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Role of MERIT40 in stabilization of BRCA1 complex: A protein–protein interaction study

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ABSTRACT

MERIT40 is a novel associate of the BRCA1-complex, thus play an essential role in DNA damage repair mechanism. It is the least implicit protein and its structural and functional aspects of regulating the stability of BRCA1–MERIT40 complex remain equivocal. Analysis of protein–protein interactions between BRCA1 and its cellular binding partners like ABRAXAS, RAP80 and MERIT40 would help to understand the role of protein complex integrity in DNA repair mechanism. The recombinant proteins were purified and their structural aspects were elucidated by spectroscopic methods. Interaction analysis was carried out to determine binding partners of MERIT40. MERIT40 showed interaction with bridging molecule, called ABRAXAS, thus generate a scaffold among various members which further stabilizes the entire complex. It acts as an adapter molecule by interacting with BRCA1-BRCT in non-phosphorylation dependent manner. The feature enlighten on structural and interaction profile of BRCA1-complex member to elucidate their role in complex stability and DNA repair process.

1. Introduction

Sensing and responding to DNA damage is the prerequisite for genomic integrity of living organism and their survival [1,2]. ATM (ataxia telangiectasia mutated) and ATR (ATM and Rad3-related) are the pair of related kinases which are activated in the response to DNA damage and acts as sensor molecules in DNA repair cascade [3–5]. Phosphorylation of the initial target H2AX (a histone variant) and MDC1 (Mediator of DNA damage complex 1) by ATM and ATR accumulate γ-H2AX at the damage site [6–8]. γ-H2AX acts as docking situate for MDC1 [8–11], a protein having the phospho binding module, which subsequently endorses the congregation of RNF8 and UBC13 [6,12–14]. Furthermost, aftereffect of this signaling event is the formation of polyubiquitin chains on the H2AX by UBC13 and RNF8 [15–18]. K-63 linked polyubiquitin chain on γ-H2AX is exclusively recognized by ubiquitin interacting motif (UIM) of RAP80 (receptor-associated protein 80) [19,20]. RAP80 is a member of BRCA1 complex which encompasses ABRAXAS, MERIT40, and BRCA1. RAP80 has two functional UIMs motifs at N-terminal and a central AIR domain (Abraxas-Interacting Region) [20,21]. ABRAXAS comprises sequence consensus of pS-X-X-F motif at the C-terminal through which it mediates the interaction with BRCA1 [22]. Similar consensus sequence is present in the BACH1, CtIP which is required for interaction with BRCA1-BRCT domains in phosphorylated dependent manner [23–25]. BRCT domains are the evolutionary conserved domain, identified in several DNA damage repair proteins and mediate protein–protein interactions by binding to phosphoproteins having pSer/(Thr)-X-X-Phe motifs [26,27]. BRCT repeats are indispensable for the tumor repressor functions of BRCA1 and obligatory for its translocation to IRIF (Ionizing Radiation Induced Foci) [28].

Genetic variants discovered in two major breast cancer susceptibility genes BRCA1 and BRCA2 are accounted approximately 5–10% of breast cancer cases [29]. Inherited mutations in the BRCA1 gene alone accounts for more than 50% familial breast cancer and 20–30% inherited ovarian cancers. However, for familial breast cancer, not more than 20% of the cases are reported for germ-line mutations in BRCA1 [30] which concentrates thoughtfulness towards the role of BRCA1 interacting protein in DNA damage repair and cancer. BRCT mutations responsible for breast and ovarian cancer found to have disrupted association between BRCA1 and RAP80 [20,31,32]. Deletion mutation at pSer/(Thr)-X-X-Phe motif in ABRAXAS prevents association of RAP80 and BRCA1, suggesting BRCA1–RAP80 complex is ABRAXAS dependent [22]. Knockdown studies with RAP80, ABRAXAS and BRCA1 have demonstrated that RAP80 is necessary for foci formation of ABRAXAS and BRCA1. As a result of UIM deletion, RAP80 mutant failed to recruit BRCA1 complex at DNA damage site. Cells knockdown for BRCA1 neither shown reduced level of RAP80 foci nor ABRAXAS

