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The Role of BRCA1 Domains and Motifs in Tumor Suppression

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The Role of BRCA1 Domains and Motifs in Tumor Suppression

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
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DEDICATION

To the memory of my grandfathers, Petar Rangelov and Jordan Velkov, and to my

family.

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LIST OF ABREVIATIONS

ATM	Ataxia Talangiectasia Mutated
ATR	Ataxia Talangiectasia Mutated and Rad3 Related
ATRIP	ATR Iteracting Protein
BACH1	BRCA1 associated C-terminal Helicase 1
BARD1	BRCA1 Associated RING Domain One Gene
BRCA1	Breast and Ovarian Cancer Susceptibility Gene One
BRCA2	Breast and Ovarian Cancer Susceptibility Gene Two
BRCT	BRCA1 Carboxy Terminal Domain
CDK	Cyclin Dependent Kinase
CHK1	Checkpoint Kinase One Gene
CHK2	Checkpoint Kinase Two Gene
CPT	Campthothecin
DDR	DNA Damage Response
DNA-PKcs	DNA Dependent Protein Kinase Catalytic Subunit
DSB	Double Strand Breaks
FHA	Forkhead Associated Domain
FLNA	Filamin A
GADD45a	Growth Arrest and DNA Damage Inducible 45 alpha

HR	Homologous Recombination
HU	Hydroxyurea
IP	Immunoprecipitation
IR	Ionizing Radiation
LOH	Loss of Heterozygosity
MRE11	Meiotic Recombination 11 Homologue
MRN	MRE11, RAD50, NBS1
NBS1	Nijmegen Breakage Syndrome One
NHEJ	Non-Homologous End Joining
NLS	Nuclear Localization Sequence
Rap80	Receptor Associated Protein 80
RDS	Radioresistant DNA Synthesis
RING	Really Interesting New Gene Domain
RPA	Replication Protein A
ssDNA	Single Stranded DNA
SMC1	Structural Maintenance of Chromosomal Protein 1
UV	Ultraviolet
VUS	Variant of Uncertain Significance
WB	Western Blot
WHO	World Health Organization
9-1-1 complex	Rad9-Rad1-Hus1

ABSTRACT

Individuals that carry deleterious mutations in the breast and ovarian cancer susceptibility gene 1 (*BRCA1*) have much more elevated risk to develop breast and/or ovarian cancer than the individuals from the general population. The *BRCA1* gene product has been implicated in several aspects of the DNA damage response, but its biochemical function in these processes has remained elusive. In order to probe BRCA1 functions we conducted a yeast two-hybrid screening to identify interacting partners to a conserved motif (Motif 6) in the central region of BRCA1. In this dissertation, we report the identification of the actin-binding protein Filamin A (FLNA) as a BRCA1 partner and demonstrate that FLNA is required for the efficient regulation of DNA repair process at its early stages. Cells lacking FLNA display a diminished ionizing radiation (IR)-induced BRCA1 focus formation and a slow kinetics of Rad51 focus formation. In addition, our data demonstrate that FLNA is required to stabilize the interaction between DNA-PK holoenzyme components such as DNA-PKcs and Ku86 in a BRCA1-independent manner. Our data are consistent with a model in which the absence of FLNA compromises homologous recombination and non-homologous end joining. Our findings have implications for our understanding of the response to irradiation-induced DNA damage.

INTRODUCTION

BRCA1 and Cancer Predisposition

Hereditary Predisposition to Breast and Ovarian Cancer

According to the American Cancer Society, 207,090 women and 1,970 men were diagnosed with breast cancer in 2010 in the US alone. Moreover, breast cancer is among the most frequent malignancies affecting women worldwide. Germline mutations in the breast and ovarian cancer predisposition gene 1 *(BRCA1)* are responsible for the majority of early-onset breast and ovarian cancers arising in families with multiple cases. Furthermore, it has been shown that mutations in *BRCA1* and *BRCA2* genes occur with frequency of 1:250, which translates into 250 000 women in the US who are carriers [4]. Therefore, it is crucial to identify individuals at risk early. These individuals and their families have the options of increased surveillance, chemoprevention, or prophylactic surgeries, all of which can potentially reduce the possibility of developing cancer.

The cloning of *BRCA1* made early identification of individuals at risk possible by genetic testing. However, there are many difficulties that leave around 10% of the women who undergo testing with uninformative results due to the finding of *BRCA1* variants for

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which the cancer association is not known. These variants are called variants of uncertain significance (VUSs). Moreover, in minority populations the percentage of women, receiving uninformative results is significantly higher (35-50%) making it a health disparity issue that needs to be addressed. These women do not have all the information to make informed clinical decisions. In addition, *BRCA1* is inactivated in some sporadic breast cancer cases, suggesting an important role for *BRCA1* in breast cancer in general.

As far as its presentation in families, breast and ovarian cancer can be considered as hereditary, familial, or sporadic. Ascertainment criteria for **hereditary** (inherited) breast and/or ovarian cancer syndrome often include but are not limited to: early age of cancer onset, multiple affected individuals within a family, development of bilateral disease, and male individuals with breast cancer. Hereditary breast and ovarian cancer cases have strong family history for the disease and can often be attributed to mutations in genes such as *BRCA1* and *BRCA2*. In **familial** cancer, there is a pattern in the family, pointing a hereditary component. However, these cases have not been ascribed to mutations in any of the known genes. **Sporadic** cancer can be defined as a type of cancer, which arises in individuals that usually do not have family history, and the disease has not been attributed to any known genetic factor. However, we should point out that this classification is arbitrary and is often difficult to make a clear-cut distinction between different classes.

In this thesis, we focus on *BRCA1* syndrome, which is defined as an "inherited tumor syndrome with autosomal dominant trait and markedly increased susceptibility to breast and ovarian tumors, due to germ line mutations in the *BRCA1* gene. Additional

organ sites include colon, liver, endometrium, cervix, fallopian tube and peritoneum" [5].

It is estimated that 5-10% of all breast cancer cases are hereditary (Figure 1).

Approximately 30-45% of the hereditary cases can be attributed to *BRCA1* and *BRCA2*

mutations [6].

According to our current understanding, hereditary breast cancer can be assigned

to three groups of susceptibility alleles: genes that have high (*e.g. BRCA1* and *BRCA2*),

moderate (*e.g. ATM, BRIP1, CKEK2,* and *PALB2*), and low penetrance alleles (*e.g.*

FGFR2, *TNRC9*, and *MAP3K1*) [7].

Early Findings in Hereditary Breast and Ovarian Cancer

The first report about hereditary breast cancer dates back to 1866. It was made by

the French surgeon Paul Broca [8] who reported that in five generations of his wife's

family 10, out of the 24 women died from breast cancer [8]. Another important discovery

in breast cancer research happened in 1950, when Gardner and Stephens examined 668

individuals from a Utah family. Their findings showed that the frequency of breast cancer

was 20 times higher in that family than in the general population of Utah over the age of

30 [9]. The researchers concluded that "the heredity is a predisposition for breast cancer,

but the nature of the inheritance is not known" [9].

The "nature of inheritance" became the focus of subsequent studies on breast

cancer. These studies explored the role of environment and family history in breast
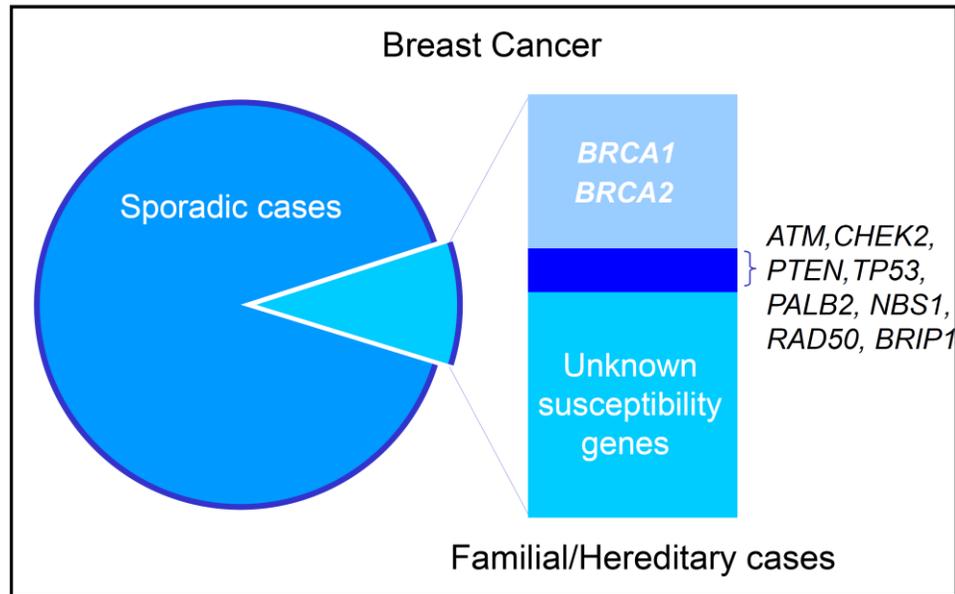
Figure 1. Breast cancer susceptibility genes. Hereditary breast cancer accounts for 5-10% of all breast cancer cases. *BRCA1* and *BRCA2* are responsible for approximately 30-45% of hereditary breast cancer cases. Mutations in *ATM*, *CHK2*, *PTEN*, *PALB2, RAD50, NBS1, BRIP1,* and *TP53* explain some of the remaining cases. The rest of the hereditary cases have not been attributed to the any known susceptibility genes. (Adapted from [1]).

cancer predisposition. Therefore, Lynch and colleagues decided to study 34 families, purposefully selected because they had two or more members affected with breast cancer. Their findings showed that in some of those families the significant predisposition was not only for breast cancer, but also for other malignancies [8]. They concluded that multiple factors including interactions of different genetic and environmental factors could contribute to cancer predisposition [8].

Further, Newman et. al analyzed data from 1579 families with multiple breast cancer cases. Based on this analysis, the researchers offered a model for the explanation of the breast cancer frequent occurrence in the participants' families [10]. According to that model, inherited breast cancer predisposition was caused by an autosomal dominant allele with high but incomplete penetrance [10]. That study was followed by mapping the gene responsible for early onset breast cancer to chromosome 17q21 [11, 12]. Finally, the seminal discovery on hereditary breast and ovarian cancer happened in 1994, when the first breast and ovarian cancer susceptibility gene *BRCA1* was cloned [13].

Breast and Ovarian Cancer Risk Associated with BRCA1

Germline mutations in *BRCA1* are responsible for 30-45% of all hereditary breast cancer cases and around 80% of the cases that have multiple breast and ovarian cancers [14]. Therefore, *BRCA1* is the major locus responsible for the breast and ovarian cancer predisposition. The lifetime risks for developing breast and ovarian cancer in the general population are estimated to be 12.7% and 1.3% respectively (American Cancer Society

2010**).** Meanwhile, women who carry deleterious mutations in *BRCA1* have the lifetime

risk of developing breast cancer of 36-82% and 16-49% for ovarian cancer (Table 1). The

wide range of those risk estimates can be explained by differences in the study design,

populations, and the age limit used to calculate lifetime risk (Table 1). Nevertheless, the

risk for women who are carriers of deleterious mutations in *BRCA1* to develop breast

and/or ovarian cancer is five to ten times higher than the risk for the women in the

general population. Finally, deleterious mutations in *BRCA1* are accepted to be the best-

known predictor for breast cancer risk [15, 16].

Table 1. Lifetime risk for breast and ovarian cancer among carriers of *BRCA1* mutations.

Breast cancer risk %	Ovarian cancer risk %	Additional information	Refs
65	39	Combines 22 studies	[17]
69	49	AJ (Ashkenazi Jewish)	[18]
43-67	14-33	AJ	[19]
36		AJ	[20]
82		Selection for strong family history	[14]
	37	AJ	[21]
68	36	Patients diagnosed with ovarian cancer in Ontario, Canada 1995-96	[22]
46		AJ	[23]
56	16	AJ	[24]
59.9		AJ, unselected for family history	[25]
72.8	40.7	Risk, estimated up to the age of 70	[26]

In contrary, in sporadic breast and ovarian cancer cases the situation is different: somatic *BRCA1* mutations are rare [27, 28] but epigenetic inactivation by promoter hypermethylation [29-31] or decreased expression of *BRCA1* [32, 33] have been found in sporadic tumors. This suggests that *BRCA1* may also play a role in sporadic breast and ovarian cancer development.

Contribution of BRCA1 to Breast and Ovarian Cancer

Breast cancer is a heterogeneous disease. Based on gene expression patterns, it can be classified into five distinct groups: luminal A and B, normal breast like, ERBB2, and basal-like breast carcinomas [34]. The majority of *BRCA1*-related breast tumors can be classified as basal-like type breast carcinomas [34, 35]. The analysis of gene expression profiles of *BRCA1*-related breast tumors led to the conclusion that they are more likely to be estrogen receptor alpha negative, progesterone receptor negative, and Her2/neu negative than the sporadic ones [34-37] (also referred as triple-negative breast cancer). Moreover, mutations in *TP53* are more frequent in *BRCA1*-related breast tumors than in the sporadic ones [38, 39]. Strikingly, triple negative breast cancer occurs more frequently in pre-menopausal African-American women when compared to the age-matched female white Americans [40]. Finally, triple negative breast cancer in African-Americans phenotypically share the same characteristics as *BRCA1*-related breast cancer [40].

Similar to the studies on the breast cancer, attempts have been made to classify ovarian tumors using a gene expression profile [41]. Unfortunately, the sample size of these studies was small. Therefore, they did not have sufficient statistical power, which precluded the classification of ovarian cancer. Based on histology, over 90% of *BRCA1*-linked ovarian tumors are serous adenocarcinomas [42].

BRCA1 Is a Tumor Suppressor Gene

Inheritance of one defective *BRCA1* copy is enough to cause cancer predisposition, but in order for cancer to develop, the remaining wild type allele needs to be inactivated [43]. Therefore, *BRCA1* fulfills the classic "two-hit hypothesis" definition for the tumor suppressor gene: both alleles are inactivated in cancer [44]. In addition, mouse models support the fact that *BRCA1* is a tumor suppressor. Although inactivation of both *Brca1* alleles causes embryonic lethality [45-48], specific inactivation of *Brca1* in mammary gland cells leads to the tumor development [49-52]. Interestingly, unlike heterozygous women, heterozygous mice did not develop tumors [51]. However, mammary tumors resembling the basal-like breast tumors of *BRCA1* mutation carriers were detected after conditional inactivation of *Brca1* in the mammary glands [50, 51, 53]. A summary of *Brca1*-related mouse models and their phenotypes is shown on Table 2.

In contrast to conditional mouse models developed to study the role of *BRCA1* in breast cancer, there are very few mouse models on the ovarian cancer progression [54-56]. In the first mouse model for *Brca1*-linked ovarian cancer, *Brca1* was conditionally

inactivated in the ovarian granulosa cells [54]. The mice developed cystic tumors, which
are thought to precede ovarian cancer [54]. Such tumors carried normal *Brca1* alleles,
which indicated a paracrine mechanism in ovarian cancer development-*Brca1* influences
tumor development in the ovarian surface epithelium indirectly through granulosa cells
[54].

Table 2. Mouse models for *BRCA1* deficiency (adapted from [57])

Allele	Phenotype	Cre-transgene	Refs
Brca1[ex2]	death at e7.5		[47]
Brca1 [5-6]	death at e7.5		[45]
Brca1 [11-]	death at e7.5-9.5		[58]
Brca1 [Δ223-763]	death at e10-13.5		[48]
Brca1 [ko]	death at e7.5-8.5		[46]
Brca1 [S971A]	none		[52]
Brca1[Tr]	lymphomas, sarcomas, breast cancer		[59]
Brca1 [1700T]	death at e10.5		[60]
Brca1 [F5-6]	thymocyte specific	Lck-Cre	[61]
Brca1 [Co/]	death at e12.5		[49]
Brca1 [Δ11]	lymphoma, ovarian tumors		[62]
Brca1[F11/F11;p53+/-]	mammary tumors	MMTV-Cre or WAP-Cre	[63]
Brca1[F11/F11;p53F5-6/p53F5-6]	mammary tumors	WAP-Cre[c]	[64]
Brca1[F22-24/F22-24;p53+/-]	basal-like breast cancer	BLG-Cre	[50]
Brca1[F5-13/F5-13;p53F2-10/F2-10]	basal-like breast cancer	K14-Cre	[53]
Brca1[F2/F2]	basal-like breast cancer	WAP-Cre	[51]

In the other mouse model, *Brca1* was inactivated in the ovarian surface epithelial cells, which resulted in pre-neoplastic changes, but not ovarian cancer [56]. In conclusion, it is not known how well these models represent the ovarian cancer in humans, and the role of *BRCA1* in the transformation of ovarian surface epithelial cells is unclear. Nevertheless, the *Brca1*-related mouse models support the evidence that *BRCA1* is a tumor suppressor gene and set the stage for studying the mechanisms by which BRCA1 suppresses tumor formation at the organism level.

Spectrum of Mutations in the BRCA1 Gene

The mutation spectrum of *BRCA1* varies in different populations. For example, genetically heterogeneous populations such as those in the United States, the United Kingdom, and Canada display big differences in documented *BRCA1* mutations (reviewed in [4] [6]). On the other hand, in geographically or historically isolated populations like Icelanders or Ashkenazi Jews, the majority of hereditary breast and ovarian cancer can be attributed to founder mutations [6]. Founder mutations are specific mutations that appear repeatedly in ethnically defined groups because of genetic drift [4]. For example, three founder mutations in *BRCA1* (185 delAG, HGVS nomenclature NC_000017.9:g.38529572_ g.38529571delAG and 5382 insC, NC_000017.9: g.38462606dupC) and *BRCA2* (6174 delT, NC_000013.9:c.24822delT) genes are responsible for most of breast and ovarian cancer cases in individuals from Ashkenazi Jewish descent [65]. Founder mutations in *BRCA1* have also been found in populations

from Germany, Norway, Poland, and Sweden [6]. The identification of founder mutations facilitates genetic testing for hereditary breast and ovarian cancer in the studied populations by decreasing the cost and the time of the test.

