Chapter 1

Introduction and Review of Literature
1.1 Cancer

Cancer is a deadly disease which arises due to abnormal cell division in the body. Instead of undergoing death, cancerous cells continue to grow and form new abnormal cells, which may undergo metastasis. Breast cancer is a malignant tumor that can invade the surrounding tissues or metastasize to other organ(s). Breast cancer can be classified into four categories, ductal carcinoma *in situ* (DCIS; also known as *intraductal carcinoma*), lobular carcinoma *in situ*, invasive (infiltrating) ductal carcinoma, and invasive (infiltrating) lobular carcinoma [22].

DCIS is the most common type of non-invasive breast cancer in which cancer cells remain in the milk ducts and do not spread through the walls into the surrounding breast tissues. Lobular carcinoma *in situ* is not considered as a true cancer. Invasive (or infiltrating) ductal carcinoma is the most common type of invasive breast cancer. It starts from a milk duct of the breast tissue and migrates through the walls of the duct, and further grows into the fatty tissue and metastasizes to other organs. Invasive lobular carcinoma (ILC) originates in the milk-producing glands (lobules) and undergoes metastasis.

It has been observed that ~ 5-10% of breast-cancer cases are hereditary in nature. The most common cause of the hereditary breast cancer is an inherited genetic alteration in the tumor suppressor genes *BRCA1* and *BRCA2* [23]. Both these genes appear to be equally responsible in the early onset of breast cancer [24-26]. The reported risk for breast and ovarian cancer is as high as 80% for members of families with *BRCA* mutations [1, 2]. *BRCA1* mutation(s) can be considered as autosomal dominant with high penetrance. However, such mutations behave recessively as per the concept of cancer
etiology [27]. The wild- type copy of brca1 gene is found to be lost in breast and ovarian cancer patients having chromosomal aberration [28, 29]. All these observations support the hypothesis that BRCA1 acts as a tumor suppressor gene.

1.2 DNA damage and genome conservation

Naturally, DNA damages happen about 10,000 to 100,000 times per day per mammalian cell [30]. DNA damage manifests as alterations in the molecular structure of DNA, such as a strand breakage or nucleotide base change. Several factors are responsible for DNA damage, including metabolic processes of the body and environmental radiations. The metabolic products such as reactive oxygen, nitrogen and carbonyl species, and lipid peroxidation products or alkylating agents can damage the DNA [31].

DNA damage can be repaired by various processes, out of which homologous recombination (HR) and non homologous end joining (NHEJ) assign a substantial role for BRCA1 [32]. Homologous recombination utilizes the coding information present on sister chromatids for accurate repair. However, NHEJ is an error-prone process because it does not consider sister chromatids to rejoin broken ends. Still, NHEJ is the major pathway of double strand break (DSB) repair in mammalian cells and happens throughout the cell- cycle. BRCA1 favours HR over NHEJ, and contributes in the NHEJ by assisting alignment of short regions of homology on either side of the DNA strand break. This process of damage repair is called as microhomology NHEJ [33, 34]. Cells prefer to take NHEJ pathway to repair their damaged DNA in the absence of BRCA1 gene, thus making the resultant repair product highly error-prone.

The task of conservation of genome is achieved by the recital action of cellular events, such as DNA replication, DNA repair, apoptosis and senescence. Most of these
processes are coordinated by the DNA-damage checkpoint, which is a complex signaling network triggered by genomic instability or DNA damage [35]. Two phospho-inositol 3-kinase-like protein kinases (PIKKs), ataxia telangiectasia mutated (ATM), and ATM- and Rad3-related (ATR), are the prime sensors of DNA damage, and act as master regulators of cell-cycle checkpoint [35]. ATM primarily responds to DNA double-strand breaks (DSBs), whereas ATR becomes activated to a much broader range of DNA damage, including DSBs [36]. The initial step in ATR activation is the recognition of single stranded DNA (ssDNA), and junctions between ssDNA and double-stranded DNA (dsDNA), which are induced by DNA damage. Although, the ATR activation at DSBs is highly dependent on ATM, it can also respond directly at interference of DNA replication [37, 38]. When ATM and ATR are activated, DSBs are recognized by MRN complex, which consists of meiotic recombination protein 11 (MRE11), RAD50 and Nijmegen breakage syndrome protein 1 (NBS1) [39]. The MRN11-RAD50-NBS1 (MRN) complex is crucial for the recruitment and stimulation of ATM at DSBs [40-42] [39]. ATM remains active throughout the cell-cycle, whereas, ATR acts often slowly and predominantly only during the S and G2 phases of the cell cycle. ATM generates long stretches of ssDNA adjacent to the breaks due to resection of DSBs by exo- and endonucleases [37]. The exo- and endonucleases involved in DSB resection include the EXO1 (an exonuclease), MRN complex (an exo- and endonuclease) and CtIP (an endonuclease and an activator of the exonuclease activity of MRN) [43] [44]. Proteins at ATR signaling cascade, including RAD17, RPA, ATRIP, 9-1-1 complex and TopBP1, get phosphorylated by ATR during checkpoint activation. Phosphorylation of ATR
substrates has a clear role in downstream processes such as cell-cycle arrest and DNA repair.

