Chapter 6

Structural Associates of BRCA1-complex

6.1 Introduction

BRCA1 interacts with numerous molecules and consequently plays an essential role in multiple pathways such as DNA damage repair, cell-cycle and transcription regulation [2, 8, 89, 162, 163]. It acts as a tumor suppressor and has been found to be associated with hereditary breast and ovarian cancers [3, 164]. Furthermore, BRCA1 BRCT deletion mutants showed perturbed behavior towards subnuclear co-localization with H2AX, thus highlighting its importance in DNA damage repair [85, 116, 147, 165].

Protein-Protein Interactions in BRCA1 complex play very important role in its structural stability, and alteration in interactions profiles lead to destabilization of whole complex [13]. MERIT40 is an essential component of BRCA1 complex, but how it stabilizes this complex remain elusive. A literature search could not find any direct association between MERIT40 and BRCA1 that stabilizes the complex. Knockdown of MERIT40 significantly reduces the RAP80 and ABRAXAS proteins levels, consequently affecting the integrity of BRCA1-complex [13, 19]. MERIT40 down regulated cells considerably lower the stability of BRCA1-complex compared to any other members [13]. Hence, MERIT40 is a mediator protein required for stable BRCA- complex formation.

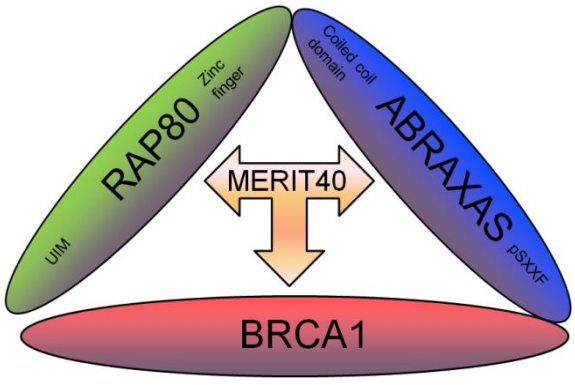

Figure 6.1: Schematic representation of members of BRCA1 complex.

MERIT40 is essential for integrity and localization of BRCA1-complex by performing multiple interactions within the complex at DSB site [13]. As, MERIT40 knockdown has a negative effect on the RAP80, BRCC36 and CCDC98 levels irrespective of BRCA1 protein level. It further demonstrates that the MERIT40 has a greater influence on the BRCA1-complex [13]. However, it remain elusive how each member form association together and bring about the stabilization of BRCA1 complex. Recombinant MERIT40, BRCA1 BRCT domain and CCDC98 were purified to establish their interacting relationship. To further explore the possibility of complex formation between MERIT40, CCDC98 and BRCA1 BRCT, GST pull-down assay and Isothermal Titration Calorimetry was performed.

The overall goal is to analyze structure of BRCA1 complex and determine the binding association among the various members

1.2 Material and Methods

In the present study, structural and binding analysis of RAP80, BRCA1, ABRAXAS and MERIT40 were carried out. The following plasmid constructs were used for the expression and purification of proteins. RAP80 (1-405), BRCA1-BRCT (1649-1859), ABRAXAS (6-409), ABRAXAS (6-373), ABRAXAS (6-301), ABRAXAS (6-268), and MERIT40 full length. Expression and purification of RAP80 (1-405) and MERIT40 have been described in chapter 3 and 4, respectively. Similarly, expression and purification of different functional domain of ABRAXAS (6-409), ABRAXAS (6-373), ABRAXAS (6-301) and ABRAXAS (6-268) have been described in chapter-5

BRCA1-BRCT was expressed in bacterial system using *E.coli* BL21 (DE3) strain. For protein expression, 100 ng/µl (1ul) plasmid construct was transformed into *E.coli* BL21

(DE3) cells and grown on LB agar plate containing antibiotic ampicillin (100 µg/ml). Single colony was inoculated in LB broth and bulk culture (10 litres) was grown, as mentioned in case of MERIT40 (chapter 4). Purification protocol of BRCA1 BRCT is the same as that of MERIT40 except the purification buffer composition.

