Figure 5.10: Binding analysis of ABRAXAS wild-type and mutant with RAP80. Histidine pull-down assay followed by western blotting [194].
5.4 Crystallization of ABRAXAS

ABRAXAS functional domain protein crystallization trial was set with 15 mg/ml protein concentration. The protein and mother liquor solution was mixed in 1:1 ratio (1 µl+1µl) and allowed to equilibrate at 22°C with 500 µl reservoir solution in a closed system. A clear drop or light precipitation was observed in most of the drops.

5.5 Conclusion

ABRAXAS is the key member of BRCA1 complex and acts as a bridging molecule of the complex. Knockout studies of ABRAXAS have shown defective recruitment of BRCA1 complex to the DNA damage site and hence defective DNA repair [6, 7]. Thus, ABRAXAS is a multifaceted molecule which plays an important role in cancer progression, and BRCA1 mediated homologous recombination repair.

Multiple sequence alignment of ABRAXAS in different species in phylogenetic order has showed highly conserved nature of Arg 361 residue (Figure 5.3). Wild-type and mutant showed similar CD spectra and secondary structural composition, while the relative orientation of Trp and Tyr were slightly disturbed. This indicates that R361Q mutation brings several localized changes in structure pattern of ABRAXAS which altogether furnish a different conformational stability of overall structure. These conformational changes are very minor and hence could not be detected at secondary structural level while their relative effect was traced during three dimension unfolding pathway. The relative redundancy of intermediate species in wild-type suggests the existence of different unfolding pathway which may be partially followed by mutant, furthermore unfolding of wild-type was found to be more cooperative. Altogether, the localized changes in the mutant structure brings down its thermal and chemical stability which
further perturbs its interaction with RAP80. The cumulative global changes in mutant structure was sufficient to disturb the critical interaction necessary for BRCA1 complex integrity and localization. Therefore in the presence of R361Q mutation, ABRAXAS could not extend its bridging interaction through RAP80 which perhaps affects the recruitment of BRCA1 complex to the DNA damage site. Consequently, the nuclear retention of BRCA1 is adversely affected which further agitates G2/M checkpoint and homology-directed DNA repair (Figure 5.11) [11].

Figure 5.11: The figure illustrates the plausible mechanism of defective homologous recombination repair and other consequences due to ABRAXAS R361Q mutation [194].

Studies of Abraxas wild-type and R361Q mutation describes the role of protein folding in disease progression such as cancer. It will further explore the opportunity of inhibitor design for therapeutic application that can recompense the effect of such adverse mutation.