The well-known substrate of ATR is the checkpoint kinase 1 (Chk1); an effector kinase involves in the ATR mediated checkpoint pathway [45]. Chk1 is not only crucial for the stability of DNA replication forks but is also important for the checkpoint response during S phase. In response to DNA damage, the Chk1 is phosphorylated by ATR at multiple sites, which stimulates the kinase activity and releases Chk1 from chromatin. Furthermore, Chk1 phosphorylates substrates such as Cdc25A and Cdc25C which are involved in cell-cycle arrest at the G1/S and G2/M transitions, respectively. The phosphorylation of Chk1 by ATR is mediated by claspin which is a component of the DNA replication fork complex [46]. Claspin and Chk1 stabilize each other even in the absence of DNA damage [47, 48]. Claspin is phosphorylated by ATR in response to DNA damage, and endorses the phosphorylation and further activation of Chk1 [49, 50]. Claspin is degraded in the G2 and M phases of the cell cycle by the Skp1–Cullin1–F-box (SCF) ubiquitin ligase [51, 52]. It is degraded in G1 by the anaphase-promoting complex (APC) while under DNA damage condition, the degradation of claspin in G2 is disrupted [53]. In response to DNA damage, claspin can accumulate itself during the S and G2 phases to ensure efficient Chk1 activation during cell-cycle. The claspin-mediated phosphorylation of Chk1 describes a good example of phosphorylation regulation of ATR substrates at multiple levels.

BRCA1 is a substrate of ATM, ATR and CHK2 protein kinases, and plays an essential role in S/G2-M checkpoint control [37]. BRCA1 has RING domain at its N-terminal, DNA binding region in the centre and two BRCT repeats at the C-terminal (Figure 1.1).
Central domain(s) of BRCA1 exhibits DNA-binding activity; the region from (452-1079) amino acids bind to branched or cruciform DNA, whereas a region from (1293–1560) amino acids independently acts as a transcription regulator [18, 54]. The BRCT domain has been found in different proteins, including RAD9, AD4, 53BP1, Crb2, RAP1, BARD1 and MDC1. It possesses phosphopeptide-binding ability through which it functions in cell-cycle regulation, DNA-damage checkpoint control and DNA repair [10, 21]. A superficial pocket at the N-terminal of BRCT motif interacts with the phosphoserine (pSer) at (0) position of the ligand, whereas a hydrophobic pocket at the interface of the two BRCT repeats accommodates (+3) position phenylalanine residue. The three dimensional structural folding at the N- and C-terminal of the BRCT pocket is similar in the all phosphopeptides-BRCTs complexes, consistent with its unbiased binding to pSer (0) [55, 56]. However, the linker region at the interface is significantly different, which determines BRCTs binding specificity.

**Figure 1.1**: BRCA1 domain organization and its cellular binding proteins.
ATM/ATR kinases hyper-phosphorylate BRCA1 at different sites, and this leads to dissociation of BRCA1 from processive RNAPII complexes [57]. Phosphorylation of BRCA1 occurs at Ser 1387, Ser 1423 and Ser 1524 positions in the central domain. Mutation on BRCA1 Ser 1387 disrupts the S-phase checkpoint, whereas mutations at Ser1423 and Ser1524 block BRCA1-mediated cell cycle G2/M checkpoints [58]. Furthermore, BRCA1 phosphorylation by CHK2 at Ser 988 position is essential for HR activity [59], G2/M cell-cycle checkpoint activation and also for suppression of genotoxicity induced mammary and uterine cancers [60].