Genetic Testing for BRCA1 and the Variant of Uncertain Significance (VUS) Problem

The cloning of *BRCA1* and *BRCA2* made the early identification of individuals at risk possible by standard genetic testing [13] [66] [67], which is done by direct sequencing [68]. The results of genetic testing influence clinical decisions such as increased surveillance, prophylactic surgery, chemoprevention, and response to therapy (both radiation and chemotherapy). In the US, genetic testing for *BRCA1* and *BRCA2* is done by Myriad Genetics Laboratories and has three outcomes:

(1) Negative ("No mutation detected" or "Favor polymorphism"). In the case of *BRCA1*, this means that no cancer associated (deleterious) mutation was detected. This result is informative for the proband, only if a *BRCA1* mutation is found in other affected family members. In this case, it excludes the person that is undergoing testing. However, it is important to point out that a negative result can be due to changes that cannot be detected by direct sequencing such as large rearrangements that lead to deletion of whole exons and mutations in the regulatory regions [69]. For these cases, there is an additional test that can be applied, called BART (BRCAanalysis Rearrangement Test), which tests for a panel of rearrangements in *BRCA1* and *BRCA2* genes. Another possible reason for an individual with a family history of breast and ovarian cancer to get a negative result is

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that the family might have a mutation in other breast cancer predisposing genes such as *CHEK2* and *ATM* [69]. A recent study has revealed that even when first-degree relatives of women, carriers of *BRCA1* or *BRCA2* mutations, are tested negative for the same *BRCA1* and *BRCA2* mutations, those relatives are still at increased risk of cancer [70].

(2) Positive ("Positive for a deleterious mutation" or "Suspected deleterious"). In this case, the risks for the individual to develop breast and ovarian cancer are much higher than the risk for the general population (Table1).

(3) Uncertain ("Genetic change of uncertain significance"). This result means a genetic change in *BRCA1* for which the impact on protein function has not been determined. These genetic changes are called unclassified variants or genetic variants of uncertain clinical significance (VUSs). It is estimated that around 10% of Caucasians, 35% of Hispanics, and up to 50% of African Americans undergoing testing receive VUS results [71-73]. Considering over 1500 different alleles of *BRCA1* in the human population (Breast cancer information core http://research.nhgri.nih.gov/projects/bic as of 3.9.11) and the rarity of the individual alleles, one of the most challenging tasks for clinical genetics is to distinguish which are benign and which are cancer predisposing. These three outcomes are similar for *BRCA2* testing.

How to Determine the Association of a Genetic Variant with a Disease?

There are two methods that can be used for determining association of a genetic variant with a disease (cancer). The first method is defined as **genetic** i.e. testing co-

segregation between the variant and the disease in families, in which this variant has been found. The second method is **epidemiological** i.e. comparing the frequency of a given variant between two groups. These groups are cases (individuals from different families that have the disease) and controls [74]. In the case of *BRCA1* VUSs, both methods have limitations.

The genetic method cannot always be applied because usually the families are small, the number of genotyped family members is limited, and there is an uncertainty about which type of cancer (diagnosis, histology, ext.) a deceased or distant family members presented. The epidemiological method relies on relatively common alleles, but individual VUS are very rare in the population. To overcome this problem, several methods to help classify the VUSs have been proposed:

1. **Functional assays** to test the effect of amino acid change on the function of BRCA1:

1.1 **Transcription activation assay** [75, 76] and reviewed in [77]

BRCA1 C-terminus functions as an activator of transcription [78, 79] when expressed as a fusion with the heterologous DNA binding domain. Cancer predisposing mutations in this region of BRCA1 impair this transcriptional activity, whereas benign polymorphisms show similar activity as the wild type BRCA1 [80, 81]. This fact served as the basis for the development of the transcription activation assay, which can test the integrity of BRCT domains and its neighboring regions at the BRCA1 C terminus. This assay is limited to the C-terminal mutants of BRCA1.

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1.2 **Yeast small colony phenotype assay** [82, 83]

Yeast small colony phenotype is based on the premise that constructs, which contain the C terminus of BRCA1, inhibit the yeast growth when expressed. This assay is limited to the C terminus of BRCA1.

1.3 **E3-ubiquitin ligase activity assay** [84]

The BRCA1/BARD1 heterodimer displays an E3 ubiquitin ligase activity [85]. Therefore, functional assays have been developed to test BRCA1 variants for their ability to bind to BARD1 and UBCH5A (the E2 ubiquitin conjugating enzyme). In addition, such functional assays test BRCA1 in vitro E3 ligase activity. This assay is domain specific and can test variants only in the N terminus of BRCA1.

1.4 **Phosphopeptide binding assay**

Phosphopeptide binding assay evaluates the ability of BRCT domains to interact with a phosphopeptide from BACH1 protein. This assay is also limited to the BRCT domains of BRCA1 [86].

1.5 **Protease sensitivity assay**

Protease sensitivity assay tests for the stability of BRCT domains upon cleavage with protease. Some subtle deleterious changes in BRCA1 may not be detected when using this assay [86] .

1.6 **Yeast recombination assay**

Yeast recombination assay tests for BRCA1 C-terminus variants effect on the recombination between the yeast HIS3 and ADE2 loci. This type of assay is restricted to the BRCT domains [87].

1.7   **Human BRCA1 BAC reconstitution into mouse embryonic stem cells**, which have conditional *Brca1* allele, followed by functional tests for cell viability, DNA repair, and mammary gland carcinogenesis [88]. The same approach can be used to rescue embryonic lethality into nullizygous for the mouse Brca1 allele embryos [88].

2.   **Co-occurrence in trans with a deleterious mutation**. This method is based on the observation that some functions of BRCA1 are essential for early development. For example, homozygous truncating mutations in *BRCA1* are embryonic lethal in mice [45, 46]. Moreover, so far no patient has been reported having two deleterious mutations in *BRCA1* gene [89]. Thus, this method is based on the hypothesis that homozygosity for deleterious mutations is either embryonic lethal or lead to recognizable phenotype [90]. If a certain VUS of *BRCA1* co-occurs with a known deleterious mutation in one tested individual, most likely this VUS is a benign polymorphism.

3.   **Bioinformatics methods** is a structure based analysis, applied to generate computation prediction models [91, 92] and analysis of BRCA1 sequence conservation among different species and the amino acid characteristics [93-95].

All the methods mentioned above have their own assumptions, advantages, and disadvantages. Therefore, none of these methods should be used alone to determine the disease association of VUSs or as a clinical application. In addition, several attempts have been made to combine different sources of data to classify VUS [91, 95-97]. Even

after using multiple sources of data, the classification of VUSs is far from being directly applied in the clinic, which is the ultimate goal of any research on the VUSs of BRCA1.

To address this problem and to improve the clarity in conveying the information from genetic testing to the patients, the scientific community recommends the usage of a five-scale classification, which includes the information about how relevant the particular *BRCA1* variant is for the clinical practice [98]. According to this scale, the results from the genetic testing for *BRCA1* and *BRCA2* mutations can fall into one of the five categories [98].

Class 1 corresponds to **"**not pathogenic/no clinical significance" or "no clinical significance" variants. Likelihood for a variant to be pathogenic in this category is below 0.1%.

Class 2 corresponds to "likely not pathogenic/little clinical significance" variants. Likelihood for a variant to be pathogenic in this category is between 0.1 and 5%.

Class 3 corresponds to "variants with uncertain clinical significance". Likelihood for a variant to be pathogenic in this category is between 5 and 95%.

Class 4 corresponds to "likely pathogenic" variants. Likelihood for a variant to be pathogenic in this category is between 95 and 99%.

Class 5 corresponds to "definitely pathogenic" variants. Likelihood for a variant to be pathogenic in this category is above 99%.

Each of these classes is accompanied by recommendations for treatment and surveillance options. As more patients are tested and the methods for classification are improving, fewer variants will fall into Class 3 [98]. Therefore, the results from the

functional assays can be a valuable addition for clinical geneticists when advising their patients. Importantly, the accumulated knowledge on functional assays to classify alleles of *BRCA1* and *BRCA2* can be applied to any other disease-predisposing gene.

In summary, information for *BRCA1* or *BRCA2* status is the most predictive factor for the risk of breast and ovarian cancer development. The increase in risk is substantial and is almost five to ten times higher than the risk in the general population. Moreover, deleterious mutations in the *BRCA1* gene have high penetrance, and the patients' response to treatment is highly influenced by their *BRCA1* status. However, despite the enormous efforts to classify VUSs and to help patients with their clinical decisions, our ability to identify at-risk women remains problematic. One of the main limitations is the lack of knowledge about mechanistic aspects of BRCA1 biology. BRCA1 is a protein with pleiotropic functions, and it is not known how many of those functions alone or in combination contribute to the functions of BRCA1 as a tumor suppressor. To bridge the gap between the lack of understanding of BRCA1 role in tumor suppression on one hand and the ability to classify the BRCA1 VUSs on the other, we set out to systematically evaluate the biochemical function of BRCA1 domains and motifs. In this dissertation, we focus on analyzing the function of two BRCA1 conserved motifs called Motif 2 and Motif 6.

Why Are Studies on BRCA1 Important?

Mutations in *BRCA1* and *BRCA2* are responsible for 5-10% of all breast cancer cases. A common critique to studies on *BRCA1* is that a relatively small number of women with inherited mutations of *BRCA1* limit their impact on the disease burden. Then, why should we study *BRCA1*? To summarize, the studies on *BRCA1* are important for **the patients that carry deleterious mutations in *BRCA1* and their families** because of the following reasons:

1.   Early detection of individuals at-risk is important so that they and their families can make informed clinical decisions about prophylactic surgery, increased surveillance, chemoprevention, and risk for other malignancies. The presence of harmful BRCA1 mutation in a family influences other decisions in life such as childbearing.

2.   Individuals that have already developed breast cancer and have deleterious mutation in *BRCA1* will respond differently to radiotherapy and chemotherapy than the individuals from the general population.

Moreover, studies on BRCA1 have much broader implications, because:

1. Approximately 10% of all genetic tests find VUS (this percentage is higher in some populations, see page 2), which translates into the fact that ~ 10 000 families in the US alone, cannot make informed clinical decisions.

2. BRCA1 is epigenetically silenced in sporadic tumors [27, 32] and may regulate mammary stem/progenitor cell fate [99, 100].

3. Many genes implicated in breast cancer function in DNA damage response pathways. Thus, an understanding of BRCA1 role will likely have an impact on other forms of breast cancer not attributable to germline mutations in BRCA1. In addition, other diseases such as Fanconi Anemia arise from defects in the BRCA1-related pathways.

4. Radiation therapy and many chemotherapy drugs act by causing DNA damage in the cells. BRCA1 is an important participant in the cellular response to DNA damage, which makes it very essential factor in determining the patient's response to therapy in many cancers including lung and ovarian cancers [101, 102].

The DNA Damage Response (DDR) Network

DDR: DNA Repair and Cell Cycle Checkpoints

In order to preserve genome integrity, different evolutionarily conserved DNA cellular processes such as the DNA damage response (DDR) and cell cycle checkpoints have evolved [103]. The DDR may be defined as a collective name for the processes, with the help of which cells detect DNA damage, initiate DNA repair, and coordinate repair with cell cycle progression. Therefore, the DDR is a complex network of different interconnected pathways. We arbitrarily divide the protein complexes involved in the DDR as sensors, signal transducers, effector kinases, mediators, and effector proteins [3, 104]. Cell cycle checkpoints represent the mechanisms by which cells stop (during G1,

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G2 phases) or slow down (during S-phase) cell cycle progression, until an earlier process such as replication or mitosis is completed [105]. After DNA repair is accomplished, cells either resume cell cycle progression or, if the damage cannot be repaired, permanently arrest, senescence, or die (apoptosis) [106].

Mutations in *ATM, TP53, NBS1, BRCA1, BRCA2, CHEK2, PALB2, BACH1,* and other genes lead to an increased risk for the development of breast cancer if compared to the risk of the general population [1]. These proteins participate in the DDR network. NBS1, p53, BRCA1, BRCA2, CHK2, and ATM itself are substrates for the ATM kinase activity, when the cells experience DNA damage (Figure 2). Moreover, cells, lacking full length functional ATM, NBS1, and BRCA1, share similar phenotypes such as cell cycle checkpoint defects, radio sensitivity, increased chromosomal breakage, and failure to phosphorylate SMC1 (cohesin subunit) after IR [107]. It is currently unclear why mutations of genes, the products of which are operating in a conserved mechanism active in every cell, lead primarily to breast and/or ovarian cancer predisposition.

Double strand break (DSB) repair can be achieved mainly by two mechanisms: non-homologous end-joining (NHEJ) and homologous recombination (HR) [106, 108] and (Figure 3). On one hand, NHEJ is dependent on DNA-PKcs, Ku86, Ku70, and DNA ligase IV [106]. It can be performed throughout all cell cycle stages and is error prone. In addition to NHEJ, there is a less studied Ku86 and DNA Ligase IV-independent alternative end-joining pathway [109]. On the other hand, DSB generated during S and
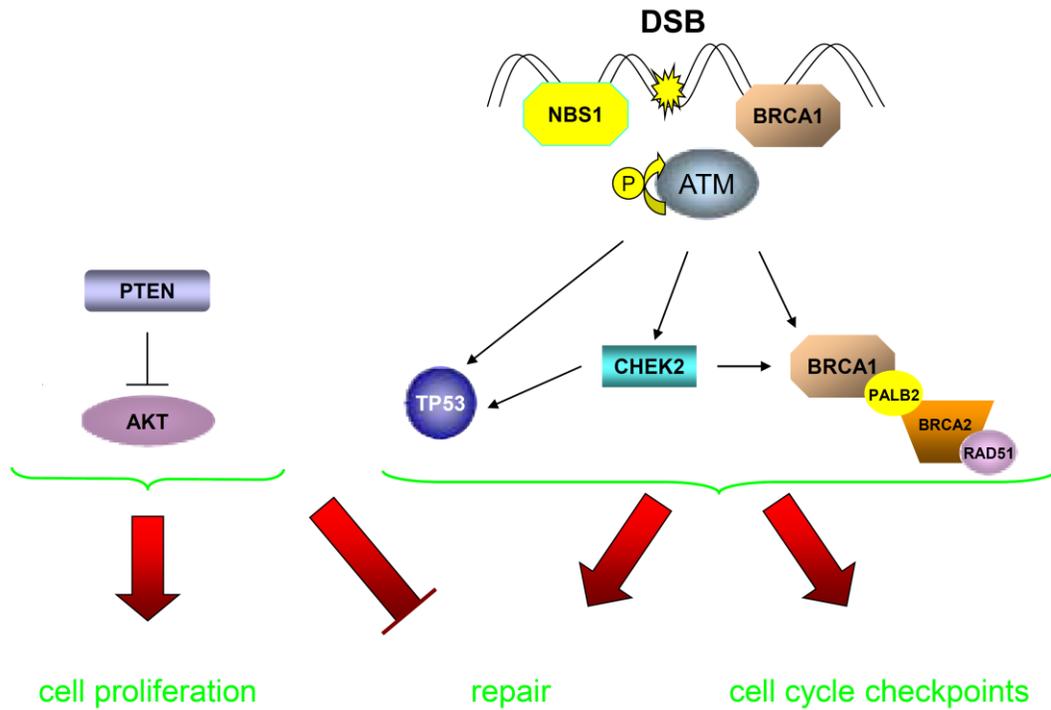
Figure 2. Breast cancer susceptibility gene products and the DDR pathway. In this simplified view ATM is activated by the presence of DSB and phosphorylate CHEK2, BRCA1, and TP53. Activated CHEK2 also phosphorylates TP53 and BRCA1. Note that BRCA1 and NBS1 contribute to the ATM activation and can function both upstream and downstream of ATM. Phosphorylation of these proteins is required for the efficient activation of various cell cycle checkpoints. BRCA2 regulates the function of the human recombinase RAD51. PALB2 act as a bridge between BRCA1 and BRCA2. Another protein implicated in breast cancer predisposition, PTEN, mediates down-regulation of AKT. (Adapted from [1]).
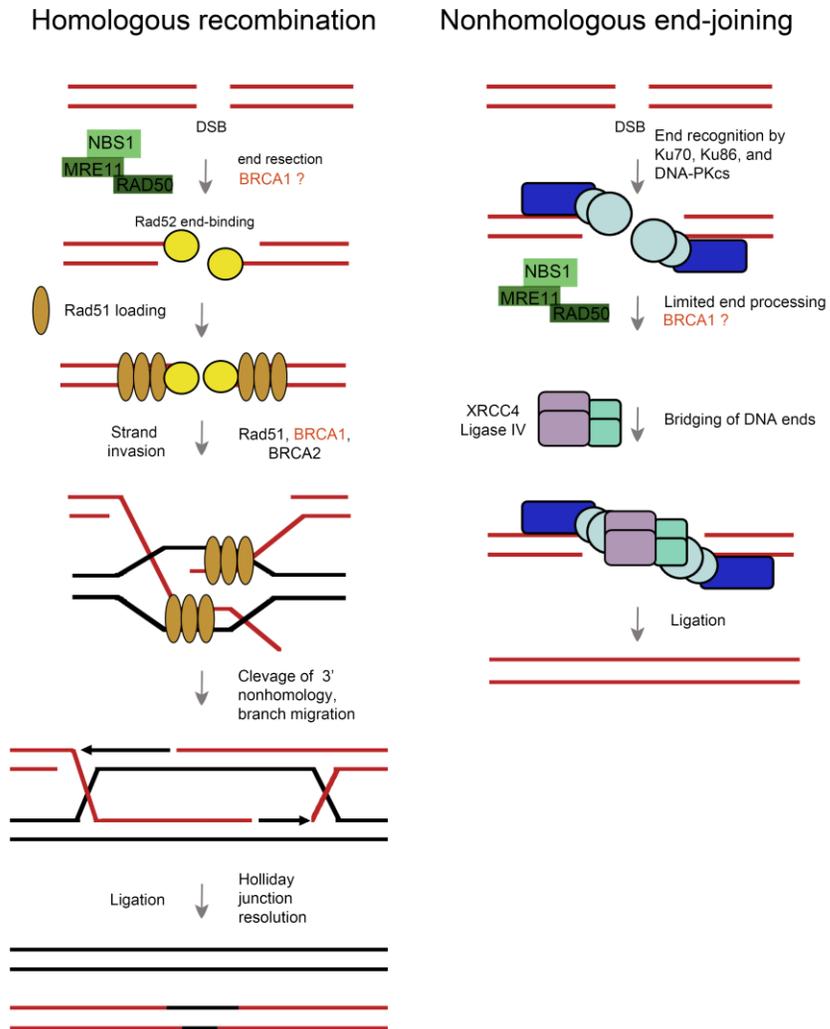
Figure 3. DNA repair by homologous recombination and non homologous end joining. The main steps of the two repair mechanisms are shown. (Adapted from [3]).