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foci formation after the DNA damage [28]. It suggests that ABRAXAS act downstream of RAP80 while upstream of BRCA1. Co-immunoprecipitation studies supported the fact that ABRAXAS acts as a linker between BRCA1 and RAP80, suggesting their co-existence in a complex [28]. ABRAXAS is also involved in BRCA1 dependent G2/M checkpoint, suggesting ABRAXAS function in a collaborative manner with BRCA1 [28]. It is currently indistinct how the RAP80 AIR facilitates DSB localization of ABRAXAS, and the entire repertoire of RAP80 AIR-associated proteins control BRCA1-complex DDR functions.

MERIT40 is a mediator required for stabilization of BRCA1-complex [33,34]. SiRNA mediated knockdown of MERIT40 reduces ABRAXAS level drastically at the RAP80 foci, suggesting aborted binding of ABRAXAS to RAP80. It highlights the importance of MERIT40 for RAP80 mediated BRCA1 recruitment at DSB [34].The indispensable role of MERIT40 in BRCA1 complex integrity was further established when cells knockdown for MERIT40 has shown reduced ABRAXAS–BRCC36 interaction. MERIT40 is also essential for localization of BRCA1-complex at DNA damage site [34]. MERIT40 knockdown unravel the negative effect on the RAP80, BRCC36 and ABRAXAS complex irrespective of BRCA1. Conversely, RAP80 or BRCC36 depletion did not appear to have any influence on the endogenous MERIT40 level, showing MERIT40 has a greater prominence in BRCA1-complex stability than others [34]. Altogether, these assumptions leads the fact which support the vulnerability of BRCA1 associated protein involvement in DNA damage repair. BRCA1-complex plays foremost role in DNA repair, and effective progression of this process sequentially depends on every member.

In the present study, an attempt has been made to understand the functional aspects of the MERIT40 in BRCA1 complex stabilization. We have purified recombinant MERIT40, BRCA1-BRCT, RAP80 and ABRAXAS to establish their role in BRCA1 complex formation. To explore the possibility of crosstalk, a qualitative and quantitative interaction relationship was explored in detail among various members. MERIT40 was found to interact with ABRAXAS and BRCA1-BRCT while no bonding with RAP80 was observed. Our findings provide insights into the stability of MERIT40 and BRCA1 complex as well as the various interactions that maintain the complex integrity. These findings will be helpful in understanding the functional interplay of DNA repair proteins. It will further elucidate mechanisms that abrogate the synergy of the BRCA1 complex members, consequently its repair function.

2. Materials and methods

All the chemicals were analytical or molecular biology grade and purchased from Sigma–Aldrich, unless otherwise specified. For buffer preparation, double distilled, 0.44 μM filtered Milli-Q water (Millipore, USA) was used. Protein and buffer solutions were filtered and degassed before proceeding to experiment.