BRCA1 is a part of BASC (BRCA1-Associated genome Surveillance Complex) complex, which when phosphorylated by ATM/ATR kinases generate binding sites for BRCA1 associated factors, such as MRE11, RAD50 and NBS1 (MRN complex) [18]. MRN complex intervenes the G2/M cell-cycle checkpoint signal, and also plays a role in DSB repair by holding two DNA ends close to each other, which is required for non-homologous end-joining function (Figure 1.2) [61]. BRCA1 specifically interacts with processive polymerase II (IIo) and acts as a part of genome surveillance complex [57] [62].

Figure 1.2: Model depicts the BRCA1 mediated checkpoint regulation. BRCA1-containing complexes interact efficiently after DNA damage with TopBP1 and the M/R/N complex. Upon DNA damage, specific kinase signaling cascade promotes BRCA1 super-complex assembly (colored circles).
BRCA1 BRCT interacts with the C-terminal of p53 and stabilizes the complex. This stable complex is essential for stimulation of transcriptional activity, as demonstrated by an increase in p53-mediated activation of responsive promoters such as p21 waf1/ cip1 and bax. Interestingly, stabilization of p53 induces a subset of p53-regulated genes involved in cell cycle arrest and DNA repair but not in apoptosis [63, 64]. The DNA damaging agents were found to stabilize p53 and promote transcription of the number of genes, including apoptosis mediators. This demonstrates that BRCA1 is involved in p53-mediated growth arrest rather than programmed cell death. The p53 acts to down-regulate BRCA1 level in a negative feedback loop thereby controlling transcription activation and cell cycle [65].

BRCA1 is a nuclear protein which contains two SV40-like nuclear localization signals (NLS; 503-KRKRRP-508 and 606-PKKNRLRRKS-615) that facilitate its nuclear import [66, 67]. Translocation of BRCA1 to the nucleus is dependent on its association with the RING structure of BARD1. Binding of BARD1 to BRCA1 masks the nuclear export signal of BRCA1, thereby retaining it into the nucleus. BRCA1 and BARD1 heterodimeric complex are also required for localization of each other to DNA damaged induced foci [68] (Figure 1.3). Ubiquitylation is a regulatory mechanism of such a shuttling of proteins from nucleus to cytoplasm and vice versa, e.g. the nuclear export of p53 [69]. Ubiquitylation of BARD1-BRCA1 complex sends a signal for nuclear export rather than for degradation [70]. BRCA1-BARD1 complex also has the unique potential to catalyze formation of Lys6-linked polyubiquitin chain, which transduces signals other than those for protein degradation [71].
Figure 1.3: Mechanism of BRCA1 Nuclear Import/Export localization. BRCA1 can enter the nucleus with two different mechanisms: 1) via the importin receptor pathway mediated by the two NLSs, and 2) through its association with BARD1 RING domain.

BRCA1 regulates a large number of chromatin remodeling activities through its activation domain 1 (AD1) and the two C-terminal BRCA1 repeats (BRCTs). These remodeling activities do not require histone acetylation and intercede through the BRCA1-dependent recruitment of a cofactor of BRCA1 (COBRA1) [72]. The COBRA1 complex binds and down regulate the transcription activity of estrogen receptor-alpha (ER-α), thus contributing to BRCA1-mediated repression of ER-α activity [73].

ZBRK1 (zinc finger and KRAB domain protein) represses the Gadd45a gene transcription through interaction with a specific DNA sequence[74]. This repression function gets inactivated in response to DNA damage due to ubiquitin-proteasome dependent degradation of ZBRK. A BRCA1-dependent C-terminal repression domain is present in ZBRK1 that mediates the oligomerization of ZBRK1, leading BRCA1 binding to DNA (Figure 1.4, Figure 1.5) [75, 76]. BRCA1 central domain interacts with ZBRK1 and unmasks BRCA1-mediated transcriptional activation of Gadd45a and other DNA
damage inducible genes [77]. BRCA1 over-expression favors the BRCA1-dependent promoter activation of DNA damage players over the ZBRK1-mediated repression of downstream proteins [74]. BRCA1 interacts and regulates transcriptional and transforming activity of c-Myc, an oncoprotein found to be over-expressed in many cancer types. BRCA1 inhibits c-Myc mediated transactivation of telomerase reverse transcriptase (TERT) and telomerase enzymatic activity [78] [79].