G2 phases of the cell cycle are predominantly repaired by HR, because sister chromatids are available to serve as repair templates. HR requires processing of DSB to generate single stranded DNA (ssDNA). ssDNA fulfills two roles: activates the checkpoint kinase ATR and is the only substrate that allows Rad51 recombinase to be loaded onto chromatin. HR is an error-free repair pathway [106].

Every day our cells are exposed to different DNA damaging agents that originate from their own metabolism (reactive oxygen species) or from their surroundings (ultraviolet (UV) radiation, IR, and different drugs) [103]. Different agents cause different types of DNA damage [3]. In this dissertation, we focus on DNA damage caused by ionizing radiation (IR), which primarily leads to double strand breaks (DSB) [110]. In some cases, we use hydroxyurea (HU) and campthothecin (CPT). To cause DNA damage, HU blocks DNA replication by inhibiting ribonucleotide reductase. Further, stalled replication forks undergo irreversible collapse and/or are processed to DSB [111]. Another effect is caused by CPT, which is a Topoisomerase I inhibitor that generates replication-mediated DSB [112]. In the current study, we investigate the extent to which different signal transduction pathways are activated after treating the cells with IR, HU, or CPT.

Sensors, Signal Transducing Kinases, Effector Kinases, and Effector Proteins

Upon DNA damage, three signal transducing Ser/Thr kinases: ATM, ATR, and DNA-PKcs are rapidly activated and recruited to the damaged areas in the DNA [113].

ATM and DNA-PKcs are mainly activated in response to DSB, whereas ATR is activated

by ssDNA and stalled replication forks [113], which consequently generate ssDNA,

coated with RPA [114]. Once at the proper place, the kinases relay the DNA damage

signals by phosphorylating numerous downstream substrates. For example, ATR and

ATM directly phosphorylate and activate the main effector kinases CHK1 and CHK2.

Activated CHK1 and CHK2 kinases then regulate the function of effector proteins such

as p53, E2F, and Cdc25 phosphatases, which in turn directly regulate cell cycle

progression [115].

When DNA damage occurs, DSB are first recognized by the MRN

(Mre11/Rad50/Nbs1) complex and the Ku70/Ku86 heterodimer [106], which are all

considered sensors for DSB. In addition, the ssDNA and stalled replication forks are

recognized by Rad9/Rad1/Hus1 complex (also known as the 911 complex) and the

ATRIP/ATR complex [106]. It has been shown that conserved motifs in NBS1, Ku86,

and ATRIP interact with and are responsible for chromatin loading of ATM, DNA-PKcs,

and ATR respectively [113].

Even though ATM and ATR overlap in the phosphorylation of their substrates,

there are differences in their relative contribution. This contribution depends on the type

of the genotoxic stress [116]. For instance, cells, that over express a kinase-dead mutant

of ATR, are hypersensitive to UV, HU, and IR, while ATM deficient cells are

hypersensitive only to IR [116]. According to current models, it is accepted that ATM

and ATR are activated by different DNA structures, rather than specific genotoxins [114].

Adding complexity to the DDR, these various DNA structures can be converted to one

another. For example, DSBs, generated after IR will primarily activate ATM. However, different enzymatic activities (nucleases, helicases, etc.) during the end processing of DSB after IR often generate ssDNA, coated with RPA, which subsequently activates ATR [117]. Furthermore, ATR is important for maintaining genomic integrity not only after genotoxic stress, but also during normal cell cycle progression.

Checkpoint Mediators

Mediators in the DDR are proteins that act directly downstream of the ATM and ATR kinases [118] such as MDC1, 53BP1, BRCA1, the MRN complex, Claspin, and MCPH1. Mediators have different roles in the DDR, e.g. control over localization, activation of other factors, and act as a scaffold for the assembly of protein complexes [118, 119]. As an example, we will briefly describe the signaling events that lead to the recruitment of BRCA1 to the sites of DSB after DNA damage (Figure 4). There, once recruited, BRCA1 can be visualized as discernible nuclear foci, called irradiation-induced nuclear foci (IRIF) [120-123].

First, after the DNA damage occurs, one of the earliest targets of activated ATM, ATR, and DNA-PKcs is the histone H2A variant H2AX [124]. It is phosphorylated at a conserved Ser 139 residue on both sides of the DSB, creating a specialized chromatin domain [125]. H2AX phosphorylated at Ser 139 is also referred as γ-H2AX. Because of its location in the nucleosomes, γ-H2AX is the most widely used DSB marker in western blots and in immunofluorescence analysis of foci formation. Our own work, which is not

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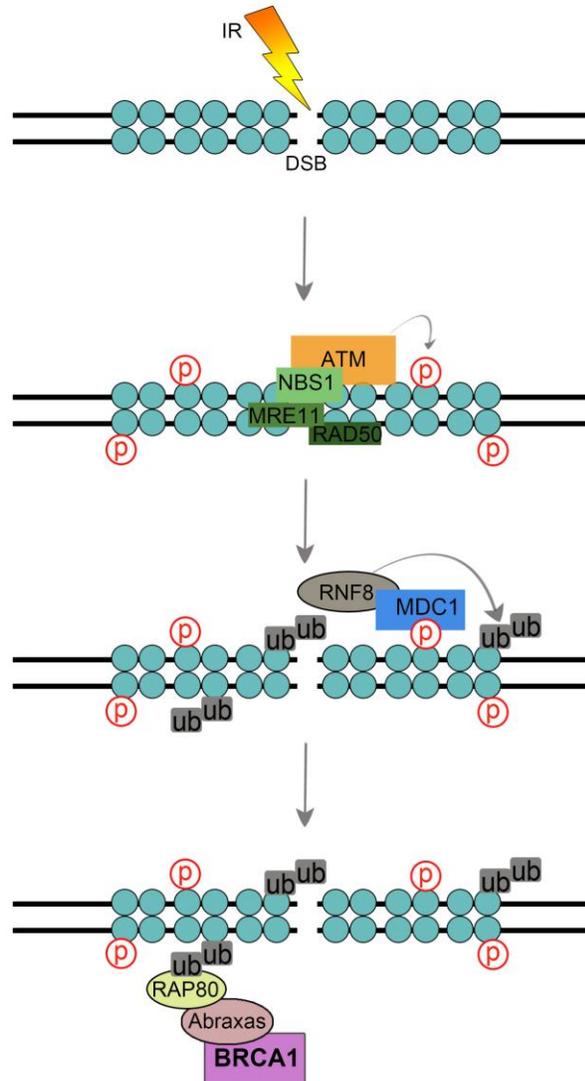
Figure 4. Current model for the BRCA1 recruitment to the IRIF. DSBs are recognized by the MRN complex, which recruits activated ATM to the DNA lesion. ATM phosphorylates H2AX (for simplicity nucleosomes are depicted as blue circles). MDC1 binds directly to the phosphorylated H2AX and recruits the ubiquitin ligase RNF8. RNF8 initiates histone ubiquitylation at the damaged sites. This chromatin modification allows the recruitment of BRCA1 to the sites of DSBs.

included in this dissertation, demonstrated the importance of H2AX post-translational

modifications in the regulation of its own turnover, cell cycle, and apoptosis [126]. After

the phosphorylation of H2AX, MDC1 is recruited to the DSBs. MDC1 possesses tandem

BRCT domains that recognize and bind directly to γ-H2AX [127]. The major function of

MDC1 is to amplify H2AX phosphorylation by recruiting additional ATM molecules or

by inhibiting γ-H2AX dephosphorylation [128]. Such amplification allows for the

recruitment of additional DDR factors to the sites of damage, most of which can be

visualized as IRIF [118].

According to the current understanding, the next event in the recruitment at the

sites of DNA damage is the interaction between the FHA domain of the E3 ubiquitin

ligase RNF8 and phosphorylated MDC1 [129-131] (Figure 4). Once recruited to the

DSB, RNF8 catalyses Lys 63-linked ubiquitination of histones and other proteins [130]

[131]. Such ubiquitination of γ-H2AX stabilizes the recruitment of 53BP1 and BRCA1 at

the DSB sites [128]. For example, a protein called Rap 80 possesses both a ubiquitin

interacting motif and a coiled-coil domain. The Lys-63 ubiquitinated histones are

recognized by the ubiquitin interacting motif of Rap80 [132] [133]. Furthermore, the

coiled-coil domain of Rap80 directly interacts with the coiled-coil domain of Abraxas

[133]. The recruitment of BRCA1 at the sites of DSB is mediated by the binding of its

BRCT domains to the phosphorylated Abraxas [132] [133] (Figure 4). Once in the proper

location at DSB chromatin, BRCA1 coordinates DNA repair, transcription, and cell cycle

checkpoints by facilitating the phosphorylation of downstream substrates such as p53,

NBS1, CHK1, and CHK2 in an ATM/ATR-dependent manner [134]. The mechanistic

details of how BRCA1 modulates the phosphorylation of such a large number of substrates are still unclear. Moreover, ATM and ATR kinases phosphorylate BRCA1 at the numerous sites, thus modulating BRCA1 activity. Further, BRCA1 interacts with ATR partner, ATRIP [135], promoting ATR-dependent checkpoint functions. However, the mechanism described above is not the only one that leads to the recruitment of BRCA1 to the DSBs sites. Even in the absence of γ-H2AX/MDC1/Rap80/Abraxas mechanism, BRCA1 still localizes to the sites of DSBs [136].

Finally, BRCA1 acts as a scaffold for the assembly of different complexes after DNA damage. It has been demonstrated that three major BRCA1-containing complexes are formed after IR: one that contains BACH1 and is involved in the intra-S-phase checkpoint, another, CtIP and MRN complex, that participates in the early G2/M checkpoint, and the third, BRCA2/Rad51 that is responsible for DNA repair by HR [137].

DDR: Spatiotemporal Regulation in Specialized Chromatin Domains

In addition to the widely used classification of DDR participants such as sensors, mediators etc., Bekker-Jensen and colleagues divide DNA repair complexes into three groups, depending on their location related to the DSB: DSB-flanking chromatin (chromatin compartment), single stranded-DNA micro compartment, and repair complexes that do not show retention at the DSB [121]. **DSB-flanking chromatin** is occupied by groups of proteins that assemble on both sides of the DSB and can spread megabases from both sides of the break [121]. Proteins that localize to this compartment

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are ATM, MRN complex, MDC1, 53BP1, and BRCA1. In addition, **single stranded-DNA micro compartment** is occupied by the proteins such as the single strand DNA binding protein RPA, ATR, Rad51, BRCA2, MRN complex, and BRCA1. Finally, **complexes that do not show retention at the DSB** are DNA-PKcs, Ku70, Chk1, Chk2, and p53.

These specialized chromatin domains perform different functions in DNA repair and checkpoint signaling. Importantly, the proteins that occupy the DSB-flanking chromatin can assemble during all cell cycle stages. Meanwhile, complexes that occupy the single stranded micro-compartment assemble in a cell cycle dependent manner only during S and G2 phases. MRN complex and BRCA1 have unique roles in DDR signaling and processing of DSB because they are the only proteins that can be found in two compartments (double and single stranded). This fact is essential when discussing dependence in the recruitment of different proteins to the DSB sites and their functional consequences. For example, the down regulation of MDC1 leads to the dissociation of BRCA1 only in the chromatin, but not in the single stranded compartment [121]. On the contrary, the down regulation of BRCA1 leads to the dissociation of BRCA2/Rad51 complex from the single strand compartment, showing that BRCA1 is upstream of BRCA2/Rad51 in the DNA repair signaling. In addition, it has been proposed that different protein complexes contribute to the recruitment of BRCA1 to the different compartments [138]. For instance, MDC1/Rap80/Abraxas complex localizes BRCA1 to the chromatin compartment (see the previous section), and MRN complex and CtIP – to the single stranded DNA micro compartment. Most likely the disruption of BRCA1

function in these different locations will have different functional consequences for the DNA repair and checkpoint signaling.

BRCA1: Structure and Functions

The Structure of BRCA1

The *BRCA1* gene locus is located on chromosome 17q21 and codes for a 1863 amino acids protein [13]. The conserved domains and motifs of BRCA1 are shown on Figure 5 and summarized in Table 3. At its N-terminus, BRCA1 has a zinc binding **RING finger domain** (aa 1-101) [13]. *In vivo,* most of BRCA1 resides in a complex with BARD1, which also contains a RING-finger domain [139, 140]. BRCA1 and BARD1 interact with each other via their respective RING finger domains and form a stable heterodimer [141]. RING finger domains possess intrinsic E3-ubiquitin ligase activity [85]. Consistent with this, BRCA1/BARD1 complex has E3 ubiquitin ligase activity [85] [142, 143].

Within the BRCA1 RING domain, cancer causing mutations lead to the abrogation of BRCA1 E3-ubiquitin ligase activity and fail to restore radiation sensitivity of a *BRCA1*-deficient cell line [143]. Moreover, BRCA1 variants at the N-terminus of BRCA1, which disrupt BRCA1 binding to the E2-enzyme, lead to the loss of ubiquitin ligase activity [84]. Thus, loss of ubiquitin ligase activity correlates with susceptibility to breast and ovarian cancer [84].

Table 3. BRCA1 domains and motifs

Domains and Motifs	Amino acids	Function	Refs
RING	1-101	E3-ubiquitin ligase	[13]
NES	81-89	Nuclear export	[144]
Motif 1	123-130	unknown	[145]
Motif 2	178-189	unknown (studied in this thesis)	[145, 146]
Ser 308	308	Aurora A phosphorylation target site	[147]
Motif 3	378-388	unknown	[145]
Motif 4	458-467	unknown	[145]
DNA binding region	452-1079	Binding to branched DNA	[148]
NLS	503-508, 651-656	Nuclear import	[149]
Motif 5	512-521	unknown	[145]
Motif 6	845-869	unknown (studied in this thesis)	[145, 146]
Ser 988	988	CHK2 phosphorylation target site	[150]
Motif 7	1147-1153	unknown	[145]
Ser 1189	1189	Cdk1 phosphorylation target site	[151]
Ser 1191	1191	Cdk1 phosphorylation target site	[151]
Motif 8	1208-1228	unknown	[145]
Coiled-coil	1369-1418	PALB2 binding	[145]
Ser 1387	1387	ATM/ATR phosphorylation target site, intra-S-phase checkpoint	[152]
Ser 1423	1423	ATM/ATR phosphorylation target site, G2/M checkpoint	[152]
Ser 1497	1497	Cdk1/Cdk2 phosphorylation target site,	[151, 153]
Ser1524	1524	ATM/ATR phosphorylation target site,	[154]
Ser1572	1572	CK2 phosphorylation target site	[155]
BRCT1 [N]	1650-1753	DNA damage signaling, transcription	[156]
BRCT2 [C]	1760-1855	DNA damage signaling, transcription	[156]
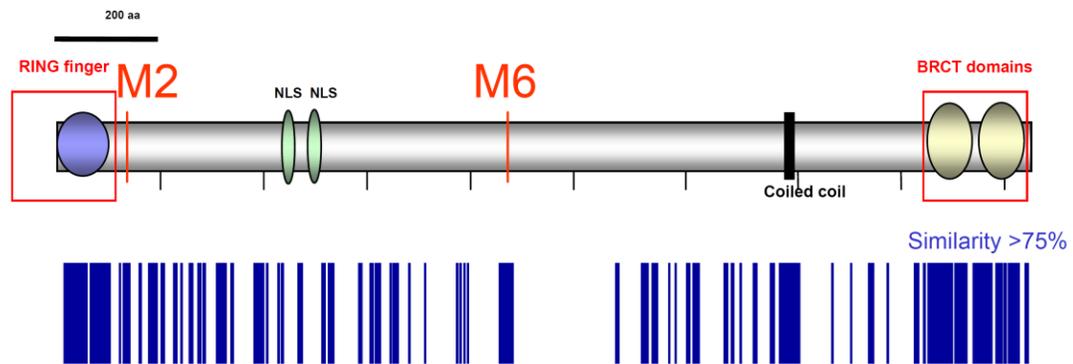
Figure 5. Structure of BRCA1. RING and BRCT domains are shown in red boxes; Coiled-coil domain is depicted as a black box; NLS, nuclear localization signal;M2, Motif 2; M6, Motif 6; The regions of BRCA1 that have evolutionary conservation above 75% are shown on the blue scale bar (Adapted from [2]).

At its C terminus, BRCA1 has two **BRCT domains** in tandem [156]. The BRCT domain was first described in BRCA1 (BRCT stands for BRCA1-C terminal domain) and was found in several proteins such as MDC1, p53BP1, and DNA ligase IV, which all participate in DNA repair and checkpoint control [156] [157]. It has been shown that tandem BRCT domains bind phospho-peptides [158, 159]. In particular, the two BRCT domains of BRCA1 behave as one functional unit that binds to phosphorylated proteins with the consensus sequence pSer-X-X-Phe (where X is any amino acid) [160]. Moreover, BRCT domains are important for the functions of BRCA1 as a tumor suppressor. According to the genetic evidence, all mutations found in cancer patients, that lead to the truncation of BRCA1 BRCT domains confer a high risk for cancer development [161]. Thus, such mutations are currently classified as deleterious variants [80].