2.1. Gene cloning, protein expression and purification

RAP80 ranging from 1 to 405 amino acids, henceforth will referred as RAP80 (kind gift from J. Chen), was PCR amplified (Thermocycler, Bio-Rad) and sub-cloned into pGEX-kT (Kind gift from John A.A. Ladias) expression vector after double restriction digestion with BamH1 and EcoR1. For PCR amplification, primers (Sigma–Aldrich) having TEV protease site (E-N-L-Y-F-Q/S) were used, and ligation was done with T4 DNA ligase. Positive clones were selected on the basis of restriction digestion and DNA sequencing. Similar cloning procedure was followed for MERIT40 and BRCA1 BRCT. Different regions of ABRAXAS ranging from 6 to 268, 6 to 301 and 6 to 373 amino acids, henceforth will referred as ABRAXAS, was sub-cloned in pET28(a+)vector. BRCA1-BRCT (1653–1853) and MERIT40 was sub-cloned into pGEX-kT. For protein expression and purification, vector construct was transformed into *Escherichia coli* Rosetta (2DE3) cells (Novagen). Protein expression was carried out by inducing bulk culture at $O.D_{600}$ between 0.6 and 0.8 with 0.4 mM IPTG (Himedia, India) for 16 h at 24 °C. Culture was harvested and re-suspended in 10 mM HEPES buffer containing 300 mM NaCl, 5 mM BME, 0.1 mM EDTA pH 7.5 (HNBE buffer). Cells suspension was sonicated (Branson Sonifier) thrice with 1 min sonication and 1 min rest in ice at duty cycle of 50 and power 60 unit. Supernatent was collected after centrifugation of culture at 18,000 rpm for 45 min. Soluble protein was passed through the pre-equilibrated glutathione resin/Ni–NTA (Novagen) with HNBE buffer. Fusion protein was allowed to bind with the affinity column and thereafter washed with 10 column volumes of HNBE buffer to remove non-specifically bound proteins. Bound fusion protein was cleaved with TEV protease to elute native protein. Elution fractions were further concentrated and passed on FPLC (AKTA, GE) through the size exclusion chromatography (Superdex 200, GE) to acquire homogenous and pure protein. Purified protein was analyzed on SDS–PAGE.

2.2. Protein estimation

Purified protein was quantified with Bradford protein estimation protocol (Expedon) and Nanodrop (Thermo scientific) as per manufacturer instructions. For Bradford method, different dilutions of BSA were prepared and absorbance was recorded at 595 nm. Concentration of protein of interest was determined by interpolation of standard curve. The experiment was performed in three sets [35], [36,37] and [38,39].

2.3. Fluorescence spectroscopy

Chemical denaturation of protein of interest (2 μM) was carried out with urea as unfolding agent. Emission maxima of native and unfolded protein (with 8 M urea) was recorded using fluorescence spectrophotometer (Horiba, USA) [40]. Protein was excited at 280 nm and fluorescence emission spectra were collected from 310 to 400 nm range. The experiment was repeated three times.

2.4. Circular Dichroism spectroscopy

Secondary structure characteristic of protein was analyzed using Circular Dichroism (CD) polarimeter (Jasco J-810, Japan). Far-UV CD scan of protein (10 μM) was collected in the 200–240 nm wavelength range. 10 μM individual protein in 2.5 mM HEPES pH 7.5, 50 mM NaCl buffer at 10 °C was scanned for secondary structure characterization. Average blank corrected data of three independent scans was submitted to DichroWeb server to predict the secondary structure composition [41–45].

2.5. GST pull down assay

GST (control), GST-BRCT and GST-MERIT40 were expressed in bacterial system (as describe above) and pellets were resuspended in buffer (10 mM HEPES, 250 mM NaCl, 10 mM β-ME, 0.1 mM EDTA and 5% glycerol) having pH 7.5 followed by sonication. Soluble fraction was separated using centrifugation and incubated with glutathione resin. Bound protein(s) (0.1 mg/ml) was used to capture prey (his-tag-ABRAXAS for GST-MERIT40, his-tag-MERIT40 for GST-BRCT) (10 μg) and RAP80 (10 μg, for GST-MERIT40), and thereafter resin was washed with buffer and loaded on SDS–PAGE. Interacting partner was probed with anti-His antibody (for ABRAXAS and MERIT40) and anti-RAP80 antibody (Abcam, USA). The experiment was repeated three times.

2.6. Isothermal titration calorimeter

Interaction studies between MERIT40 and ABRAXAS, BRCT and RAP80 was performed using Isothermal Titration Calorimetry (MicroCal200, GE). All the samples were degasses thoroughly before setting up titration reaction. 25 μM BRCT was kept in cell and allowed to titrate with MERIT40 (250 μM) through repeated injection (2 μl each) at 25 °C with spacing of 2 s. Stirrer speed was constant at 1000 rpm and equilibration time was 4 s in between successive injections. 25 μM of RAP80 was kept in cell and allowed to interact with 250 μM MERIT40. Similarly, MERIT40 was taken as titrant (30 μM) and ABRAXAS (290 μM) as titer with the above listed parameters. Data fitting was done using in-built origin 5.0 software and binding affinity was determined. The experiment was repeated twice independently.