**Figure 1.4:** BRCA1-mediated transcription regulation in response to DNA damage.

**Figure 1.5:** Transcription regulatory functions of BRCA1.
On DNA damage, cell sequesters the Cdc25C protein in the cytoplasm to prevent the activation of cyclinB–cdc2 kinase complex. Cdc25C is one of the targets of 14-3-3σ chaperone protein, and is transcriptionally regulated by BRCA1 [80]. Similar to 14-3-3σ, BRCA1 depleted cells could not elicit G2/M arrest in response to ionizing radiation (IR) [81]. BRCA1 also inhibits phosphorylation of cdc2 by upregulating the transcription of the wee-1 kinase leading to subsequent inhibition of cyclinB–cdc2 kinase [80].

BRCA1-BARD1 complex targets γ-tubulin for ubiquitination thereby regulating centrosome during cell division [55, 82]. Absence of ubiquitination due to mutation in γ-tubulin at the residues Lys-48 or Lys-344 leads to centrosome hyperactivity. BRCA1 restrains the association of γ-tubulin with centrosomes and inhibits microtubule nucleation function, thus making centrosomes quiescent [83]. BRCA1 prevents the hypertrophy and aneuploidy by acting as a member of the surveillance complex to maintain centrosome fidelity. In this case, BRCA1 acts as a sensor of microtubule disorganization caused by drugs, and promotes apoptosis through JNK activation (Figure 1.6). BRCA1 interacts with cdc20/Anaphase Promoting Complex which controls the transcription of MAD2 and Spindle Assembly Checkpoint [84].

1.3 DNA damage response (DDR) and BRCA1 Associated proteins

The moment DSB takes place, phosphorylation on the histone H2A variant, H2AX, occurs which results in its accumulation within chromatin at the sites of DSBs to form γ-H2AX foci [85, 86]. ATM and ATR mediated phosphorylation of histone H2AX at S139 position promotes the direct recruitment of MDC1 through MDC1-BRCT domains. This leads to the recruitment of a ubiquitin ligase RNF8/UBC13 to the damage sites [17, 87].
MDC1 then further interacts with γ-H2AX and recruits additional activated ATM to the sites of DSBs [88], resulting in polyubiquitin chain formation at DSBs. This positive feedback loop promotes further modification of H2AX and allows expansion of regions surrounding DSBs, thus providing binding sites for DNA damage and repair factors, including the MRN complex and BRCA1. The polyubiquitin chains are subsequently recognized by RAP80 in the BRCA1 complex, thereby facilitating the formation of BRCA1 foci at DSB [35]. BRCA1 further recruits downstream proteins and promotes homologous recombination repair.

BRCA1 interacts with a variety of molecules through its various functional domains and is potentially responsible for multiple functions in cell-cycle regulation, DNA damage repair and transcription [89]. For example, BRCA1 interacts with cellular proteins via their pSXXF motifs and forms three distinct complexes-BRCA1 A, BRCA1 B and BRCA1 C [90]. The unique proteins present in BRCA1 A, BRCA1 B and BRCA1 C complexes are ABRAXAS, BACH1 and CTIP, respectively. However, the binding motif
of each molecule occupies similar conformation at the phosphopeptide binding site of BRCA1 BRCT [16] [91]. Interestingly, it has been reported that doubly phosphorylated Abraxas binds to BRCA1 BRCT in a mutually exclusive manner of BACH1 and CTIP [55]. BRCA1-BRCA2-containing complex (BRCC36) is a member of the four-subunit of BRISC (BRCC36-isopeptidase complex) that contains ABRAXAS paralog, KIAA0157/Abro1 [92]. The BRISC complex contains three additional protein components RAP80, BRCC45 and MERIT40. In addition, the BRCA1-complex contains a five-member stoichiometric complex consisting of RAP80, ABRAXAS, BRCC36, BRCC45 and MERIT40 (Figure 1.7) [13].