Purification buffer composition: 300 mM NaCl, 50 mM Tris pH 7.5

FPLC buffer composition: 300 mM NaCl, 10 mM HEPES

Purified proteins were passed through gel filtration column (Superdex 200) in order to get pure and homogenous protein. They were further used in various bio-physicochemical experiments. The complete details of protocol have been discussed earlier in chapter 3 (material and methods).

6.3 Results and discussion

MERIT40, BRCA1-BRCT, RAP80 and ABRAXAS native proteins were FPLC purified and analyzed on SDS-PAGE (**Figure 6.2**). Purified proteins were gel extracted and subjected to MALDI-TOF/TOF mass spectrometry analysis for identification [197] [198]. MERIT40, BRCA1, RAP80 and ABRAXAS have shown correct identity in Expasy server (http://www.expasy.org/) through mascot analysis (**Table 6.1**). It illustrates the authenticity of purified protein for further use in structural and functional analysis.

Table 6.1: Mass spectrometry profiles of MERIT40, BRCA1, ABRAXAS and RAP80 using Mascot analysis [199]

Protein	Match	Score	Nominal Mass (M_r, Dalton)	Calculated pI	Sequence Coverage (%)
MERIT40	h-MERIT40	119	37050	4.6	35.0
ABRAXAS	h-ABRAXAS	164	47033	6.6	31.5
RAP80	h-RAP80	85	80875	5.3	20.1
BRCA1	h-BRCA1	63	208000	5.0	24.0

h: Human

6.3.1 Structural Characterization: Secondary and tertiary structural characteristics are the important benchmark for defining protein folding and functional properties. A well-folded protein shows typical secondary structure signature as well as localized behavior of aromatic and hydrophobic residues. Tyrosine and tryptophan are the most extensively used intrinsic fluorophores to study the micro-environmental changes induced in the protein due to external stimulus such as temperature and chaotrophs [187]. In a folded protein, these residues are generally buried inside the hydrophobic core and are extremely sensitive to modification around their micro-environment.

In order to evaluate the secondary structural characteristics of MERIT40, RAP80, ABRAXAS and BRCA1, far-UV CD spectra were recorded. Secondary structural component of purified

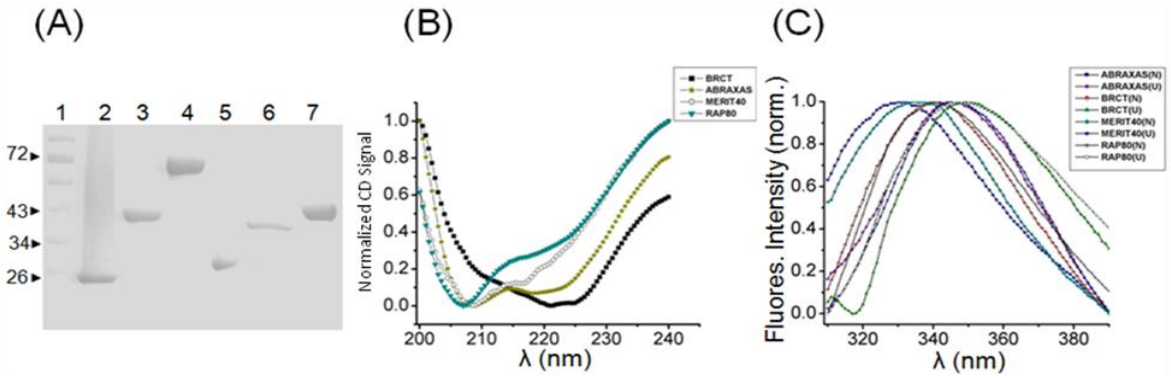

Figure 6.2: Protein purification and structural characterization of ABRAXAS, BRCA1-BRCT, MERIT40 and RAP80. (A) Lane 1-marker, purified proteins in lane 2-BRCA1-BRCT, 3-MERIT40, 4-RAP80, 5-ABRAXAS (6-268), 6-ABRAXAS (6-301) and 7-ABRAXAS (6-373). (B) Overlay of far-UV CD spectrum and (C) emission maximum of BRCA1-complex proteins. N-native, U-unfolded with urea [199].