The remaining central region of BRCA1 between the RING and BRCT domains has not been studied in depth. However, this region of BRCA1 contains several significant features. They include nuclear localization (aa 503-508), nuclear export sequences (aa 81-99) [144, 162], the coiled coil domain, and BRCA1 aa 452-1079 region, important for binding to branched DNA structures [148]. The **coiled-coil domain** encompasses BRCA1 exons 12 and 13 (aa 1391-1424) (see 5) [94, 163]. It has been found to interact with two proteins: JunB [163] and PALB2 [164, 165]. Importantly, mutations within the coiled-coil domain found in breast and ovarian cancer patients modulate the ability of BRCA1 to activate transcription [75]. Moreover, mutations such as L1407P and M1411T, mapped to the BRCA1 coiled-coil domain, disrupt the binding

between BRCA1 and PALB2 and are defective in homologous recombination repair assays [164]. The evidence described above suggests a potential role of the coiled coil domain in tumor suppression.

Furthermore, the biological role of some BRCA1 phosphorylation sites has already been studied. For example, BRCA1 double mutant Ser 1423Ala/Ser 1524Ala cannot rescue the radiation sensitivity of BRCA1 deficient cells, showing that these two sites are important for survival after IR [154]. In addition, phosphorylation of Ser 1423 is required for the IR-induced early G2/M checkpoint [152] and Ser 1387 – for the intra-S-phase checkpoint activation after IR [166]. Finally, phosphorylation of Ser 988 was reported as important for the role of BRCA1 in homologous recombination repair [167] and in mitosis [168]. Importantly, only one of these sites, Ser 1524, has been found mutated in patients with breast and ovarian cancer. However, its association with cancer has not been determined [2].

Finally, **nine additional conserved segments** [94] [145] were revealed during the analysis at the conserved positions in BRCA1 orthologs. Such segments are called Motifs 1 to 8, and the coiled-coil domain of BRCA1 is referred to as Motif 9. The contribution of these nine motifs to different BRCA1 functions is still under researched. Analyzing the function Motif 2 and Motif 6 is the main focus of this dissertation.

BRCA1: Biochemical Activities and Biological Processes

BRCA1 participates in many biochemical activities and biological processes and, thus, has a pleiotropic role in the cell. In fact, there is often a confusion between BRCA1's involvement in various activities and processes.

First, BRCA1 participates in such central processes in the life of a cell as DNA repair, control of the cell cycle, transcription, and mitosis [169] (Figure 6). In addition, many biochemical activities, ascribed to BRCA1, including transcription activation and repression [79, 170], chromatin remodeling [171], and ubiquitination [4]. However, it is not clear, which of these functions and activities, alone or in a combination, contribute to BRCA1 role as a tumor suppressor. Though many of these BRCA1 functions and activities are not tissue specific, and BRCA1 is a ubiquitously expressed protein, it is not clear why individuals heterozygous for BRCA1 mutations develop mainly breast and/or ovarian cancer. Thus, understanding of BRCA1 biology is ultimately linked to our ability to provide risk assessment and better treatment options for the patients with BRCA1-associated breast and ovarian cancer.

Second, research on BRCA1 functions has shown that BRCA1 activities in the cell are regulated by different post-translational modifications such as phosphorylation [122], ubiquitination [172], sumoylation [173, 174], and interactions with close to 100 different cellular proteins. Another level of such regulation happens when BRCA1 expression is tightly controlled during cell cycle progression. For example, BRCA1 protein level is low in G0 and G1 phases of the cell cycle and increases as cells enter S-

phase [149, 175]. Furthermore, BRCA1 localizes to discrete nuclear foci (dots) during normal S-phase, but it re-locates to PCNA-containing replication foci after DNA damage [122].

Finally, BRCA1 participates in many aspects of DNA damage signaling pathways such as intra-S-phase checkpoint [107, 166] [152], G2/M checkpoint [176] [152], spindle assembly checkpoint [169, 177], end processing [178, 179], and DNA repair both by HR and NHEJ (Figures 3, 6, and Table 4). In this dissertation, we focus on the functions of BRCA1 in the DDR.

G1 Checkpoint

The signal transduction pathway, that is responsible for the cell cycle arrest in the G1 stage of the cell cycle includes, ATM, ATR, CHK2, CHK1, MDM2, TP53, and p21 [103]. The ultimate goal of this pathway is to stop the cell cycle by increasing the stability and transcriptional activity of TP53 protein [103]. TP53 controls the expression of CDK inhibitor p21, which in turn inhibits the kinase activity of cyclin E/Cdk2 [103]. The main mediators of G1 checkpoints are the tumor suppressors TP53 and pRB. BRCA1 has been implicated in interactions with both p53 and pRB, and it is thought that BRCA1 participates in G1 checkpoint control by its numerous interactions with ATM, ATR, RB, p53, and p21 [180] [181]. However, the molecular details and the end point effects of BRCA1 deficiency with regard to G1 checkpoint are not clear.

Intra-S-Phase Checkpoint

The intra-S-phase checkpoint is defined as the checkpoint "activated by DNA double strand breaks that are generated in genomic loci outside the active replicons" [182]. For example, it can be triggered by the IR-induced DSBs. The activation of intra-S-phase checkpoint leads to a rapid decrease in DNA synthesis after DNA damage because of the inhibition of new origin firing [115]. Cells with defective intra-S-phase checkpoint control fail to down-regulate DNA synthesis and display a radio-resistant DNA synthesis (RDS) phenotype [115, 182]. The RDS phenotype has been observed in cells deficient in proteins such as: ATM, ATR, CHK1, CHK2, NBS1, 53BP1, BRCA1, and BRCA2 [182]. This suggests these proteins' participation in the activation or maintenance of the checkpoint. Finally, it has been demonstrated that the intra-S-phase checkpoint is cooperatively controlled by at least two parallel ATM-dependent mechanisms: the ATM-NBS1-BRCA1-SMC1 [107, 182] axis and ATM-CHK2 - CDC25A-Cdk2 axis [183] and (Figure 7).

In the first pathway, ATM-dependent phosphorylations of SMC1 at Ser957 and 966, NBS1 at Ser343, and BRCA1 at Ser1387 are necessary for the intra-S-phase checkpoint activation [152, 166]. However, it is not clear how phosphorylated SMC1 leads to down regulation in DNA replication. In addition, the functional consequences of NBS1 and BRCA1 phosphorylations are poorly understood. To complicate this matter
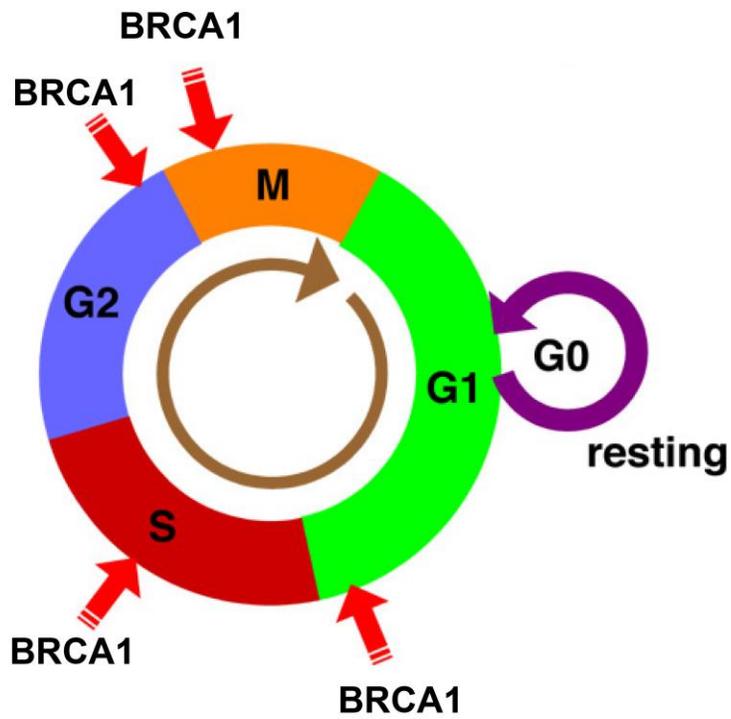
Figure 6. Cell cycle stages and participation of BRCA1 in cell cycle checkpoints.
G1, gap phase 1;G2, gap phase 2; S, DNA synthesis stage; M, mitosis. Participation of BRCA1 in different cell cycle checkpoints is shown with red arrows. (Adapted from http://homepage.mac.com/enognog/checkpoint.htm).
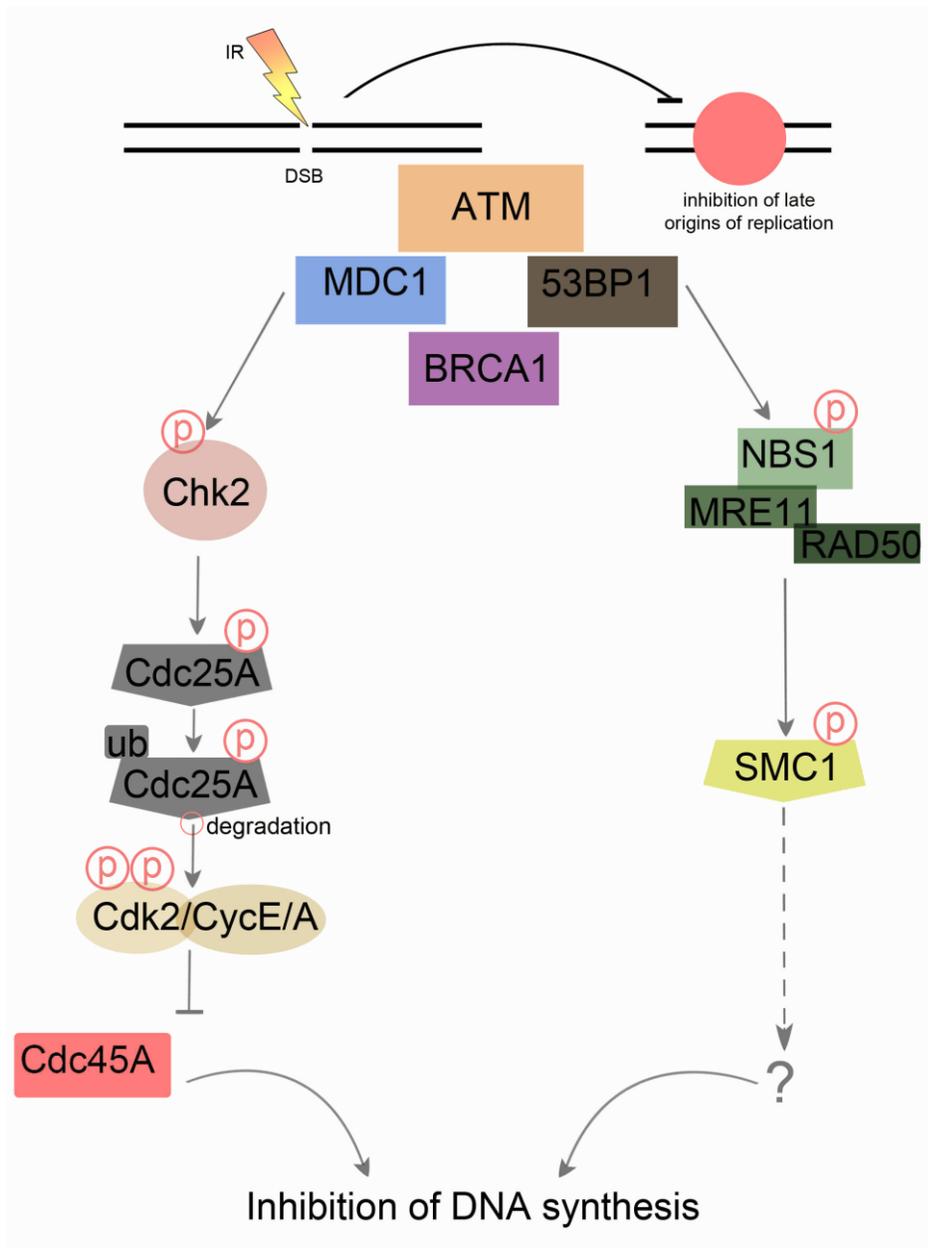
Figure 7. The intra-S-phase checkpoint signaling. The two parallel pathways that cooperatively control this checkpoint are depicted. (Adapted from [3]).

further, it has been demonstrated that the phosphorylations of BRCA1 at Ser1387 and

NBS1 at Ser343 are not necessary for the ATM-dependent phosphorylation of SMC1 at

the Ser 957 and 966 sites after IR. Instead, it has been hypothesized that BRCA1 and

NBS1 might be needed as scaffold proteins in order for SMC1 phosphorylation to occur

properly after DNA damage [184].

According to the current understanding, the recruitment of BRCA1 and NBS1 to

the DSBs happens independently of ATM phosphorylation. Moreover, the initial

activation of ATM occurs independently from BRCA1 and NBS1 [107]. However,

BRCA1 and NBS1 are required for the proper localization of once activated ATM to the

break sites [107]. Only then, ATM can phosphorylate substrates such as BRCA1, NBS1,

and SMC1. Moreover, BRCA1 and NBS1 phosphorylations are dependent on each other

and on the proper ATM recruitment to the DNA DSB sites [107].

The other signaling pathway, implicated in the intra-S-phase checkpoint, is ATM-

CHK2 -CDC25A-Cdk2 [183]. Signaling through this pathway leads to Cdc45 inability of

being loaded at the replication origins. However, such loading is necessary for the

initiation of DNA replication [183]. Interestingly, BRCA1 interacts with BACH1 helicase

and TopBP1. BRCA1, BACH1, and TopBP1 appear to control Cdc45 origin-licensing

factor after DNA damage through poorly understood mechanisms [137]. Thus, in

addition to ATM-NBS1-BRCA1-SMC1 pathway, BRCA1 might also participate in the

ATM-CHK2 -CDC25A-Cdk2 axis of the intra-S-phase checkpoint by controlling Cdc45.

In conclusion, ATM initiates two parallel pathways that are responsible for the

activation of intra-S-phase checkpoint by phosphorylating proteins such as CHK2,

BRCA1, NBS1, and SMC1. The examples discussed above illustrate the importance of BRCA1 in the regulation of different steps in DDR: functioning both upstream and downstream of ATM and acting as a scaffold for the assembly of multiple protein complexes. In addition to BRCA1, ATM, and NBS1, and another BRCT domain containing protein – DNA replication factor C has been purified as a part of a large super complex named BASC (BRCA1 associated genome surveillance complex) [185]. This shows a potential link between BRCA1 and replication-associated repair and signaling. Clearly, more research is necessary to elucidate mechanisms behind the complex role of BRCA1 in the intra-S-phase checkpoint. Any molecular details on this process will likely translate not only into better surveillance options for the patients with harmful BRCA1 mutations, but also into designing improved treatments for cancer.

G2/M Checkpoint

ATM-CHK2 and ATR-CHK1 pathways control the checkpoint responses in the G2-phase of the cell cycle by phosphorylating the mitosis promoting phosphatase CDC25C (Figure 8). This phosphorylation inhibits the CDC25C, and it cannot dephosphorylate and thus activate cyclin B/CDK1 kinase complex [115]. Consequently, these events block the cells in G2/M phase of the cell cycle. Moreover, the maintenance of the G2/M checkpoint relies on the transcription initiated by p53 and BRCA1. Thus,
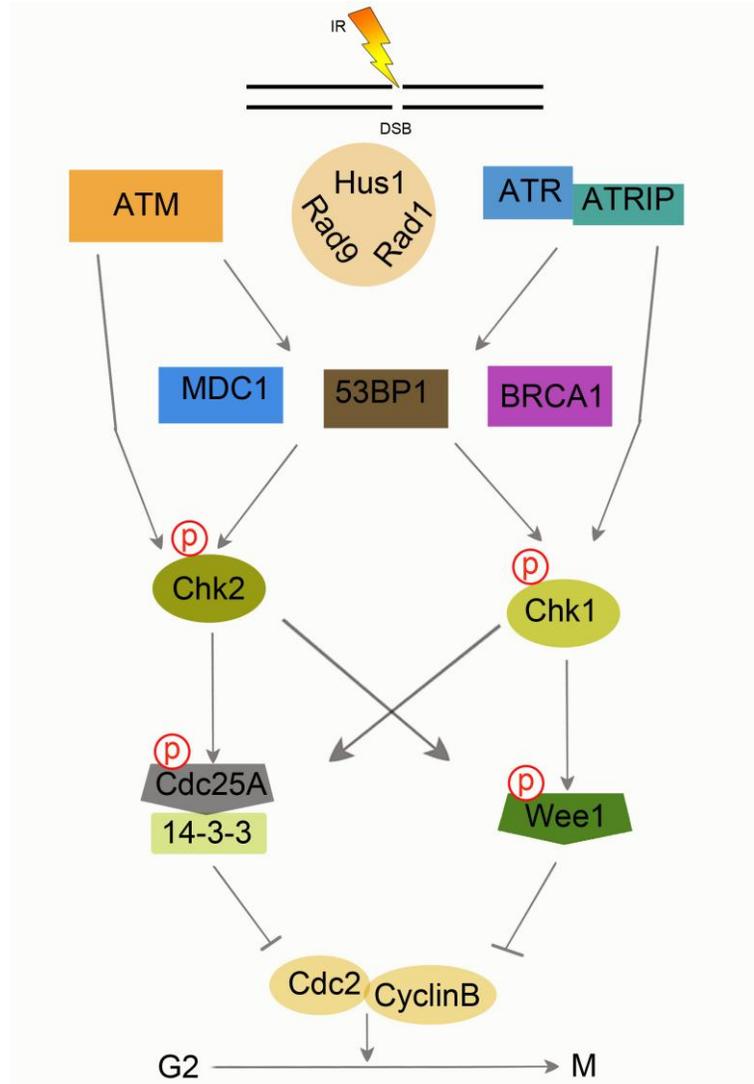
Figure 8. The G2/M checkpoint signaling. Main sensor (911 complex), mediators (MDC1, BRCA1, and 53BP1), effector kinases (CHK1 and CHK2), and effector protein (Cdc25 phosphatase) are shown. (Adapted from [3]).