**Figure 1.7**: BRCA1 complex and its recruitment at the site of DNA damage in response to IR.

The RING domain at the N-terminal of BRCA1 binds to the RING domain of BARD1, and together they form a heterodimeric E3 ubiquitin ligase complex. The C-terminal of BRCA1 binds to RAP80, ABRAXAS, MERIT40 and other DNA damage response proteins to accomplish an effective damage repair process at the site of DNA damage. Mutations which delete or disrupt the C-terminal BRCT domain have been shown to cause significant relocalization of the BRCA1 from nucleus to cytoplasm [93]. Most of
the cancer-causing missense mutations which destabilize the structure of BRCTs have been identified at the interface between the two BRCT repeats of BRCA1 [94].

1.3.1 BRCA1-Associated RING Domain (BARD1): The BRCA1-associated RING domain protein 1 (BARD1) is the obligatory binding partner of the tumor suppressor BRCA1 and plays a central role in the regulation of its cellular localization, stability and function [95, 96]. It has been reported that Bard1-null mice have shown a characteristic phenotype which is identical to Brca1-null animals, and cellular levels of BARD1 and BRCA1 are reciprocally regulated. Furthermore, BARD1 and BRCA1 are always found in close association during various processes such as DNA damage repair [97]. BARD1 is a putative tumor suppressor, and is found to be mutated in a subset of breast and ovarian cancer patients [98]. Human BARD1 comprises different functional domains, including RING finger (46-90 residues) at the N-terminal, three ankyrin repeats (420-525 residues) at the central domain, and two tandem BRCT domains (568-777 residues) at the C-terminal (Figure 1.7). The RING domain of BARD1 belongs to the class of zinc finger domains having ubiquitin ligase activity[99, 100]. BARD1 exists as a heterodimer with BRCA1, which is important for cell viability, and their absence results in accumulation of chromosomal aberration and early embryonic death (Figure 1.7, 1.8) [97]. BRCA1-BARD1 heterodimeric complex is stabilized by four-helix bundle of α-helices found at the edge of RING domains of both polypeptides [101]. Ankyrin repeats comprise of a helix-turn-helix and a beta-hair-pin which together staking in multiple numbers from loops that protrude from one face of the structure to constitute the protein interaction domain.
BARD1 translocates between nucleus and cytoplasm; however, an optional localization occurs during apoptosis. BRCA1/BARD1 complex can undergo auto ubiquitination, as well as catalyze Lys6-linked polyubiquitin chain formations, which send other than degradation signal to its substrate [71]. However, BRCA1-BARD1 ubiquitinate RNAP II which targets it for proteasome-intervene degradation, and further inhibition of transcription and RNA processing in response to DNA damage [102].

BARD1 coordinates the proapoptotic stress signals and p53-dependent apoptosis, and its depletion annihilates p53 upregulation and apoptotic response to stress, further leads to genomic instability and a premalignant tumor [103].

**Figure 1.8:** Functional domains of BARD1 and its associated binding proteins.

BARD1 performs few cellular functions without BRCA1 coordination. In the testis, BARD1 was found in premeiotic cells to regulate apoptosis in the absence of BRCA1 [104]. BARD1 and BRCA1 expressions have been differentially regulated in an ovulatory cycle in case of breast, ovarian and uterine tissue [95]. BARD1 expression was found to be upregulated from dioestrus to postoestrus, whereas reverse expressions were found in BRCA1 from oestrus to postoestrus [105]. BARD1 expression is also associated with
apoptosis as seen in hypoxia induced by artery blockage, which suggests differential expression of BARD1 as compared to BRCA1 in the tissues undergoing apoptosis. Furthermore, overexpression of exogenous BARD1 leads to apoptosis that is associated with p53 stabilization and activation of caspase 3. BARD1 performs p53 dependent apoptosis function, which is inhibited by BRCA1 in p53 or BRCA1-deficient cell lines [103].