3. Results

MERIT40 is an indispensible partner of the BRCA1 and helps in complex stability. It perpetuates the level of RAP80 and ABRAXAS and facilitates recruitment of BRCA1 at the site of DNA damage, hence favors homologous recombination repair. With an objective to understand the role of MERIT40, we have studied structural and functional characterization of BRCA1 complex members. MERIT40, BRCA1-BRCT, RAP80 and ABRAXAS native proteins were purified and analyze on SDS–PAGE (Fig. 1A). Purified protein were gel extracted and subjected to MALDI-TOF mass spectrometry for identification [46,47]. MERIT40, BRCA1, RAP80 and ABRAXAS showed their correct identity in Expasy server (http://www.expasy.org/) through mascot analysis (Table 1). It illustrates the faithfulness of purified protein for further use in structural and functional analysis.

3.1. Structural characterization

Secondary and tertiary structural characteristics are the important benchmark for defining folded-ness and functional upshot of a protein(s). A well folded protein showed a 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 [48]. In a well folded conformation of 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 spectrum were recorded. Secondary structural component of purified protein was analyzed using DichroWeb server (http://dichroweb.cryst.bbk.ac.uk) [41–45]. 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 (Fig. 1B). 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%) (Fig. 1B). Tertiary structure of purified proteins were evaluated with respect to the position of Tyr and Trp residues. Emission spectrum of native and unfolded protein was monitored. Native MERIT40, RAP80, ABRAXAS and BRCA1 showed their emission maxima at 338, 340, 332 and 339 nm respectively, while undergoes a substantial red shift when unfolded (Fig. 1C). It indicates Trp and Tyr residue are buried in three dimensional hydrophobic environments [48]. Altogether, these results also deciphered a well folded state of MERIT40, RAP80, ABRAXAS and BRCA1.

3.2. Interaction analysis

Protein–protein interaction is immensely important to unravel the stability of protein complex at molecular level. BRCA1/BARD1 co-interdependency is an excellent notable example where BRCA1 cannot perform its regular functions in the absence of BARD1 [49]. ABRAXAS also interacts with BRCA1 BRCT through its sequences containing pS-X-X-F motif, and abrogation of this interaction cause severe DNA repair defect [22]. Interaction network in BRCA1-complex is highly crucial and alteration in even a single

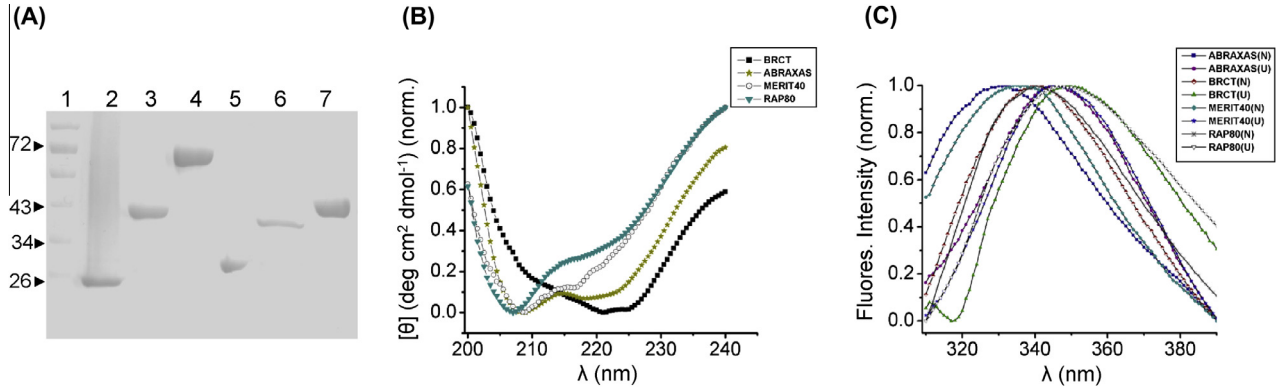

Fig. 1. Purification and structural characterization of ABRAXAS, BRCA1-BRCT, MERIT40 and RAP80. (A) Proteins were purified by affinity and gel filtration chromatography, and loaded on SDS–PAGE. Lane 1-marker, 2-BRCA1-BRCT, 3-MERIT40, 4-RAP80, 5-ABRAXAS (6–268), 6-ABRAXAS (6–301) and 7-ABRAXAS (6–373). (B) Overlay of far-UV CD spectrum and (C) emission maximum of BRCA1-complex proteins. N-native, U-unfolded with urea.