BRCA1 participates in G2/M checkpoint signaling via the transcriptional regulation (both activation and repression) of genes responsible for G2/M checkpoint control [186]. These genes include Cyclin B, 14-3-3 proteins, the Wee 1 kinase, and p21[186]. Finally, BRCA1 regulates the transcription of GADD45a [103], which in its turn regulates cyclin B/CDK1 kinase complex by the inhibition of its kinase activity [187].

However, besides its role in transcription, BRCA1 participates in the G2 phase checkpoint responses by being a target of ATM and ATR kinases. They both phosphorylate BRCA1 on multiple residues (see Table 2). According to Kastan and colleagues, there are two distinct G2 checkpoints: **early G2/M checkpoint** and **G2 accumulation**, which are measured by different methods and at different time points after IR [188]. Importantly, both checkpoints require BRCA1 participation [152, 188]. In particular, the expression of BRCA1 S1423A mutant in a BRCA1-deficient breast cancer cell line HCC1937 can reconstitute early G2/M checkpoint, but not G2 accumulation [188]. This indicates that the two checkpoints are controlled by different signaling events.

The **early G2/M checkpoint** [188] occurs early after IR (1-2h) and is ATM-dependent. Furthermore, it occurs in cells in the G2/M boundary at the time of IR that fail to progress to mitosis [188]. On the contrary, the **G2 accumulation checkpoint** is dose-dependent and ATM- independent [188]. It measures the cells that have been in S- and G1-stages at the time of irradiation. In addition, it represents these cells' accumulation in G2 stage [188]. The G2 accumulation checkpoint is usually measured at a later time after IR (i.e. 24h). It is important to point out, that BRCA1- or NBS1- deficient cells, that have

a defect in IR-induced intra-S-phase checkpoint, usually display G2 accumulation phenotype [188].

Another checkpoint, called the **Human decatenation checkpoint,** can be related to the G2 checkpoint responses in the cell, although it is not a response to IR. **The Human decatenation checkpoint** monitors chromatid decatenation in cells that progress from the G2 phase of the cell cycle to mitosis [189]. If sister chromatids are insufficiently decatenated, the progression from G2 phase to mitosis is blocked [189]. In particular, ATR and BRCA1 work together to control G2 decatenation checkpoint by excluding cyclin B1/Cdk1 complexes from the nucleus [189]. On the molecular level, BRCA1 ubiquitinates topoisomerase II alpha and regulates its decatenation activity [190]. Finally, Human decatenation checkpoint seems to be independent of ATM [189].

Importantly, failure in any given checkpoint by itself does not lead to radio sensitivity [188]. This can be exemplified by BRCA1 mutants, expression of which affect only one checkpoint function of BRCA1 and do not influence cells' survival after irradiation [152, 166, 188]. For example, the expression of BRCA1 S1387A mutant reverses the early G2/M checkpoint defect but not the intra-S phase checkpoint defect [152, 166]. On the contrary, S1423A mutant reverses the intra-S checkpoint defect but not the early G2/M phase checkpoint defect [152]. Therefore, radiation sensitivity as a phenotype is not necessarily a consequence of the defective checkpoint signaling; rather it represents a more complex phenotype because of the failure in more than one stage in the DDR. Thus, the fact that cell lines that do not express functional BRCA1 have

severely compromised long-term survival after irradiation, illustrates that BRCA1

functions on several stages in the DDR and performs multiple functions after IR.

Spindle Assembly Checkpoint

The spindle assembly checkpoint monitors the accuracy of chromosome

segregation by preventing cells with misaligned chromosomes from exiting mitosis [180,

191]. BRCA1 regulates the transcription of several spindle assembly checkpoint genes

(including Mad2) [177, 180]. Mouse embryonic fibroblasts, isolated from $Brca1^{\Delta11/\Delta11}$

mice, displayed chromosomal abnormalities (bridges and lagging chromosomes), which

are hallmarks of spindle assembly checkpoint defects [177]. The studies of spindle

assembly checkpoint related to BRCA1 in mammalian cells have been difficult because

the inhibition or over expression of BRCA1 leads to cell cycle arrest or apoptosis [180].

The down regulation of BRCA1 in human prostate cell line DU-145 (which

expresses wild type BRCA1) and human breast cancer cell line MCF7 (hemizygous for

wild type BRCA1 expression), followed by microarray analysis revealed that BRCA1

controls the transcription of many genes that are required for mitotic progression [192].

Moreover, it has been demonstrated that checkpoint kinase Chk2 and its target BRCA1

(phosphorylated at Ser 988) function independently of p53 and DNA damage in mitosis.

Chk2 kinase and BRCA1 are required for normal progression of mitosis and,

subsequently, maintaining chromosomal stability [168].

DNA-Repair by HR

HR is an error-free DNA repair mechanism, which uses the sister chromatid or less often the homologous chromosome as a repair template [193] and (Figure 3). BRCA1 participates in the regulation of HR. The research on such regulation involved the measurement of HR efficiency in *BRCA1*-deficient cell lines [194, 195], association of BRCA1 with other proteins participating in different stages of HR (see below), and the decrease of Rad51 foci formation in the absence of functional BRCA1 [121, 137, 196]. As an example, mouse cell lines, which do not express functional Brca1, have decreased HR efficiency and increased genomic instability [194, 195]. In addition, several BRCA1-interacting partners participate in the control of HR repair. These include a growing number of proteins such as BRCA2 [4], PALB2 [164], BACH1 [197], CtIP [178, 198], 53BP1, and the MRN protein complex [3, 198]. Several of these proteins function as enzymes, which facilitate HR repair. For example, the MRN complex [199] has nuclease activities and BACH1 [197] and BLM [200] are DNA helicases.

The interaction of BRCA1 with PALB2 is of particular interest because the latter is also a breast cancer predisposition gene [164, 201-203]. Furthermore, biochemical studies on the BRCA1/PALB2 complex demonstrated that PALB2 not only binds to the coiled-coil domain of BRCA1 through its own coiled-coil domain [204], but also interacts with BRCA2 via the WD40 domain [205]. Thus, PALB2 interacts with both BRCA1 and BRCA2 and acts as a bridge between them [164, 205].

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Lastly, the participation of BRCA1 in the HR is demonstrated by the relation between BRCA1 and Rad51 recombinase. According to the current understanding, there is a hierarchy in the recruitment of the aforementioned proteins to the DNA damage sites. BRCA1 localization to the DSBs does not depend on PALB2, BRCA2, or Rad51 recombinase. PALB2 is recruited to the sites of damage, at least in part, via its interaction with BRCA1 [193]. Furthermore, PALB2 interacts with BRCA2, the protein that loads the Rad51 recombinase on chromatin. The role of BRCA2 in HR is a direct control of Rad51 nucleoprotein formation [206], while the role of BRCA1 seems to be more complex. BRCA1 functions earlier in HR and DNA damage checkpoint signaling by interacting with PALB2, BACH1, CtIP, and MRN complex.

In addition to their role in HR, BRCA1, BRCA2, and PALB2 participate in the intra-S-phase checkpoint. Cells defective in the expression of these proteins do not have intact intra-S phase checkpoint [152, 205, 207]. Therefore, the intra-S-phase checkpoint is another possible process except for HR, in which the protein complex that consists of BRCA1, BRCA2, and PALB2 can potentially be involved. It is equally possible that BRCA1 participates in the intra-S-phase checkpoint signaling independently of BRCA2-PALB2 complex.

At this stage, the mechanism by which BRCA1, BRCA2, and PALB2 carry out their tumor suppression activities is not clear. One possibility is that BRCA1 is localizing PALB2, BRCA2, and Rad51 at the DSBs, and, consequently, facilitating HR [121, 137]. Another possibility is that BRCA1, PALB2, and BRCA2 alone or in combination are responsible for the intact intra-S-phase checkpoint signaling. Finally, it may happen that

all of the above mentioned possibilities are working together. However, it is important to point out that the coiled-coil domain of BRCA1 seems to be necessary both for its role in HR [164] and for the intra-S-phase checkpoint function (Velkova and Monteiro unpublished results).

In addition to PALB2, another important interacting partner of BRCA1, involved in the control of HR, is CtIP. In mammalian cells, CtIP is phosphorylated by CDKs, and it exists in a complex with BRCA1 in a cell cycle (during G2 phase) dependent manner [208]. Studies performed on the avian B-cell line DT40 demonstrated that CtIP participates in micro-homology directed end joining (during G1 phase) and in HR (during S and G2 phases) [178]. Importantly, the function of CtIP exclusively in HR is dependent on its interaction with BRCA1 [178, 208, 209]. Moreover, BRCA1 promotes CtIP ubiquitination and its localization to the sites of DSBs [210].

Both CtIP and BRCA1 control RPA foci formation after DNA damage, thus controlling ATR activation [198, 209, 211]. In addition, BRCA1 co-localizes with the MRN complex at the IR-induced foci [120, 212] and inhibits its exo-nuclease activity [148], while CtIP stimulates the nuclease activity of the MRN complex [198]. Based on these data, it has been proposed that BRCA1/CtIP/MRN complex participates and controls the initial processing and/or resection of DSBs [138].

Furthermore, BRCA1 might participate in the end-resection by its relation to 53BP1. Only recently, the details about their role in the DDR became clear. Two recent papers explored the effect of *53bp1* deletion in *Brca1*-deficient mouse cells. In the first report, the deletion of *53bp1* rescues several phenotypes associated with *Brca1* deficiency

such as the proliferation defect, hypersensitivity to DNA cross-linking agents, spontaneous formation of DSB, and activation of the DDR [213]. In the second report, *53bp1* deletion rescues the HR defect of Brca1$^{\Delta11/\Delta11}$ mouse cells by a mechanism dependent on ATM-controlled end resection [179]. Thus, it has been hypothesized that BRCA1 and 53BP1 might regulate the choice between HR and NHEJ [179], at least partially by their role in end-resection. The manner, in which BRCA1 in complex with CtIP, MRN, 53BP1, or other proteins participates and controls end resection and DNA damage signaling remains obscure and is a subject for active research.

Taken together, these data suggest that BRCA1 participates in HR by controlling protein localization and different activities during the end-resection step. Despite the evident contribution of the studies cited above, they should be interpreted with caution. The experiments described above have been carried out in avian B cell line DT40 (in the case of CtIP) and in mouse embryonic fibroblasts (in the case of 53BP1). DT40 cell line has a short cell cycle taken up mostly by S-phase and high levels of HR [108]. In addition, the survival of mouse embryonic stem cells is dependent on HR. However, the relative contribution of HR and NHEJ in repair and cellular responses to damage varies between species and in different cells [108]. Thus, the physiological importance of the effects of BRCA1 deficiency on breast and ovarian epithelial cells remains to be tested.

NHEJ is considered to be the major DNA repair pathway responsible for fixing IR-induced DSB in mammalian cells [214]. It is operational throughout all cell cycle stages [214] and dependent on the Ku (Ku70/86) heterodimer, DNA-PKcs, XRCC4, DNA Ligase IV, Artemis, and XLF [214] (Figure 3).

It is generally accepted that NHEJ has several stages: detection of DSB, end processing, and ligation [214]. In the process of detection of DSBs, the Ku heterodimer is the one recognizing the DSBs. Upon DNA damage, Ku86 interacts with a conserved motif on DNA-PKcs C-terminus, which leads to the recruitment of DNA-PKcs to DSBs and its activation [214]. Over 16 phosphorylation sites have been described on DNA-PKcs, although their role is still poorly understood. Nevertheless, DNA-PKcs phosphorylation status is believed to influence its activity [215]. The unphosphorylated form of DNA-PKcs interacts with Ku86 [216]. Further, DNA-PKcs autophosphorylation is required for NHEJ progression and, subsequently, for end processing [217]. It has also been shown that Ku86 interacts with BRCA1 N-terminus, and this complex formation is necessary for the rapid recruitment of BRCA1 at DSB sites [218]. However, the downstream events in the DDR signaling, in which BRCA1 participates, are not clear.

Furthermore, the reports for the role of BRCA1 in NHEJ have been conflicting (Table 4), ranging from no significant difference (slight elevation) to compromised NHEJ. However, the cited reports used different cell lines (mouse versus human, embryonic stem cells versus breast epithelial cells), which express various BRCA1

mutants such as the Δ11 isoform versus truncating mutation in HCC1937 cell line.

Moreover, to measure the role of BRCA1 in NHEJ, different assays were used: from gene

targeting and single chromosomal DSB at the specific position to plasmid-based in vivo

and in vitro assays (Table 4). In addition, plasmid-based assays account for the total end-

joining activity and do not discriminate between classical and alternative end joining.

Thus, based on the available data, the role of BRCA1 in NHEJ regulation might be cell

and DNA break type specific.

Table 4. Comparison of different assays for BRCA1 repair function.

Cell type	Assay	HR	NHEJ	Refs
Mouse embryonic stem cells line 236.44	Gene targeting	Decreased	Proficient, Elevated	[194]
Mouse embryonic stem cells	Gene targeting	Decreased	Increased	[219]
Mouse embryonic stem cell line 236.44	I-Sce I induced chromosomal break	Decreased	Proficient, Elevated	[194]
Mouse embryonic stem cells	Cell free assay-total end joining		Decreased	[220]
HCC1937	Plasmid base in vivo end joining		Decreased precise end joining	[221]
HCC1937	Plasmid base in vivo end joining		Decreased precise end joining	[222]

Hypotheses for the Tissue Specific BRCA1 Functions

Several hypotheses have been proposed to address the tissue specific functions of BRCA1. First, Scully and Livingston [223] hypothesized that some estrogen metabolites could potentially form DNA adducts. Specifically, breast and ovarian epithelial cells are estrogen-responsive. Thus, BRCA1 may play an essential role in the repair of estrogen-induced DNA damage that cannot be compensated by other proteins.

Second, Elledge and Amon [224] proposed a hypothesis, according to which the loss of *BRCA1* as an essential gene has two different end results. In various tissues except for the breast and ovary, the loss of *BRCA1* leads to cell death or proliferation defects. Nevertheless, in the breast and ovary, *BRCA1*-negative cells receive the necessary survival signals (hormones, growth factors, *etc*.) and continue to divide. This growth advantage increases the possibility of the secondary mutations to be accumulated over time, which in some cases will lead to the development of breast and/or ovarian cancer.

In addition, Foulkes extended the Elledge and Amon's hypothesis by proposing that BRCA1 functions as a major regulator for breast stem cells differentiation [225]. In this role, BRCA1 regulates differentiation of the breast epithelium. Failure in this regulatory mechanism results in loss of control over cell growth and potential block in the proper differentiation of the breast epithelial cells. This hypothesis explains some of the phenotypes of BRCA1-associated breast cancer (see pages 7-8).

Finally, Monteiro proposed an alternative mechanism to explain the tissue specific tumor suppressor role of BRCA1 [226]. According to this hypothesis, BRCA1

locus is the subject to increased loss of heterozygosity rates only in the breast and ovarian epithelial cells, but not in the cells from the other tissues. Currently, none of these models has been experimentally tested and verified.

Summary and Rationale

Women who are carriers of deleterious mutations in *BRCA1* are at a greater risk to develop breast and ovarian cancer than women from the general population. The 17 years after the cloning of *BRCA1* have been fruitful for breast cancer research. The results from such research benefit patients not only when they make informed clinical decisions, but also when they undergo therapy for their *BRCA1*-related breast cancer. However, the experience from the bench and from the clinical practice shows that much more remains to be learned about *BRCA1* and the mechanism through which it participates in one of the most universal processes in life — maintenance of the genome stability.

Considering that there are over 1500 alleles of *BRCA1* in the human population, one of the most challenging tasks for genetic counseling remains to distinguish which are benign and which are cancer predisposing. Previous research has indicated that the likelihood of a BRCA1 variant being deleterious is higher when the variant is located in structurally and functionally defined protein domains such as the RING and BRCT domains. Consequently, the identification of other functional domains, besides the well-studied ones, is critical for the understanding of BRCA1 function and for classifying variants as cancer predisposing or benign. To approach this problem, we formulate our

central hypothesis: poorly characterized, conserved domains in the central region of BRCA1 (Motif2, Motif 6, and coiled-coil domain) directly participate in the tumor suppression functions of BRCA1.

This dissertation aims at testing our hypothesis and determining how specific domains and motifs of BRCA1 act to promote tumor suppression. In particular, we set out to determine the functional significance of the two poorly characterized domains of BRCA1. This is a step further in our long-term goal to understand the mechanisms, by which BRCA1 is required for maintaining genomic stability, and to use this information to develop a system of assays for testing the functional role of any mutation in BRCA1. Importantly, our research has much broader implications because the gene products involved in breast cancer seem to cluster around DNA damage response pathways. Thus, the understanding of BRCA1 role may have an impact on other forms of breast cancer, not attributable to germline mutations in BRCA1. Moreover, both radiation therapy and most of the drugs used for cancer treatment rely on introducing DNA damage in the cells. BRCA1 is the main participant in the cellular response to DNA damage, which makes it an important factor in determining the patient's response to therapy.

MATERIALS AND METHODS

Constructs and Cloning

GST-fusion fragments of BRCA1 in the mammalian expression vector pEBG BF 1-6 were a gift from Toru Ouchi. BRCA1 fragments BF1A (aa 1-70), BF1B (aa 71-140), BF1C (aa 1-101), BF1D (aa 141-240), BF1D1 (aa 160-190), BF1D2 (aa 190-210), BF1D3 (aa 160-210), BF1E (aa 241-324), and BF1F (aa 1-302) were obtained by PCR using pEBG BF1 as template. The PCR products were digested, cloned into pEBG vector, and sequenced. Construct BF1D Y179C was obtained by site directed mutagenesis using BF1D as template for the PCR reaction. FLAG FLNA-Bf was obtained by cloning a PCR fragment of Filamin A (FLNA, aa 2477-2647) in frame to FLAG in pCMV2-FLAG vector.