1.3.2 BRCA1/BRCA2-Containing Complex 36 (BRCC36): BRCC36 is a member of BRCC multiprotein complex, and is found to be associated with BRCA1 and BRCA2 (Figure 1.7). It plays an important role in the regulation of the ubiquitin E3 ligase activity of BRCC complex. The BRCC36 gene is located at the Xq28 locus, a chromosomal break point in patients with prolymphocytic T cell leukemia. BRCC is considered as E3 ligase complex and performs E2 mediated ubiquitination of the tumor suppressor p53 [106].

BRCC36 shares sequence homology with the human Poh1/Pad1 and Jab1 which are the subunits of 26S proteasome and COP9 signalosome [107]. Cancer-associated mutations of BRCA1 disrupt the interactions with BRCC36 [107]. Moreover, the four-subunit of BRCC complex (BRCA1/BARD1/BRCC45/BRCC36) has enhanced E3 ligase activity compared to the two subunit BRCA1/BARD1 complex. Furthermore, anomalous expression of BRCC36 was observed in the majority of breast cancer cell lines and invasive ductal carcinomas [107].

1.3.3 Receptor Associated Protein 80 (RAP80): RAP80 was originally identified as a protein associated with retinoid receptor (RTR) which plays an important role in embryonic development as well as in the adult [108]. RTR expresses in embryonic stem
cells and is regulated during differentiation of embryonal carcinoma and embryonic stem cells [109]. RAP80 mRNA is expressed most abundantly in testis, and encodes a 79.6-kDa (719 amino acids) nuclear protein [108]. RAP80 comprises different functional domains, including Cys-X2-Cys-X11-His-X3-Cys zinc fingers near the C-terminal and UIMs (ubiquitin-interacting motif) at its N-terminal. UIMs are typically found in proteins with roles in (de)ubiquitination, endocytosis, transcription, replication and DNA repair, and are also known to bind ubiquitin [110]. The UIMs bind to K63-linked ubiquitin chain of H2AX, thereby recruiting the BRCA1 complex to the DNA damage site (Figure 1.7) [16, 111]. RAP80 acts upstream of BRCA1 and ABRAXAS and regulates localization and function(s) of BRCA1 after DNA Damage [112]. RAP80 deficient mice were found cancer prone and showed hypersensitivity to ionizing radiation [113].

RAP80 is found to be involved in DSB repair and cell cycle checkpoint control in response to IR exposure [111]. The reported crystal structure of the mouse RAP80-UIM1-UIM2 complex with K63-linked di-ubiquitin (PDB ID: 3A1Q) has a continuous 60Å long α-helical structure for the two UIMs. Individually, the UIMs are weak binders, but together they generate higher binding affinity through an avidity based mechanism. The linker region that joins the two UIMs determines the selectivity for the K63-linked chains [114]. The inter-UIMs region forms a 12Å-long α-helix and is an important determinant of specific binding with Lys 63-linked di ubiquitin. The binding affinity between UIMs and mono- ubiquitin varies from (Kd) ~0.1–2mM. However, the observed strong interactions between UIMs and Lys 63-linked polyubiquitin substrates is probably accomplished by co-operative binding among multiple UIMs. The binding of first UIM to one ubiquitin makes possible for the second UIM to adopt a conformation favorable for
interactions with a nearby ubiquitin in the chain through an avidity-based mechanism [114].

1.3.4 Coiled-Coil Domain Containing Protein 98 (CCDC98): CCDC98 is a 45 kDa nuclear protein which plays an important role in DNA damage repair mechanism. It is also called as ABRAXAS because of 39% sequence identity with a protein KIAA0157. Recent finding has also predicted that ABRAXAS directly binds to BRCA1 BRCT repeats through its pSer-X-X-Phe motif to the mutual exclusion of earlier reported binding partners like BACH1 (BRCA1-associated C-terminal helicase) and CtIP(CtBP-interacting protein) [115]. ABRAXAS acts downstream to RAP80 but upstream of BRCA1 in the DNA damage response pathway [7]. ABRAXAS acts as a bridging molecule that mediates the interactions for both RAP80 and BRCC36 with BRCA1 (Figure 1.7). ABRAXAS binds to RAP80 through the N-terminal domain and to BRCC36 through its coiled-coil domain. Downregulation of ABRAXAS or RAP80 in cells displayed hypersensitivity to UV and IR (Ionization radiation), G2-M cell cycle checkpoint defects and defective homologous recombination (HR) repair [7].