Table 1
Mass spectrometry profiles of MERIT40, BRCA1, ABRAXAS and RAP80 using Mascot analysis.

Protein	Match	Score	Nominal mass (M_r, Dalton)	Calculated pI	Sequence Coverage (%)
MERIT40	h-MERIT40	119	37,050	4.6	35.0
ABRAXAS	h-ABRAXAS	164	47,033	6.6	31.5
RAP80	h-RAP80	85	80,875	5.3	20.1
BRCA1	h-BRCA1	63	208,000	5.0	24.0

h: Human.

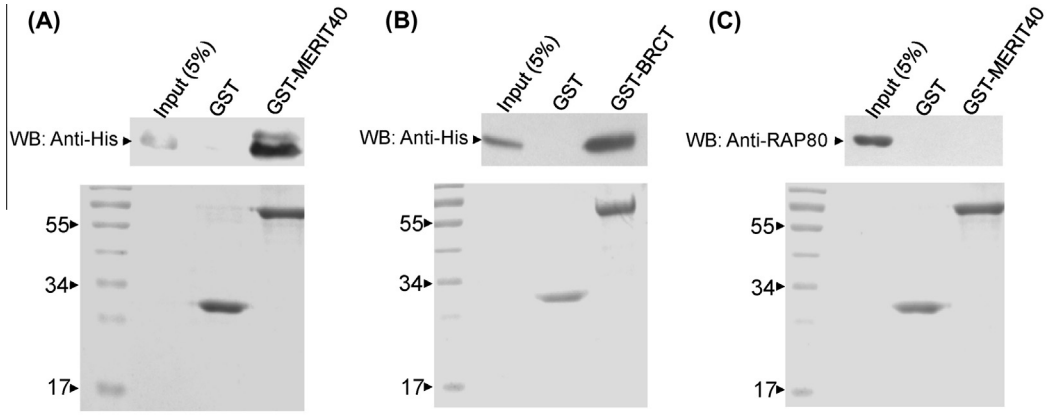

Fig. 2. Pull down assay to determined direct binding partner(s) of MERIT40. Protein–protein interaction analysis of MERIT40 with (A) ABRAXAS, (B) BRCA1-BRCT and (C) RAP80. Lower panel showing the loaded bait protein on SDS–PAGE stained with ponceau. In panel (A)–(C); GST-MERIT40, GST-BRCT and GST-MERIT40 were taken as bait and western blotting was performed against the his-ABRAXAS (prey), his-MERIT40 (prey) and RAP80 (prey), respectively.