protein was analyzed using DichroWeb server (http://dichroweb.cryst.bbk.ac.uk) [186, 200-203]. In MERIT40, percentage of α-helices and β-sheets were found to be 20 and 16.6% while BRCA1 BRCT showed it 22 and 27.1%, respectively (**Figure 6.2B**). RAP80 and ABRAXAS showed the presence of fewer α-helices (21.1 and 15%), and displayed

the prevalence of β-sheets (30.7 and 24%) (**Figure 6.2B**). Tertiary structures of purified proteins were evaluated by monitoring positions of Tyr and Trp residues. Emission spectrum of native and unfolded protein was monitored. Native MERIT40, RAP80, ABRAXAS and BRCA1 showed emission maxima at λ=338, 340, 332 and 339 nm respectively, and these maxima undergo substantial red shift when the protein is unfolded (**Figure 6.2C**). It indicates Trp and Tyr residues are buried in three dimensional hydrophobic environments [187]. Altogether, these results also deciphered a well folded state of MERIT40, RAP80, ABRAXAS and BRCA1.

6.3.2 Qualitative interaction analysis of MERIT40 with BRCA1, RAP80 and ABRAXAS: Pull-down interaction analysis suggested direct interaction of MERIT40 with BRCA1-BRCT or ABRAXAS (**Figure 6.3**). However, we did not observe significant binding between MERIT40 and RAP80, indicating their interaction is either absent or highly transient.

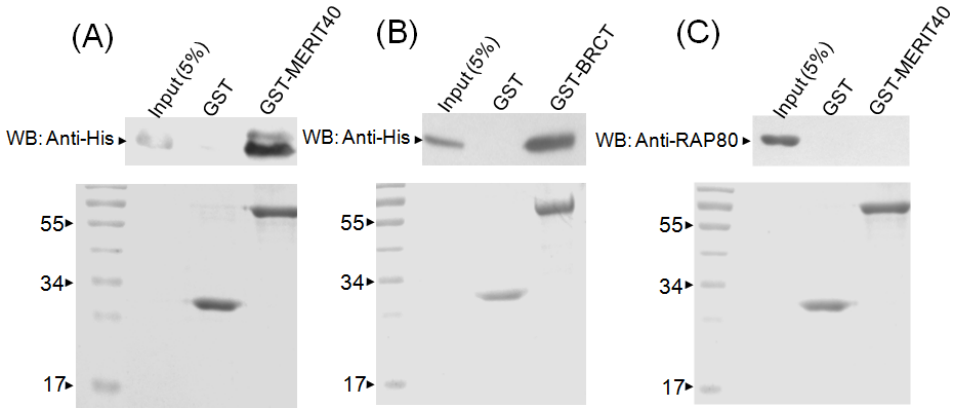

Figure 6.3: Pull-down assay to determine direct binding partner(s) of MERIT40. (A) ABRAXAS, (B) BRCA1-BRCT and (C) RAP80. Lower panel showing the loaded bait protein on SDS-PAGE stained with ponceau. Western blotting was performed against the his-ABRAXAS (prey), his-MERIT40 (prey) and RAP80 (prey), respectively [199].

6.3.3 Quantitative Interaction analysis of MERIT40 and BRCA1: To establish role of MERIT40 in stability of BRCA1-complex, interaction study between MERIT40 and

BRCA1 was performed using MicroCal ITC 200. All the samples were degassed thoroughly (30 min) before proceeding to the titration reactions, and a control/standard was run before performing the individual experiment. 30 µM BRCA1 BRCT in cell was titrated with 300 µM MERIT40 in the syringe, the reaction was allowed to proceed at 25°C. A total of 22 injections (spacing of 2 seconds) were given with constant stirring at 1000 rpm to achieve equilibrium in the system. The interaction between BRCA1-BRCT domain and MERIT40 was found with an affinity constant of K_d =521±7 µM. Hence, MERIT40 mediates a direct interaction with BRCA1-BRCT, however, the affinity of interaction was found to be weak (**Figure 6.4**).