1.3.5 Mediator of RAP80 Interaction and Targetting 40 (MERIT40): MERIT40 (C19orf62) is the recently discovered member of BRCA1- complex [19]. The putative C19orf62 gene product consists of 329 residues (36.5 kD), although it migrates as a 40 kDa protein on SDS-PAGE. So far, no known functional domains of MERIT40 are reported. However, the central region (93–320) is highly conserved from sea anemone and Arabidopsis to human, and is surrounded by acidic residues [19]. MERIT40 has associations with RAP80, ABRAXAS, BRCC45, BRCC36 and BRCA1 as assessed by
coomassie staining of the purified complex [13]. MERIT40 is an integral member of the BRCA1 complex and mediates BRCA1 DSB recruitment through RAP80. IR induced foci formation of ABRAXAS and BRCC36 was also strongly reduced in MERIT40 downregulated cells, suggesting that MERIT40 mediates interaction of ABRAXAS to BRCC36 and RAP80 (Figure 1.7). MERIT40 knockdown reduces protein levels of ABRAXAS and BRCC36 as well as RAP80, thereby suggesting that MERIT40 is essential for the stability of BRCA1–RAP80 complex. The reported cancer-causing mutation of BRCA1 M1775R disrupts binding of MERIT40 with BRCA1 and further disrupts DSB localization of BRCA1 to IR-induced foci (IRIF) [116]. MERIT40 largely influences RAP80 stability and DSB localization. Down-regulation of MERIT40 results in loss of RAP80-associated DUB activity compared to BRCC36 depletion. MERIT40 or RAP80 knockdown significantly increases the sensitivity to IR in BRCA1 mutated HCC1937 cells [13]. This least characterized protein, MERIT40, opens the vast perspective to understand the structural and functional mechanism of stability of BRCA1 complex. It would be interesting to look whether it could be the main determinant of BRCA1 complex stability through its binding partners.

1.3.6 BRCA1/BRCA2-Containing Complex (BRCC45): BRCC45 acts as an adapter in the BRCA1-A complex and bridges the interactions between MERIT40 and the other members of the complex (Figure 1.7). It modulates the E3 ubiquitin ligase activity of the BRCA1-BARD1 heterodimer. It is also the component of BRISC complex that specifically cleaves 'Lys-63'-linked ubiquitin. It plays a role in cellular homeostasis and differentiation, and acts as a death receptor-associated anti-apoptotic protein, which
inhibits the mitochondrial apoptotic signaling pathway [107]. It regulates TNF-alpha signaling either directly or indirectly via its interactions with TNFRSF1A [107].

1.4 Ubiquitination and its role in cell signaling

Ubiquitin is a 76 kDa molecule and comprises seven lysine residues involved in lysine specific linkage during chain assembly [117, 118]. Most of the proteins undergo degradation by K48-linked chains in which second ubiquitin forms the isopeptide bond with the previous one [119]. These proteins undergo degradation because of specific recognition of K48 isopeptide linkage by 26 S proteasomes [120]. However, K63-linked chain present non-proteolytic signals that are involved in protein trafficking. [121].

Ubiquitination is a sequential process which involves at least three classes of enzymes: the E1 (ubiquitin-activating enzyme), an E2 (ubiquitin-conjugating enzyme) and an E3 (ubiquitin ligase). At the last stage of this process, an ubiquitin molecule get attached covalently to a Lys residue of the target substrate protein by an isopeptide bond. Monoubiquitinated substrate Lys residue then serves as a target for E3 to assemble next ubiquitin molecule and thereby forming polyubiquitin chain. Eucaryotes produce various types of E3 to ensure the specificity of ubiquitination [117]. E1 forms a thioester linkage between their active site cysteine and the C-terminus of ubiquitin through ATP molecule. The ubiquitin molecule is then transferred to the active site cysteine of E2 (Figure 1.9) e.g. In HECT family, ubiquitin is transferred from E2 to cysteine residue of HECT domain of the E3 before being attached to a substrate. Most E3s with the help of their RING domain or a structurally related Ubox bring a substrate and a charged E2 together, thereby activating the E2 to ligate ubiquitin molecule to a Lys in the substrate [122]. Ubiquitin generally attaches to the ε-amino group of Lys in substrates, and
Figure 1.9: E2 Catalytic mechanism for addition of ubiquitin. The model illustrates isopeptide bond formation during ubiquitin chain synthesis. Transfer of a single ubiquitin can occur to one (monoubiquitylation) or multiple (multimonoubiquitylation) sites to recruit binding partners; this promotes or inhibits interactions, protein localizations and activities (Figure 1.10) [123].