For the yeast two hybrid interactions assays BRCA1 Motif 6 fragment (aa 845-869) was obtained by PCR and cloned in frame with the GAL4 DNA binding domain into pGBKT7 vector. Similarly, FLNA repeat 23/Hinge/repeat 24 (aa 2477-2647), FLNA repeat 23 (aa 2477-2516), FLNA Hinge (aa 2517-2549), FLNA repeat 23/Hinge (aa 2477-2549), FLNA repeat 24 (aa 2550-2647), FLNA repeat 24 (aa 2517-2647) were

obtained by PCR and cloned in frame with GAL4 activation domain into pGAD 424 vector.

The constructs containing BRCA1 Motif 6 mutants: S864L, F861C, Q855P, R866C, and R866H were obtained by site directed mutagenesis using as a template for the PCR reaction pGBKT7 Motif6 (aa 845-869). The products were digested, cloned in frame with GAL4 DNA binding domain into pGBKT7 vector, verified by sequencing, and used for the transformation of yeast strain AH109.

Yeast Two Hybrid Assays

For deletion analysis the yeast two hybrid was performed using the MATCHMAKER Two-Hybrid System 3 (Clontech laboratories). In brief, pGBKT7 Motif 6 and pGAD 424, containing FLNA deletion mutants were co-transformed in AH 109 yeast strain. Transformations were plated on SD –Leu – Trp selective media to access the efficiency of the transformation and SD – Leu – Trp – Ade – His to map the minimal domain of FLNA necessary for binding to BRCA1.

For the liquid culture growth assay the AH109 transformants were inoculated in liquid media lacking either leucine and triptophane (-leu,-trp, double selection) or lacking leucine, tryptophan, histidine, and adenine (-leu-trp-his-ade, quadruple selection). The vectors used for the expression confer growth in the absence of leucine (pGAD424) or tryptophan (pGBKT7). Samples were taken every two hours and quantified by spectrophotometer at a wavelength of 600nm. The growth on double selection was used

to ensure that the constructs tested are not toxic for the yeast. If any of the Motif 6

constructs interact with FLNA construct activation of the expression of histidine and

adenine will follow and the yeast will grow in media lacking histidine and adenine.

Cell Lines and Transfections

The FLNA-deficient M2 melanoma cell line and its isogenic cell line, A7,

reconstituted with full length FLNA cDNA [227](gift from T. Stossel) was grown in

MEM (Sigma) with 8% newborn calf serum (Sigma) and 2% fetal bovine serum (FBS;

SAFC Biosciences, Lenexa, KS). A7 cells were grown in the presence of 0.2 mg/ml

G418 (Fisher). HeLa (ATCC, Manassas, VA) was grown in DMEM with 5% FBS

(Sigma). HCT116 (ATCC) was grown in McCoy's with 10% FBS. 293FT (InVitrogen)

cells were grown in DMEM media (Sigma) with 10% FBS. Tissue culture media was

supplemented with penicillin and streptomycin. Transfections were performed using

Fugene 6 (Roche) according to the manufacturer's instructions.

Antibodies

The following antibodies, peptides, and beads were used: α-BRCA1 mouse

monoclonal antibody MS110 (Ab-1; Calbiochem; San Diego, CA) and SG11 (gift from

D. Livingston); α-Filamin A mouse monoclonal antibody PM6/317 (Chemicon

International); α-FLAG M2 mouse monoclonal antibody (Sigma); 3xFLAG-peptide

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(Sigma); α-GST goat polyclonal antibody (Pharmacia Biotech); GT-sepharose 4B beads (GE Healthcare); α-Ku86 monoclonal antibody B-1 and α-Rad51 rabbit polyclonal antibody H-92 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); α-TP53BP1 mouse monoclonal antibody and α-phosphoserine 343-NBS1 mouse monoclonal antibody (Upstate Biotechnology); α-p34 RPA mouse monoclonal antibody Ab-1 and α-DNA-PKcs mouse monoclonal antibodies Ab-2 (Neomarkers, Freemont, CA); α-phosphoserine 2056 DNA-PKcs rabbit polyclonal antibody (Abcam, Cambridge, MA); α-phosphoserine 2609 DNA-PKcs rabbit polyclonal antibody (Novus Biologicals); α-MDC1 (SIGMA); γ-H2AX rabbit polyclonal;α-H2AX; α-phosphoserine 1981 ATM; α-ATM; α-ATR; α-phosphothreonine 68 CHK2; α-phosphoserine 317 CHK1 (Cell Signaling); α-β actin (Sigma). Conjugates for immunofluorescence were Alexa fluor 488 or 555 Molecular Probes.

Immunoprecipitation, Pull-Downs, Western Blot Analysis, and Densitometry

Whole cell extracts were prepared by lyzing cells in a mild RIPA buffer (120mM NaCl, 50 mM Tris pH 7.4, 1% NP40, 1mM EDTA, protease inhibitors, 4 mM PMSF) lacking harsher SDS, sodium deoxycholate, and Triton X-100 detergents. The same buffer was used for immunoprecipitation. For high stringency immunoprecipitations the RIPA buffer was supplemented with 0.5 % SDS. Antibodies (1μg) were pre-incubated with protein A/G agarose beads (Santa Cruz Biotechnology, Inc.), washed twice in RIPA buffer and incubated with the cell extracts overnight at 4°C. After incubation, the slurry

were pelleted by centrifugation (2,000 rpm) and washed twice by removing the supernatant. Sample buffer was added to the beads and boiled for 10 min. For GST-pull downs, cell extracts were incubated with GT-beads, washed in RIPA buffer, and boiled.

Samples for western blot analysis were separated by SDS-PAGE and gels were electroblotted on a wet apparatus to a PVDF membrane. The PVDF membrane was blocked with 5% milk in TBS buffer containing 0.1% Tween (TBS-Tween) for 1h. The membrane was washed three times in TBS-Tween and the antibody was added in 0.5% milk in TBS-Tween. The membrane was washed three times in TBS-Tween and incubated with the appropriate conjugate. After final washes Blots were incubated with ECL (Millipore, Billerica, MA).

Chromatin fractions were obtained by lyzing the cells with mild RIPA buffer and centrifuging at 14,000 rpm for 5 min. The pellet was then washed twice in mild RIPA and extracted with acid extraction buffer (0.5M HCl, 10% Glycerol, 100mM BME) and subsequently neutralized using 40mM Tris pH 7.4 with protease inhibitors and NaOH. Western blot data was quantified by densitometry using AlphaEaseFC v 3.1.2. Each lane was normalized using the corresponding loading controls and then expressed as a fold change relative to the untreated FLNA$^+$ cells in each blot.

Immunofluorescence

For BRCA1 analysis cells were fixed with 4% formaldehyde for 5 min followed by 5 min incubation with 100% ethanol. Cells were permeabilized with 0.25% Triton X-100 in PBS for 10 min, washed with PBS, and then blocked for 30 min with 5% BSA in PBS at room temperature (RT). After blocking, BRCA1 monoclonal antibody (SG11; kind gift from David Livingston) was added to 1% BSA in PBS for 1h RT. Cells were washed and goat α-mouse Alexa Fluor 488 (Molecular Probes) was added for an additional 1h RT.

For all other antibodies, cells were plated onto chamber slides and after 24h they were washed with cytoskeleton buffer (10mM HEPES/KOH pH 7.4, 300mM sucrose, 100mM NaCl, 3mM $MgCl_2$) and fixed with 4% formaldehyde for 30 min RT. For analysis of chromatin bound RPA cells were pre-extracted for 2 min on ice with cytoskeleton buffer supplemented with 0.5% Triton X-100 before fixation [228]. After fixation cells were permeabilized with 0.25% Triton X-100 in PBS for 5 min RT and then washed and blocked with 5% BSA in PBS for 30 min RT. Primary and secondary antibodies in 1% BSA in PBS were added for 1h each.

Cells were washed and mounted with Prolong Gold medium (Molecular Probes). Images were taken on a Leica Confocal Microscope. For quantification of BRCA1 and Rad51 immunofluorescence foci approximately 100 cells were scored per each time point. Cells were scored as foci-positive if they presented with more than 10 foci per cell (an example can be found in Figure 34). For γ-H2AX and NBS-P-343 at least 50 cells per

time point were counted for each condition. Cells were scored as foci-positive if they presented with more than 20 foci per cell. A threshold of 20 foci was chosen based on the number of foci found in unirradiated samples using the described antibodies. Determination of foci number per cell was done using Definiens Developer XD 1.1 (Definiens AD, Germany). A rule set was developed to segment nuclei based on the DAPI stain and then segment foci within the nucleus based on an intensity threshold. Representative results from at least two independent experiments are shown instead of statistical data on a small number of measurements with variability as recently recommended [229] .

Comet Assay

Comet assays were performed in neutral conditions using a comet assay kit (Trevigen, Gaithersburg, MD) according to manufacturer's instructions. Briefly, cells were collected at the indicated time points, combined in low melting agarose (Trevigen, Gaithersburg, MD), spread over the comet slide area and allowed to set. Then, slides were immersed in lysis buffer for 30 min at 4°C. Electrophoresis was run in TBE buffer for 20min at 1V/cm voltage. Image analysis was done with Comet Analysis System 2.3.3 software (Loats Associates Inc., Westminster, MD).

RESULTS

Characterization of the Interaction between FLNA and BRCA1

Identification of FLNA as a Binding Partner of BRCA1 Motif 6 in Yeast

Our laboratory has focused on the systematic analysis of domains and motifs in BRCA1 as a means to understand its biochemical functions [81]. Here we analyzed a conserved region, called Motif 6, spanning amino acids 845-869, coded by BRCA1's large exon 11 (Figure 5) [94, 145].

In order to identify interactors for the conserved Motif 6 of BRCA1 spanning amino acid residues 845-869 (Figure 9), we performed a yeast two-hybrid screening against a human mammary gland cDNA library. Two overlapping clones coding for human Filamin A (FLNA; OMIM # 300017), spanning amino acid residues 2443-2647 and 2477-2647 (Figure 9), were identified. This region includes repeat 23, the hinge region, and repeat 24 in the C-terminus FLNA (Figure 9) [230]. We mapped the minimal region of FLNA, which interacts with BRCA1 Motif 6, by testing binding of a series of FLNA deletion mutants (Figure 9). Only the fragment aa 2477-2647 was able to bind to BRCA1 Motif 6 (Figure 9). Interestingly, FLNA has been shown to interact with BRCA2

and to participate in the DDR [231-233]. Cells lacking FLNA exhibit prolonged

checkpoint activation, leading to the accumulation of cells in G2/M after ionizing

radiation [231].

Interaction between FLNA and BRCA1 in Mammalian Cells

Next, we tested whether endogenous FLNA interacted with endogenous BRCA1

in mammalian cells. Immunoprecipitation, using a specific monoclonal antibody against

BRCA1, pulled down FLNA in HeLa and HCT116 cells (Figure 10). In addition,

immunoprecipitation using an antibody against FLNA, was able to pull down BRCA1

(Figure 10). Thus, BRCA1 and FLNA interact in vivo, and the interaction is mediated by

the C-terminus of FLNA.

Because FLNA and BRCA1 have been demonstrated to be primarily cytoplasmic

and nuclear respectively, we biochemically fractionated HCT116 cells to determine in

which subcellular compartment the interaction occurs (Figure 11). We found that FLNA

is expressed in the nucleus and cytoplasm, and BRCA1 can be co-immunoprecipitated by

FLNA in the nuclear fraction (Figure 11). We also determined that the interaction is

direct as bacterially expressed GST-tagged BRCA1 (aa 141-302) can pull down

bacterially expressed His-tagged FLNA C-terminus (Figure 12).
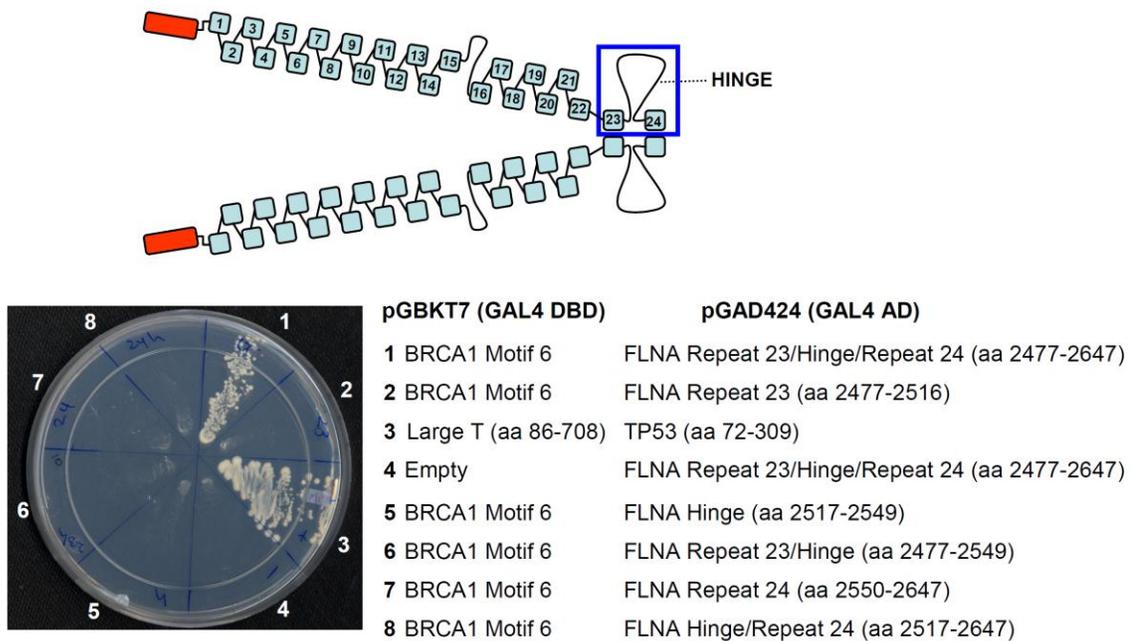
Figure 9. BRCA1 Motif 6 binds to Filamin A (aa 2477-2674) in yeast. *Top panel:* Diagram showing a Filamin A dimer. Red box, Actin binding domain; blue box indicates the minimal region that interacts with Motif 6 of BRCA1. Note that this region contains the dimerization domain for two Filamin A subunits. *Bottom panel:* Yeast two-hybrid experiments testing Motif 6 interaction with Filamin A fragments, as well as positive (Large T and p53) and negative (FLNA and empty vector) controls. Smaller regions of FLNA such as repeat 23, hinge region, repeat 23 and hinge, repeat 24, and repeat 24 plus hinge failed to interact indicating that both repeats and the hinge region are necessary for a stable interaction.

64
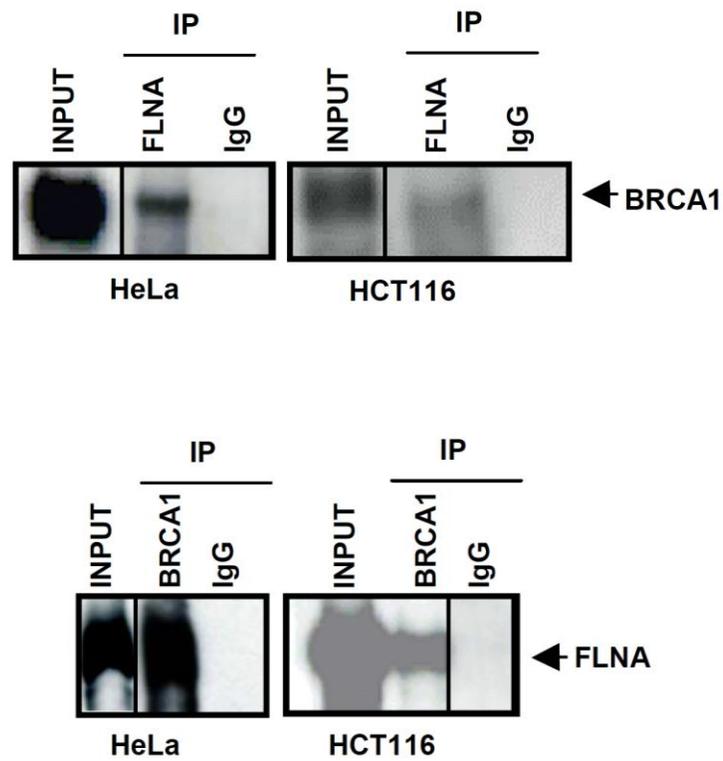
Figure 10. BRCA1 and Filamin A interact in mammalian cells. *Upper panels,* co-immunoprecipitation of endogenous BRCA1 with FLNA in HeLa and HCT116 cells showing interaction of endogenous BRCA1 and FLNA. *Lower panels,* reverse reaction showing co-immunoprecipitation of endogenous FLNA with BRCA1.
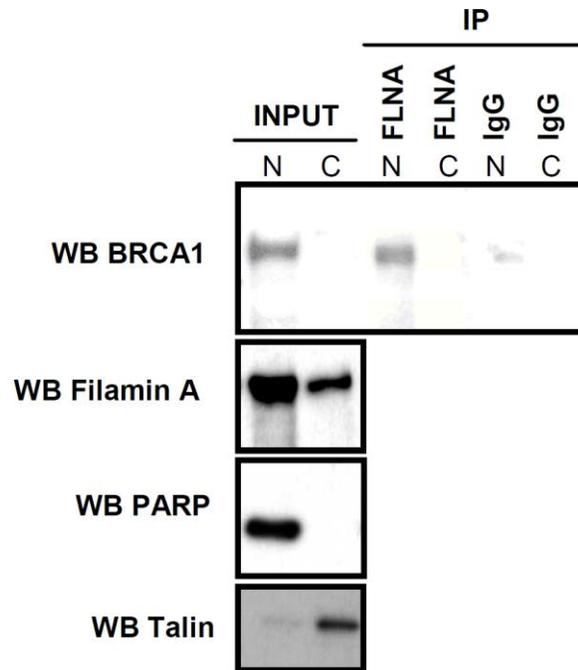
Figure 11. FLNA interacts with BRCA1 in the nucleus. HCT116 cells were fractionated into nuclear and cytoplasmic fractions using PARP and Talin as controls, respectively. Top panel shows that BRCA1 can be immunoprecipitated using FLNA antibody in the nuclear fraction.
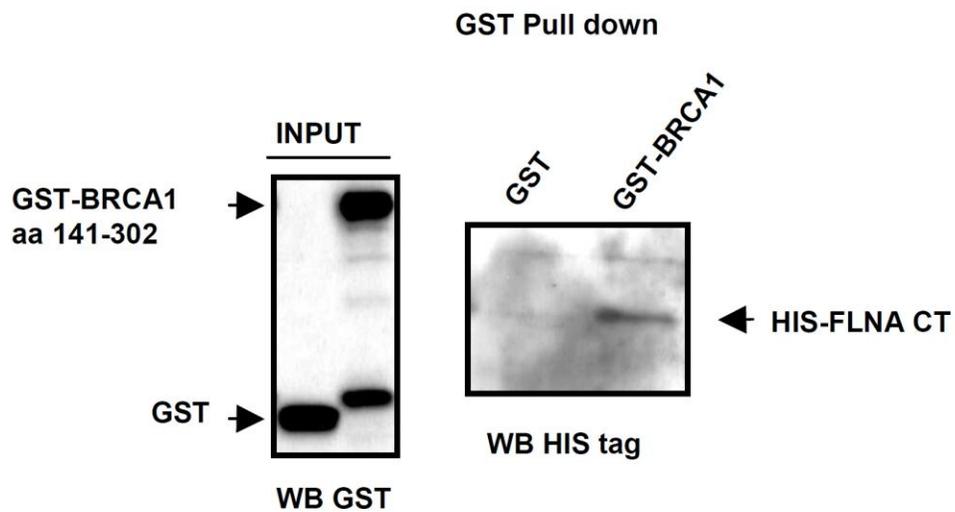
Figure 12. FLNA directly binds to BRCA1 in vitro. GST and GST-BRCA1 (aa 141-302) were used in pull down in vitro assays. GST-BRCA1 but not GST can pull down bacterially expressed His-tagged FLNA C-terminal region.