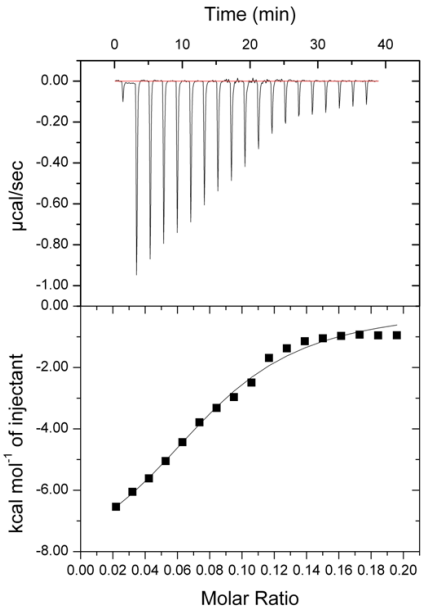

Figure 6.4: ITC thermogram for interaction analysis between MERIT40 and BRCA1-BRCT [199].

6.3.4 Interaction analysis of MERIT40 and ABRAXAS: Interaction analysis of MERIT40 with ABRAXAS (6-409) was performed using Isothermal Titration Calorimetry. MERIT40 was titrated with ABRAXAS (10-fold molar ratio) at constant temperature and heat changes were determined.

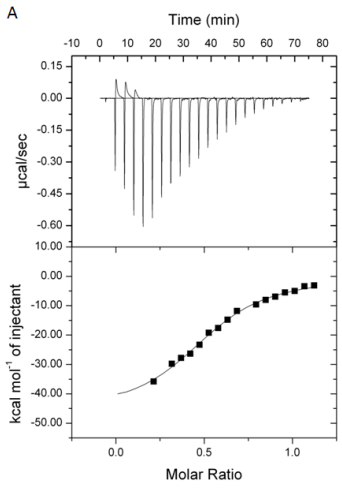

Figure 6.5: Interaction analysis of MERIT40 with ABRAXAS using Isothermal Titration Calorimetry [199].

MERIT40 was taken as titrant (30 µM) and ABRAXAS (290 µM) as titre in a Microcal ITC200. The reaction was allowed to proceed at 25°C with a stirring speed of 1000 rpm. A total of 23 injections were given (each 2 µl) with an equilibration time of 4 seconds. MERIT40 showed direct interaction with ABRAXAS as indicated with significant heat change during reaction. The binding affinity between MERIT40 and ABRAXAS was found K_d =2.5 ± 0.24 µM (**Figure 6.5**). Pull-down assay also confirmed the interaction between MERIT40 and ABRAXAS (**Figure 6.5**).

6.3.5 Determining the minimal region of ABRAXAS binding to MERIT40: In order to determine the minimal binding region of ABRAXAS , isothermal titration calorimetry was carried out using different deletion regions of ABRAXAS, such as (6-373), (6-301) and (6-268) amino acids. ITC of different ABRAXAS fragment was performed with full length MERIT40. Interaction profile of MERIT40 and ABRAXAS (6-373) suggests that ABRAXAS C-terminal region might be involved in interaction with MERIT40 with a binding affinity K_d= 1.7 ± 0.28 µM (**Figure 6.6**). To further narrow down the minimal

binding domain of ABRAXAS with MERIT40, we have performed interaction analysis with shorter region 6-301, 6-268 amino acids. However, no binding was observed between MERIT40, and ABRAXAS (6-301), (6-268) amino acids. From these experiments we conclude that the BRCA1 BRCT binding domain of ABRAXAS lies in the C-terminal region, while N-terminal of ABRAXAS might be mainly involved in interaction with RAP80 (**Figure 6.6**) [7].