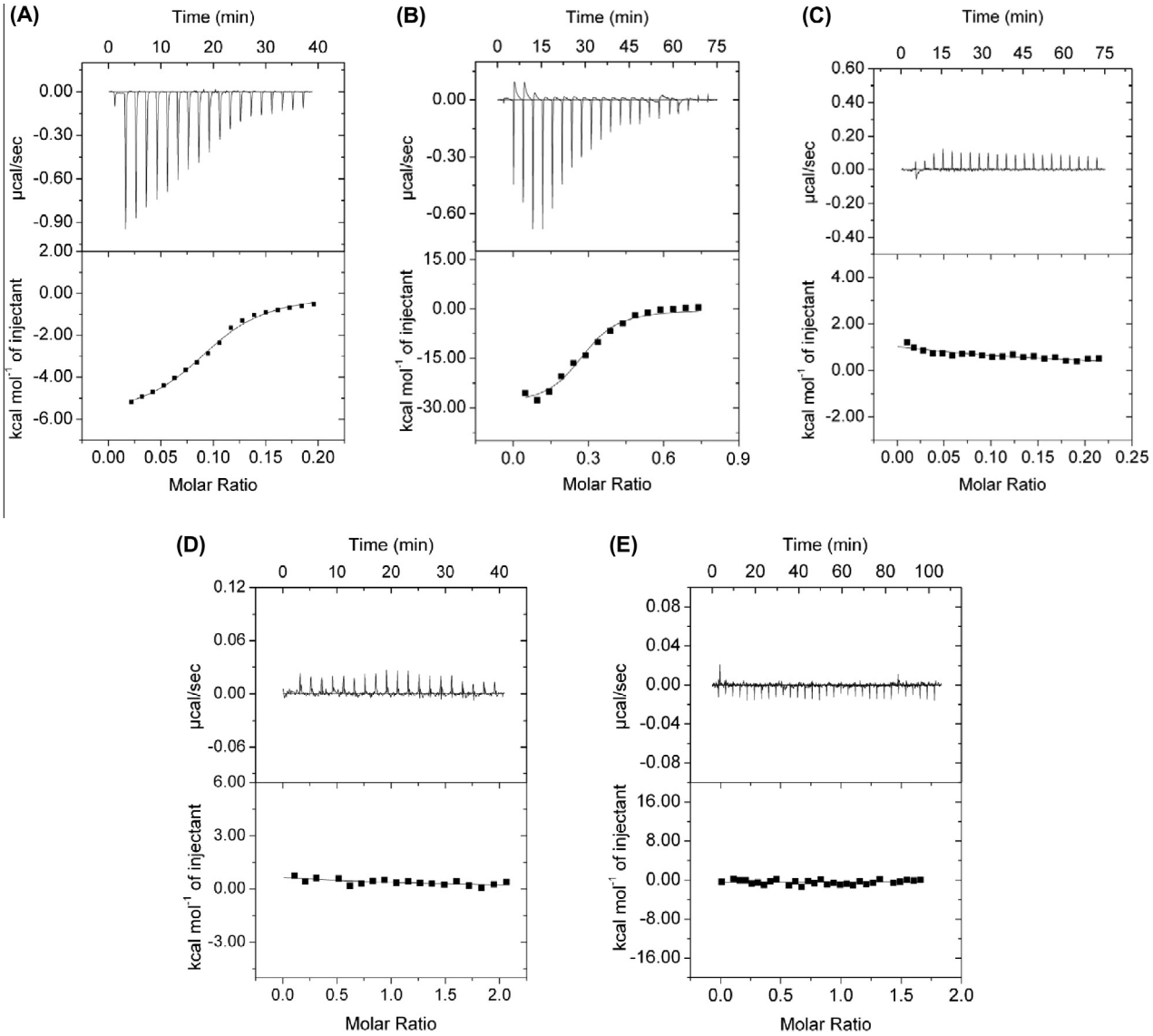

Fig. 3. Isothermal titration calorimetry of MERIT40 with BRCA1-complex members to determined binding interactions and affinity constants. Protein–protein interaction analysis of MERIT40 with (A) BRCA1-BRCT, (B) ABRAXAS (6–373), (C) ABRAXAS (6–301), (D) ABRAXAS (6–268) and (E) RAP80. The binding isotherm showed direct interaction of MERIT40 with ABRAXAS and BRCA1-BRCT.