BRCA1 Binding to FLNA is Mediated Primarily by BRCA1 Motif 2 not Motif 6

We searched the Breast Cancer Information Core Database (BIC,

http://research.nhgri.nih.gov/projects/bic) and identified five missense variants in BRCA1

Motif 6 region: S864L, F861C, Q855P, R866C, and R866H. All five mutants were

classified as VUSs. Next, we tested the extent to which the indicated variants interact

with FLNA. We checked the ability of the yeast strain AH109 co-transformed with

constructs, coding for FLNA and Motif 6 to grow on liquid media under stringent

selection. If any of the Motif 6 constructs interacted with FLNA construct, the activation

of histidine and adenine expression would follow, and the yeast would grow in media,

lacking histidine and adenine. In this experiment a strong interaction should have similar

profile as the positive control. However, even the wild type Motif 6 did not display such a

profile, indicating that the interactions between FLNA and all Motif 6 constructs tested

were relatively weak (Figures 13 and 14). This observation raises questions about the

significance of the results obtained with the Motif 6 mutants. Thus, we hypothesized that

other regions in BRCA1, besides Motif 6, might contribute to BRCA1 binding to FLNA.

We co-expressed in-frame fusions of GST to deletion fragments of BRCA1 and a FLAG-

tagged FLNA fragment (aa 2477-2647) in 293FT cells (Figure 15) to assess each region's

contribution to binding.

We immunoprecipitated FLAG-FLNA using α-FLAG agarose beads, and the

eluate with FLAG-peptide was separated by SDS-PAGE. Western blot against

endogenous FLNA confirmed that FLAG-FLNA was properly folded, because FLAG-

M6/FLNA double selection

Legend:
- Large T/p53
- FLNA/pGBKT7
- M6/pGAD424
- S864L
- F861C
- Q855P
- R866C
- R866H
- M6wt
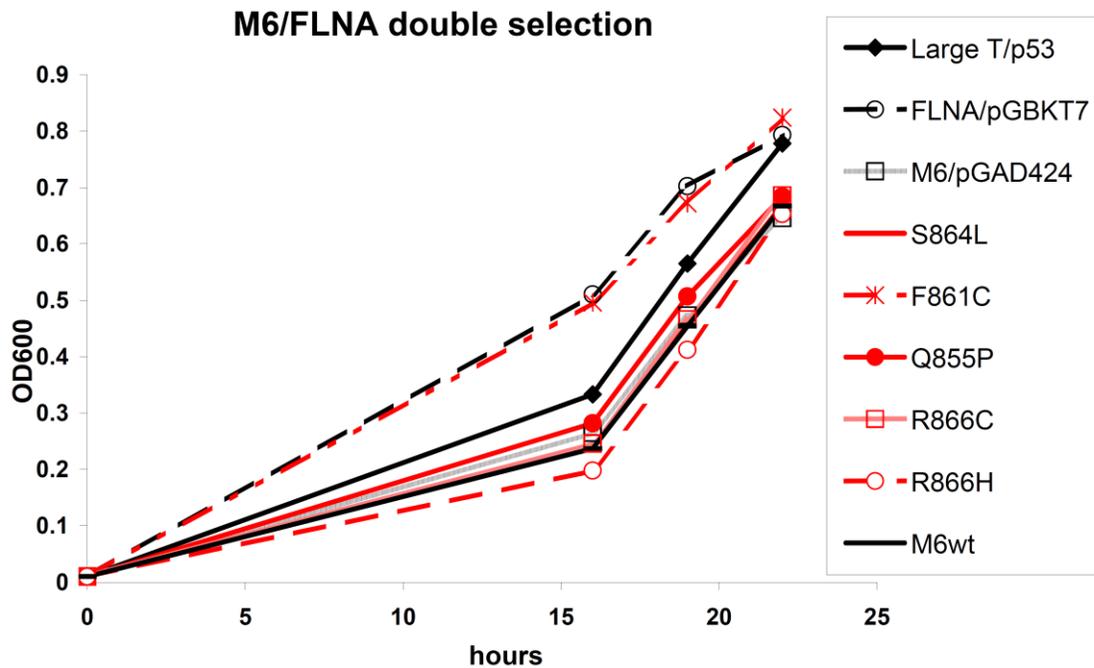
Figure 13. Growth curve in medium lacking leucine and tryptophan. *S.cerevisiae* strain AH109 (Clontech) was transformed simultaneously with pGBKT7/Motif 6 mutants (aa 845-869) and pGAD424/FLNA (aa 2477-2647) constructs. Growth curves in medium lacking leucine and tryptophan (-leu,-trp, double selection) are shown. OD_{600} of the cultures (y axis) is plotted against time (x axis). Large T Antigen (aa 86-708) and p53 (aa 72-309) were used as a positive control and pGBKT7/Motif6+pGAD424 and pGAD424/FLNA+pGBKT7 as negative controls. The growth on double selection was used as a control to ensure that the constructs tested are not toxic for the yeast.

69
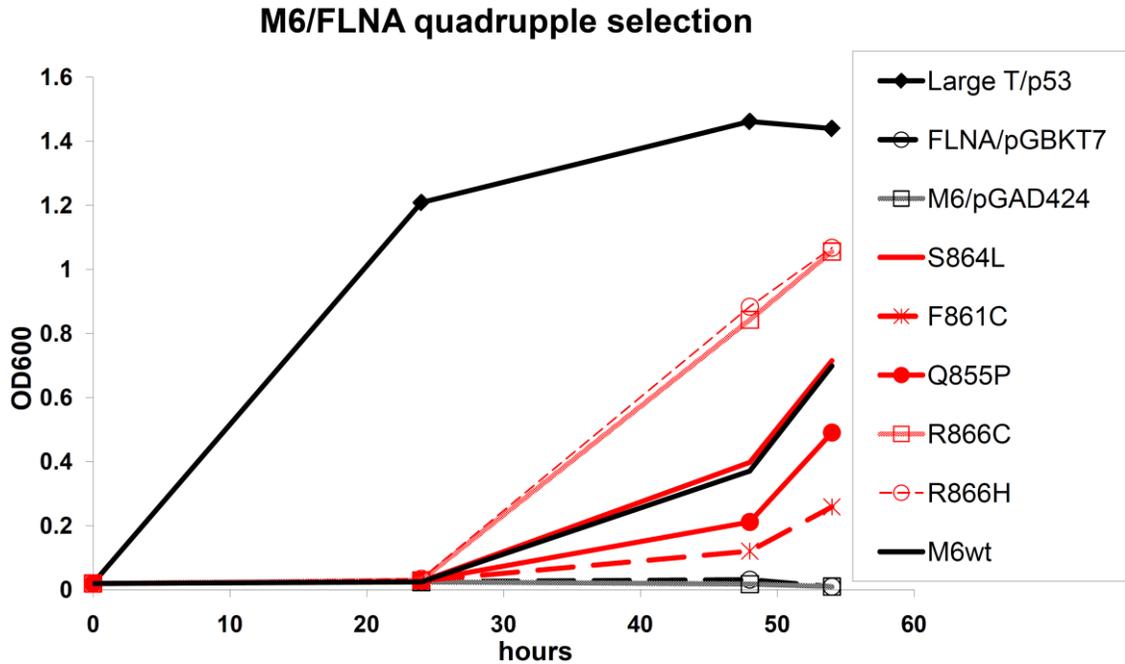
Figure 14. Interaction between pGBKT7 Motif 6 mutants (aa 845-869) and pGAD424 FLNA (aa 2477-2647) constructs. *S.cerevisiae* strain AH109 (Clontech), transformed with the indicated constructs, was grown in medium lacking leucine, tryptophan, histidine, and adenine (-leu-trp-his-ade, quadruple selection). OD_{600} of the cultures (y axis) is plotted against time (x axis). Large T Antigen (aa 86-708) and p53 (aa 72-309) were used as a positive control and pGBKT7/Motif6+pGAD424 and pGAD424/FLNA+pGBKT7 as negative controls. Note that none of the yeast, transformed with the M6/FLNA constructs grew within the first 24 hours, indicating that the interaction between BRCA1 Motif 6 and FLNA is weak.

70
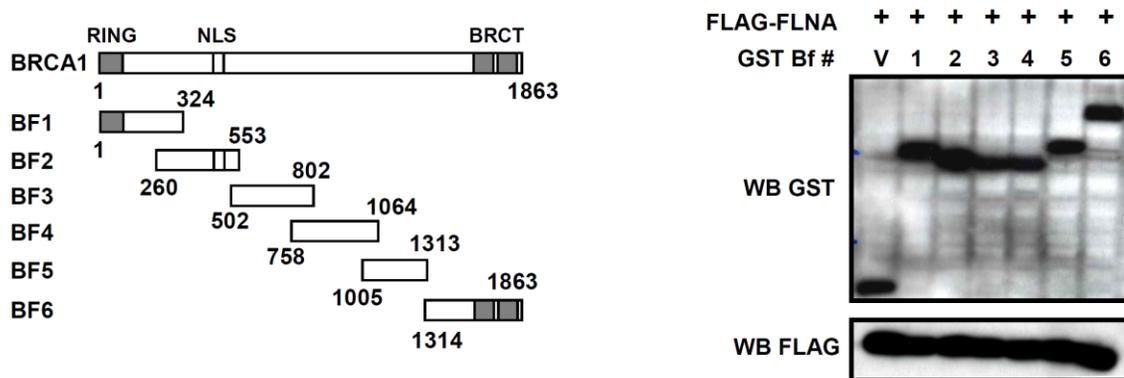
Figure 15. Expression of BRCA1 fragments in 293FT cell line. *Left panel,* diagram of deletion constructs used to map the BRCA1 interaction site to FLNA. RING, RING finger domain; NLS, nuclear localization signals; BRCT, BRCA1 C-terminal domains. *Right panels,* co-expression of GST-fragments of BRCA1 and FLAG-FLNA (aa 2477-2647) in 293FT cells. Lower molecular weight band obtained in the empty vector (V) transfection corresponds to GST.
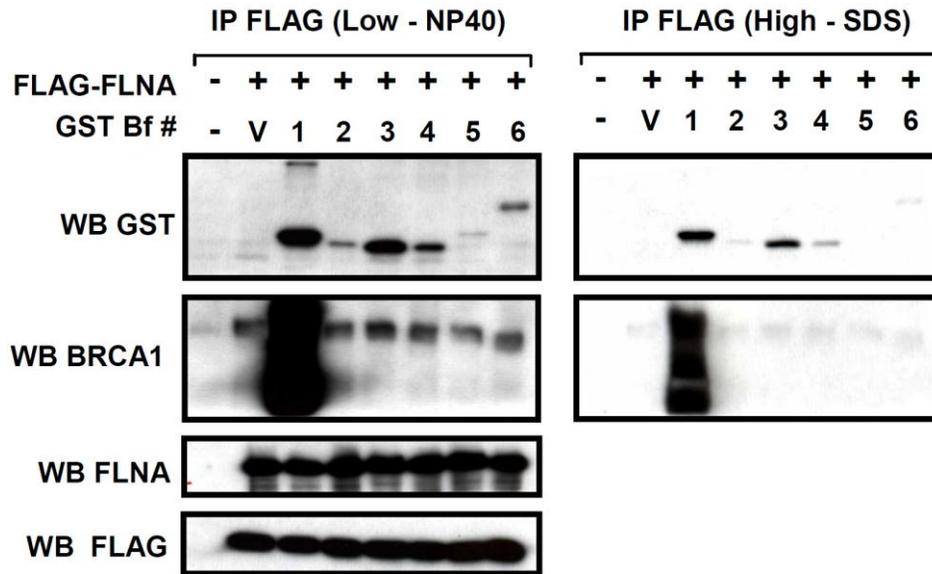
Figure 16. FLNA interacts with multiple sites in BRCA1. Co-immunoprecipitation of BRCA1 fragments (WB GST), endogenous BRCA1 (WB BRCA1) with FLAG-FLNA (aa 2477-2647). Note that endogenous FLNA is also immunoprecipitated by FLAG-FLNA (aa 2477-2647) confirming it is in the native conformation (WB FLNA). Strong reactivity shown for GST BF1 is due to recognition to the GST-BRCA1 fragment that contains the epitope for the antibody.
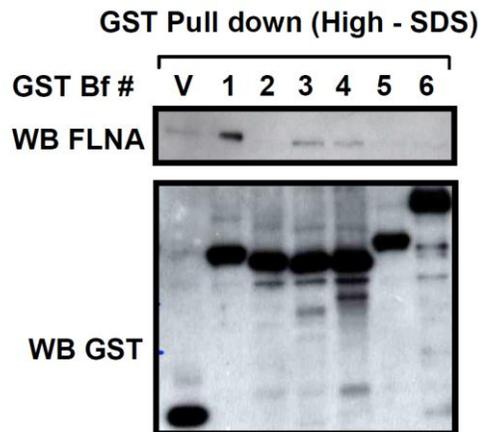
Figure 17. GST-pull down experiments show that GST-BRCA1 fragments 1, 3, and 4 can precipitate endogenous FLNA. Co-immunoprecipitation of BRCA1 fragments (WB GST) and endogenous FLNA (WB FLNA).

73
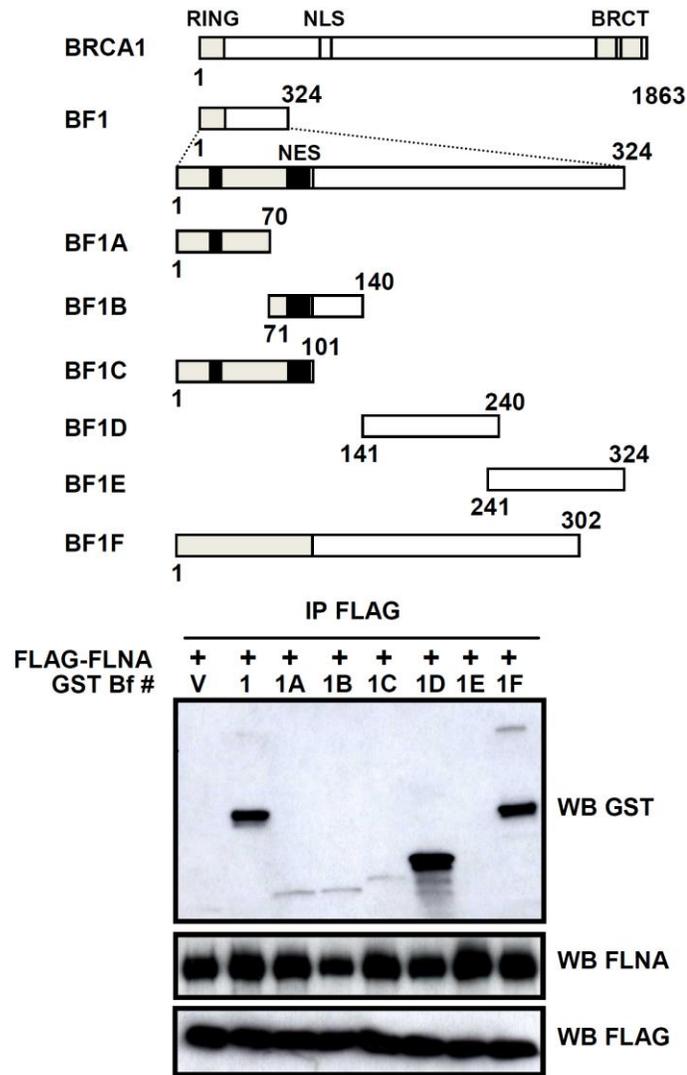
Figure 18. FLNA binds to aa 141-240 in BRCA1. *Upper panel,* diagram of deletion constructs used to map the interaction site to FLNA. NES, nuclear export sequence (black boxes). *Lower panels,* FLAG-FLNA (aa 2477-2647) interacts strongly with GST-BRCA1 fragments BF1 (aa 1-324), BF1D (aa 141-240), and BF1F (aa 1-302).
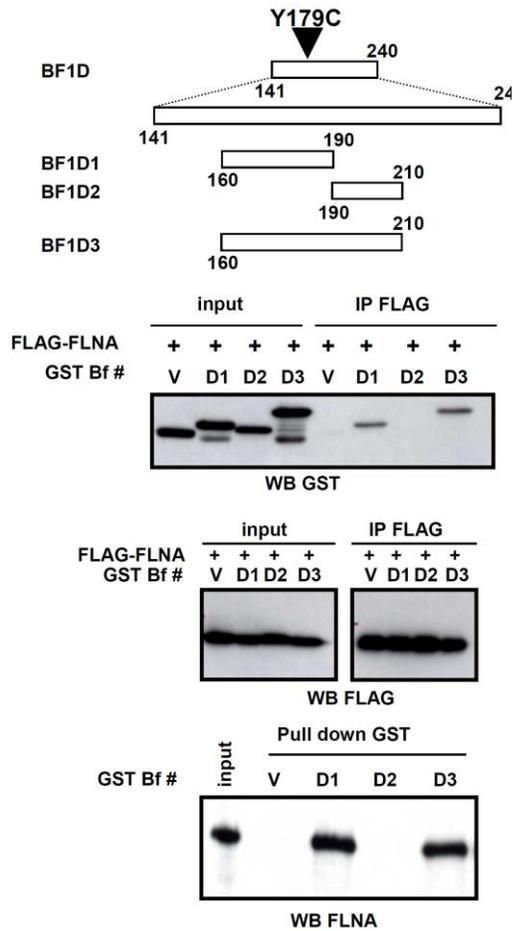
Figure 19. FLNA binds to aa 160-190 in BRCA1. *Upper panel,* diagram of deletion constructs of fragment aa 141-240 used to map the interaction site to FLNA. The location of the missense variant Y179 is indicated. *Middle left panel,* FLAG-FLNA (aa 2477-2647) co-immunoprecipitates with GST-BRCA1 fragments BF1D1 (aa 160-190) and BF1D3 (aa 160-210). V, GST; D1, GST-BRCA1 fragment BF1D1 (aa 160-190); D2, GST-BRCA1 fragment BF1D2 (aa 190-210); D3, GST-BRCA1 fragments BF1D3 (aa 160-210). *Bottom panel,* control for expression levels. *Middle right panel,* GST-BRCA1 fragments BF1D1 (aa 160-190) and BF1D3 (aa 160-210) can pull down endogenous FLNA.