6.3.6 Interaction analysis of MERIT40 and RAP80: RAP80 is an upstream player

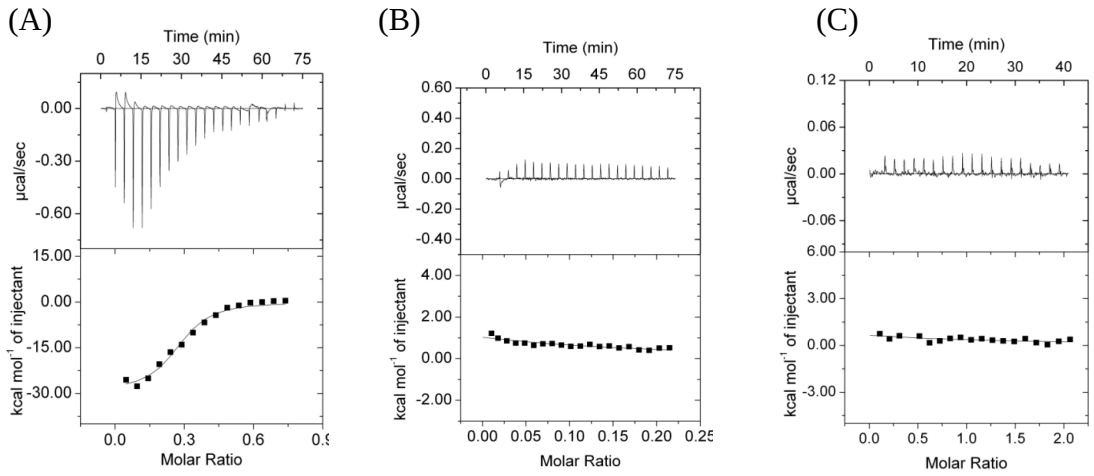

Figure 6.6: Isothermal titration calorimetry of MERIT40 with (A) ABRAXAS (6-373), (B) ABRAXAS (6-301), (C) ABRAXAS (6-268) amino acids [199].

in BRCA1-complex, hence it would be interesting to observe the binding between RAP80 and MERIT40. Surprisingly, MERIT40 did not show interaction with RAP80 in isothermal titration calorimetry (**Figure 6.7**).

6.4 Co-crystallization of MERIT40 with ABRAXAS and BRCA1 BRCT

For co-crystallization, both the proteins were mixed in equal proportion (0.8 mM) and crystallization trials were set. 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.

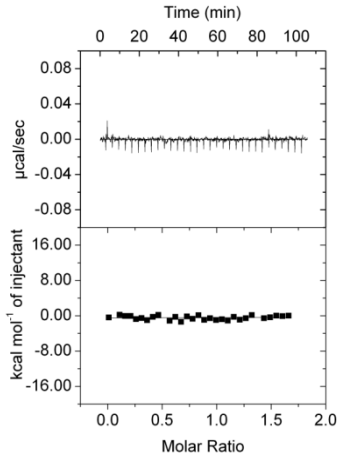

Figure 6.7: Isothermal titration calorimetry of MERIT40 with RAP80 (1-405) amino acids [199].

6.5 Conclusion

A direct interaction of BRCA1-BRCT and ABRAXAS with MERIT40 was observed which list MERIT40 as an essential molecule in BRCA1-complex. It perhaps set up an interaction network in BRCA1 complex which is being utilized for stabilization of ABRAXAS, since knockdown of MERIT40 significantly reduces the ABRAXAS and RAP80 levels (**Figure 6.8**)

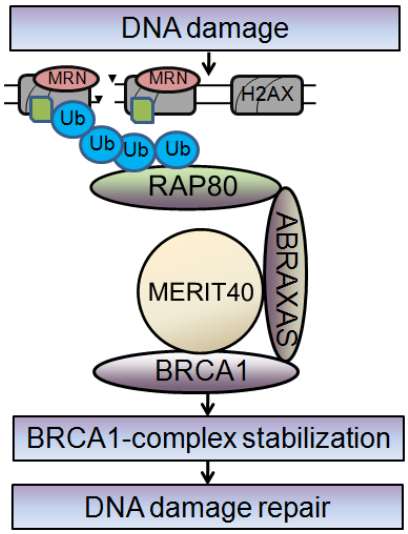

Figure 6.8: Anticipated model of MERIT40 mediated BRCA1-complex stabilization [199].

[13, 19]. Stabilization of ABRAXAS further helps in maintaining the structural integrity of BRCA1-complex. This study will provide insights into the diverse interactions involved among various members which are essential for DNA repair function of BRCA1 complex. These findings will be further helpful in understanding functional interplay of DNA damage repair proteins and mechanisms that abrogates the synergy of the protein complex, consequently, its DNA damage repair function.