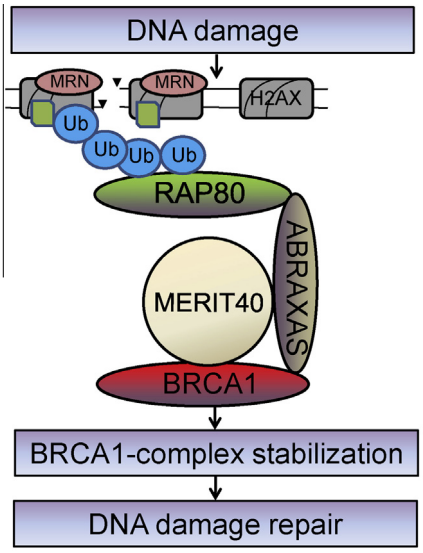

Fig. 4. Anticipated model of MERIT40 mediated BRCA1-complex stabilization. The representative illustrate a possible mechanism of complex stabilization through direct interaction of MERIT40 with BRCA1 and ABRAXAS. Ionization radiation induced DNA double strand break (pointed arrowhead) causes ATM/ATR kinase activation followed by assembly of various damage repair proteins at nucleosome complex. In the sustenance of MERIT40, the polyubiquitin chain(s) formed on H2AX are recognized by RAP80 thereby recruiting the intact BRCA1-complex to the DNA damage site.

amino acids of any of the member in the complex leads to destabilization of whole complex [34]. MERIT40 is a novel associate of this complex, however it remain elusive how MERIT40 establish a network among various member which maintain the BRCA1-complex integrity. To appraise this, we have carried out the interaction analysis of MERIT40 with RAP80, ABRAXAS and BRCA1 BRCT using pull down assay and Isothermal Titration Calorimetry.

A quantitative pull down interaction analysis has established the direct interaction between MERIT40 and BRCA1-BRCT, ABRAXAS (Fig. 2A, B). However, we have not observed significant binding between MERIT40 and RAP80, indicating their interaction is either absent or highly transient (Fig. 2C). To validate these findings, we have perform isothermal titration calorimetry among members of BRCA1 complex. The observed ITC isotherm of MERIT40 and BRCA1-BRCT interaction depict a low binding affinity (K_d = 9.8 ± 0.66 μM) but found somewhat cooperative (Fig. 3A). This further creates a possibility of MERIT40 existence in neighborhood of direct BRCT binding proteins, such as ABRAXAS. Interaction profile of MERIT40 and ABRAXAS (6–373) suggest that ABRAXAS C-terminal region involve in interaction with MERIT40 with a moderate binding affinity (K_d = 1.7 ± 0.28 μM) (Fig. 3B). To further narrow down the minimal binding domain of ABRAXAS with MERIT40, we have performed interaction analysis with shorter region (6–301, 6–268). Absence of interaction between MERIT40, and ABRAXAS (6–301) and (6–268) amino acids showed that probable binding domain of ABRAXAS resides are at the C-terminal regions only while N-terminal of ABRAXAS mainly interacts with RAP80 (Fig. 3C, D) [22]. Since RAP80 is an upstream player in BRCA1-complex, it would be interesting to observe the binding profile of RAP80 and MERIT40. Surprisingly, MERIT40 does not showed interaction with RAP80 (Fig. 3E). Collectively, these binding profiles suggest that MERIT40 showed direct interaction with BRCA1-BRCT as well as ABRAXAS.

4. Discussion

Interactive relationship of MERIT40 with ABRAXAS as well as BRCA1-BRCT mediates the formation of stable BRCA1 complex. However, MERIT40 does not exhibit consensus sequence pS-X-X-F motif as ABRAXAS, therefore, it might be interacting with BRCA1-BRCT through some other motif. Direct interaction of MERIT40 with ABRAXAS and BRCA1-BRCT categories it as a mediator molecule required for the stabilization of the BRCA1-complex. Knockdown of MERIT40 leads to reduced ABRAXAS foci as well as abrogating ABRAXAS–RAP80 interaction. However, BRCA1 level remain unaffected implying MERIT40 is required for integrity and stability of the BRCA1-complex at DNA damage site. Hence, it can be concluded that MERIT40 is the core mediator molecule and obligatory for organization of stable BRCA1-complex through binding with ABRAXAS and BRCA1-BRCT. Crosstalk between MERIT40 and ABRAXAS probably help in bridging the interactions among BRCA1-complex (Fig. 4). Our study will be helpful in better understanding the accomplishment of MERIT40 in BRCA1-complex and hence DNA damage repair. It will further divulge the molecular organization of this complex and novel associate require maintaining its integrity and stability.

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