Figure 1.10: Various type of Lys residue specific linkage during (poly)ubiquitination and their fate in various cellular events.

One of the important functions of E2 is to ensure that it receives ubiquitin only and not the ubiquitin like modifiers (UBLs) on its active site (Figure 1.10). UBLs and ubiquitin are structurally similar, and the former can also be conjugated to the substrate Lys.
residues with the help of E1s, E2s and E3s. Both can compete for the same Lys residue in substrates but modification with UBLs usually amends protein activation or localization, e.g. monoubiquitylated proliferating cell nuclear antigen (PCNA) endorses translation, DNA repair, whereas blocking same lysine with the UBL small ubiquitin like modifier (SUMo) blocks recombination between sister chromatids [124].

\[\text{Figure 1.11: Importance of E2 in ubiquitylation. E2 interacts with the E1 that is preloaded with two ubiquitin molecules, one is Cys at its active site and other at adenylation domain as adenylate (~AMP). The activated ubiquitin is relocated to the Cys in the active site of E2. The E2 then dissociates from the E1 before it holds with E3, which further recruits substrates. After dissociation of E2 from E3, the former can be recycled for the next round of recharging with ubiquitin.}

Once E2 is charged with a ubiquitin molecule, it employs catalysis of substrate ubiquitylation mediated through E3s (**Figure 1.11**). E2 can act together with several kinds of E3s, and is most predominantly seen in UBE2D family members. E2s recognize its counterpart E3s through the L1 and L2 loops and the N-terminal α-helix present on E2 surface, and sequence variations in these motifs determine the specificity of E3 binding (e.g. E2 UBE2L3) [125]. BRCA1 also undergoes polyubiquitination that is
independent of its own enzymatic activity, which involves other E3 ubiquitin ligases [126]. However, this Ubiquitination enhances BRCA1/BARD1 stability rather than targeting it for degradation [127].

Different proteins that contain ubiquitin binding motifs such as the ubiquitin-associated (UBA) domains and ubiquitin-interacting motif (UIM) can recognize substrates modified with ubiquitin [128]. These ubiquitin binding proteins may preferentially unite with different monoubiquitin or a multiubiquitin chain species to bring effectors downstream signaling pathways and thereby couple ubiquitylation to the particular biological function. For example, RAP80 binds to K-63 linked polyubiquitin chain and perform the protein trafficking function in DNA damage repair, whereas those bind to Lys 48-linked chains go for degradation (γ-tubulin) [129].

1.5 Conclusion

BRCA1-complex contains a five-member stoichiometric complex consisting of RAP80, ABRAXAS, BRCC36, BRCC45 and MERIT40. Although, BRCA1 is the most studied gene, but least characterized for its association with cellular binding partners. It remains elusive how a downstream acting protein, MERIT40 affects the RAP80 and CCDC98 protein’s levels. What are the binding partners associated with MERIT40 that are present in BRCA1 complex, through which it may extend its interaction network and maintain genomic stability of BRCA1-complex? Furthermore, the interacting regions of RAP80 and ABRAXAS are not well defined at the atomic level. Structural and functional mechanisms of disease susceptibility due to RAP80 and ABRAXAS mutations remain imprecise. These ambiguities inspired us to explore the structural aspects of wild type and mutant protein, and interaction analysis of BRCA1-complex members. Our findings
suggest that MERIT40 directly interacts with BRCA1 and ABRAXAS, thus helping in the stabilization of BRCA1-complex. These studies will help in elucidating the mechanism of repair defects, and their remuneration for structure based drug lead discovery.