FLNA contains the dimerization domain for two FLNA subunits (Figure 9). In addition, western blot against BRCA1 showed that FLAG-FLNA interacts with endogenous BRCA1 (Figure 16, left panel). Moreover, western blot against GST revealed the interaction of FLNA with different fragments of BRCA1 under low stringency (Figure 16, left panel). Interaction with Fragments 1 (aa 1-324), 3 (aa 502-802), and 4 (aa 758-1064) was detected even under high stringency conditions (Figure. 16, right panel). Reverse pull-downs of endogenous FLNA using GT-beads confirmed that the interaction is mediated by BRCA1 Fragments 1, 3, and 4 (Figure 17). In both experiments, BRCA1 Fragment 1 showed the strongest interaction (Figures 16 and 17).

Fragment 1 (aa 1-324) includes the RING finger (aa 1-101) [13] and nuclear export signals (aa 22-30 and aa 81-99) [144, 234]. To determine whether the interaction was mediated by these motifs, we used deletion mutants of BRCA1 Fragment 1 (Figure 18). Initially, we identified BRCA1 residues 141-240 as the interacting region to FLNA (aa 2477-2647) (Figure 18). Further mapping identified residues 160-190 as the minimal region required for binding (Figure 19). This region, called Motif 2, had been previously identified as a conserved motif in *BRCA1* orthologs [94, 145].

BRCA1 Variant Y179C, Found in Breast and Ovarian Cancer Patients, Disrupts the

Binding between BRCA1 and FLNA

To assess whether BRCA1 and FLNA interaction might contribute to breast cancer, we searched the Breast Cancer Information Core database (http://research.nhgri.nih.gov/bic/) for variants in this region. Variant Y179C is a frequent missense change recorded in the database (BIC Database). The introduction of BRCA1 Y179C mutant significantly reduced BRCA1 interaction to FLAG-FLNA aa 2477-2647 and to endogenous FLNA (Figure 20, upper and middle panels), further demonstrating the specificity of the interaction. Because other regions in BRCA1 except for Motif 2 also contributed to the binding, we investigated whether the Y179C mutation would disrupt binding to FLNA in the context of full length BRCA1. The introduction of Y179C mutation significantly reduced the interaction in the full-length context as compared to wild type BRCA1 (Fig. 19, bottom panel). In summary, these experiments demonstrate that Motif 2 primarily mediates the interaction with FLNA. Taken together, these data raised the possibility that lack of FLNA might impair BRCA1 foci formation after DNA damage. Thus, the following experiments were directed at assessing the role of FLNA/BRCA1 interaction in the DNA damage response.
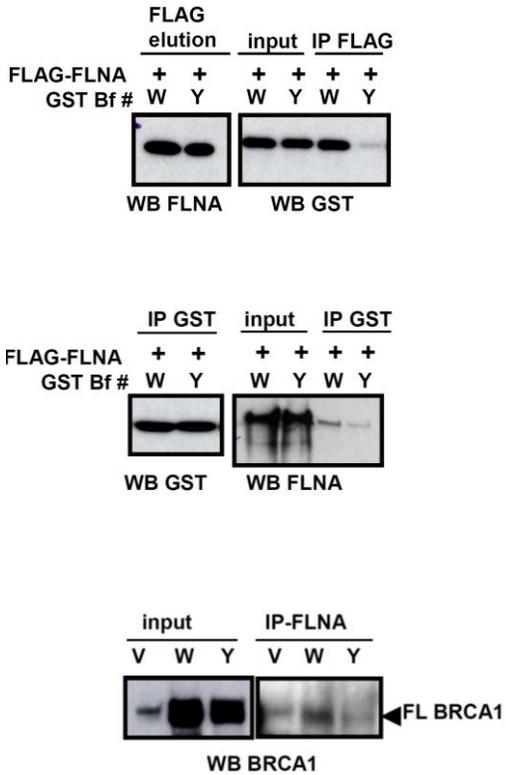
Figure 20. Introduction of BRCA1 Y179C mutation significantly reduces BRCA1 interaction to FLAG-FLNA aa 2477-2647 and to endogenous FLNA. W, wild type GST-BRCA1 fragment BF1D (aa 141-240); Y, GST-BRCA1 fragment BF1D with Y179C mutation. *Bottom panels*, Introduction of BRCA1 Y179C mutation into a full length BRCA1 context significantly reduces interaction to endogenous FLNA. W, wild type full length BRCA1; Y, full length BRCA1 carrying a Y179C mutation.

Analysis of the DNA Damage Response Signaling in FLNA Positive and Negative Cell Lines

FLNA Deficiency Does Not Cause a Defect in Sensing DNA Damage

To further characterize the functional significance of FLNA/BRCA1 interaction, we obtained the M2 melanoma cell line, which lacks FLNA, and its counterpart A7, which was obtained by reconstituting M2 cells with full length FLNA cDNA [227]. First, we assessed the kinetics of DSB repair after IR. We irradiated or mock treated the FLNA$^-$ and FLNA$^+$ cell lines and collected cells at several time points after IR. We monitored the presence of DSB with an antibody against histone H2AX phosphorylated at Serine 139 (γ-H2AX), a marker for DSBs [125] . Whereas the FLNA$^+$ cell line efficiently repaired DSBs, and by 8h after IR there was no detectable γ-H2AX (Figure 21), FLNA$^-$ cells had a sustained high level of γ-H2AX for up to 32h after IR. We confirmed this observation using Comet assays (Figure 22).

Figure 21. FLNA-null cells are deficient in DNA repair. FLNA[+] (A7) and FLNA[-] (M2) cells were irradiated with 8 Gy or mock-treated (U) and harvested at the indicated time points. While FLNA^{+} cells repair most of the DSBs (as measured by γ-H2AX) by 8h, FLNA^{-} cells show significant unrepaired DSBs even after 32h post-IR (*Top panel*). *Bottom panel* shows total levels of H2AX as a loading control.
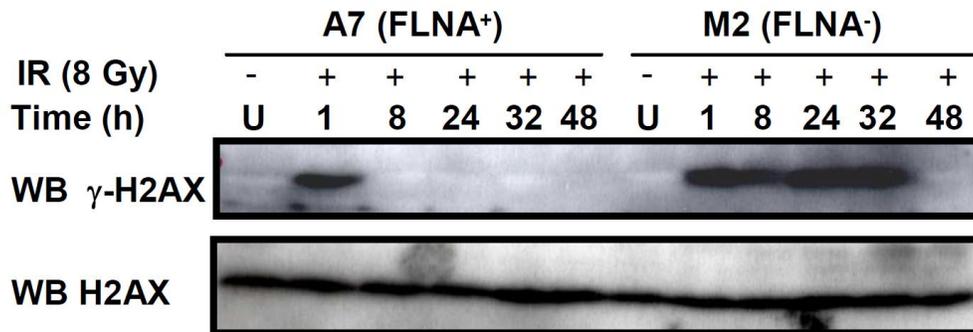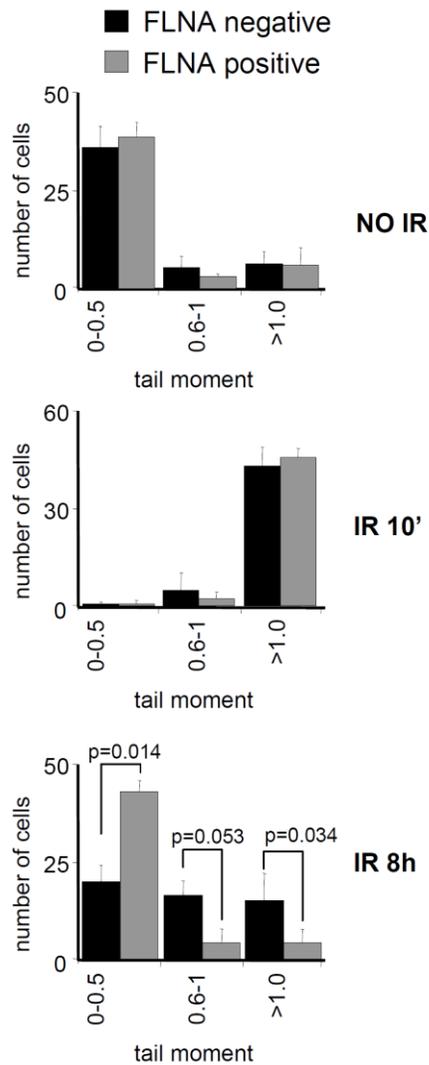
Figure 22. Neutral comet confirms that FLNA-null cells are deficient in repair. FLNA$^+$ (A7) and FLNA$^-$ (M2) cells were irradiated with 8 Gy or mock-treated (NO IR) and harvested at the indicated time points and comet assays were performed under neutral conditions. A two-tailed Student's T test was performed and *p* values are shown for statistically significant differences.

Further, we assessed whether cells lacking FLNA had a compromised DNA damage signaling. Thus, we tested whether ATM and ATR were properly activated upon DNA damage. The phosphorylation of ATM S1981 was not compromised in FLNA⁻ cells (Figure 23, Top panels). Likewise, the phosphorylation of CHK2 T68 and CHK1 S317, markers of ATM and ATR activation respectively, did not show a defect (Figure 24). Surprisingly, we consistently observed higher levels of phosphorylation of ATM, CHK2, and CHK1 in cells lacking FLNA (Figures 23, 24), which indicated the upregulation of ATM and ATR signaling. These results confirmed the previous data by Meng et al [231], showing the sustained activation of CHK2 and CHK1 in FLNA-deficient cells following damage.

The Defect in FLNA-Deficient Cells Is not Restricted to Ionizing Radiation

To determine whether the repair defect we observed was restricted to double strand breaks caused by IR, we tested a panel of agents that cause DNA damage by different mechanisms. First, we tested Camptothecin (CPT), a specific topoisomerase I inhibitor, by incubating cells with the drug for 1h and then removing it. The cells were then collected at several time points after the drug removal. Similar to what was found with IR, we noted that at early time points FLNA⁻ cells displayed increased and sustained activation of ATM (Figure 25). CHK2 also retained high levels of phosphorylation up to 6h after the drug removal in FLNA⁻ cells (Figure 25). Next, we investigated the phosphorylation status of DNA-PKcs at residues S2056 and S2609, which represent the

two main SQ/TQ clusters of phosphorylation [214]. Interestingly, while levels of phosphorylation in S2069 are similar in both cell lines, phosphorylation levels of S2056 were markedly diminished in FLNA⁻ cells (Figure 25). In addition, we saw the increased and sustained phosphorylation of CHK1, an indirect measure of ATR activation, and persistent phosphorylation of NBS1 even at 6h time point after the drug removal in cells lacking FLNA (Figure 26). The phosphorylation of RPA, which can be seen as a slower migrating band above the main band (Figure 27), was also increased and sustained up to 24h in FLNA⁻ cells.

Further, we tested the effect of Hydroxyurea (HU), a replication inhibitor that targets ribonucleotide reductase, by incubating cells with the drug for 24h and then removing it. The cells were then collected at several time points after the drug removal. In general, we verified that FLNA⁻ cells, treated with HU, displayed the increased and sustained phosphorylation of CHK2, CHK1, NBS1, and RPA (Figures 28, 29). These data are consistent with the abnormal activation of the DNA damage checkpoint after CPT and HU treatments.
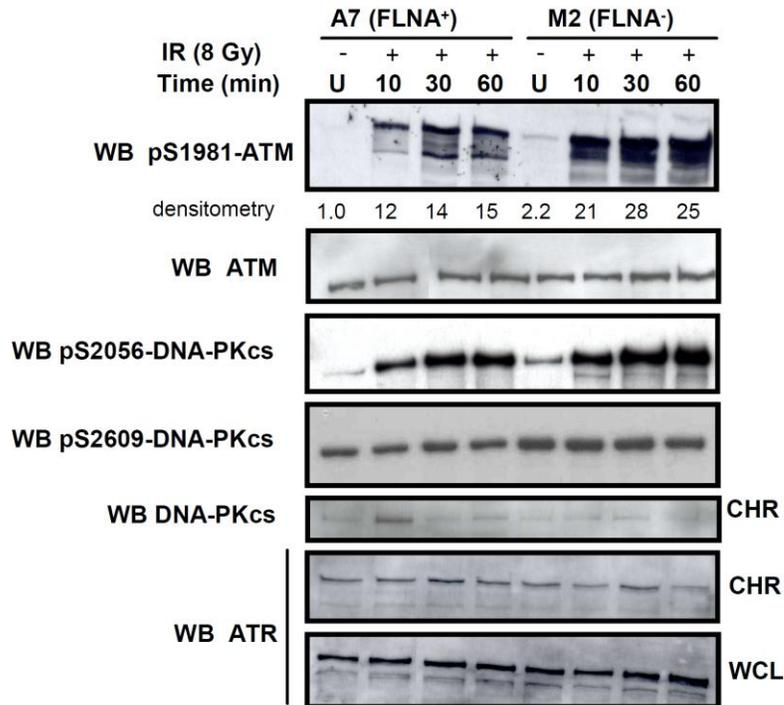
Figure 23. FLNA-null cells show no impairment in activating the DNA damage response. *Top two panels,* ATM activation as measured by phosphorylation of S1981 is not compromised in FLNA⁻ cells. Blot for total ATM is used as a loading control. Note significantly higher levels of pS1981-ATM in FLNA⁻ cells. *Middle three panels,* no significant difference was observed in levels of DNA-PKcs S2056 or S2609 phosphorylation but recruitment of DNA-PKcs to chromatin is defective in FLNA⁻ cells. *Bottom two panels,* ATR presence in chromatin (CHR) is shown. Blot for ATR levels in whole cell lysates is used a loading control.
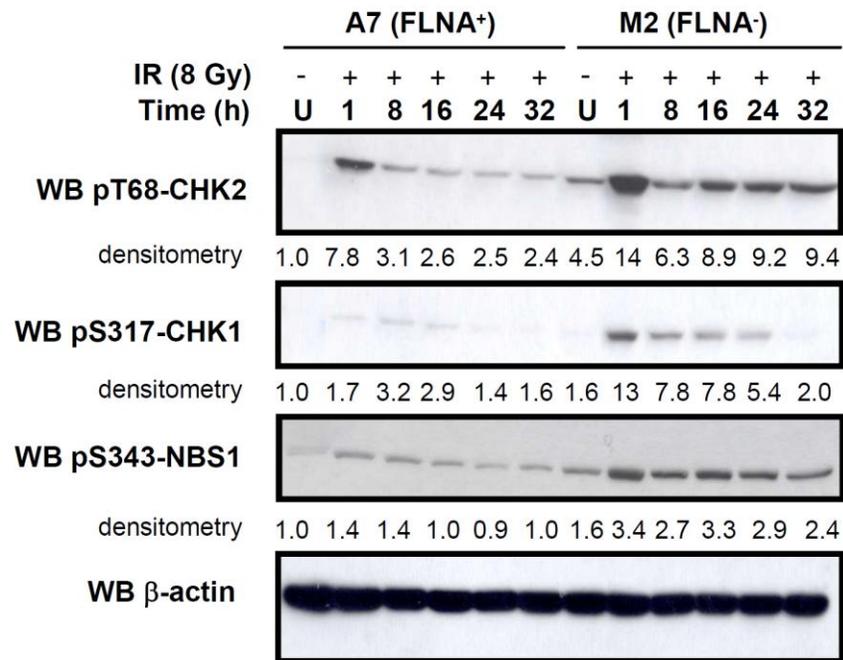
Figure 24. CHK2, CHK1, and NBS1 activation as measured by pT68-CHK2, pS317-CHK1, and pS343-NBS1, respectively is not compromised in FLNA⁻ cells. Note consistently higher levels of pT68-CHK2, pS317-CHK1, and pS343-NBS1 in FLNA⁻ cells. β-actin is used a loading control.
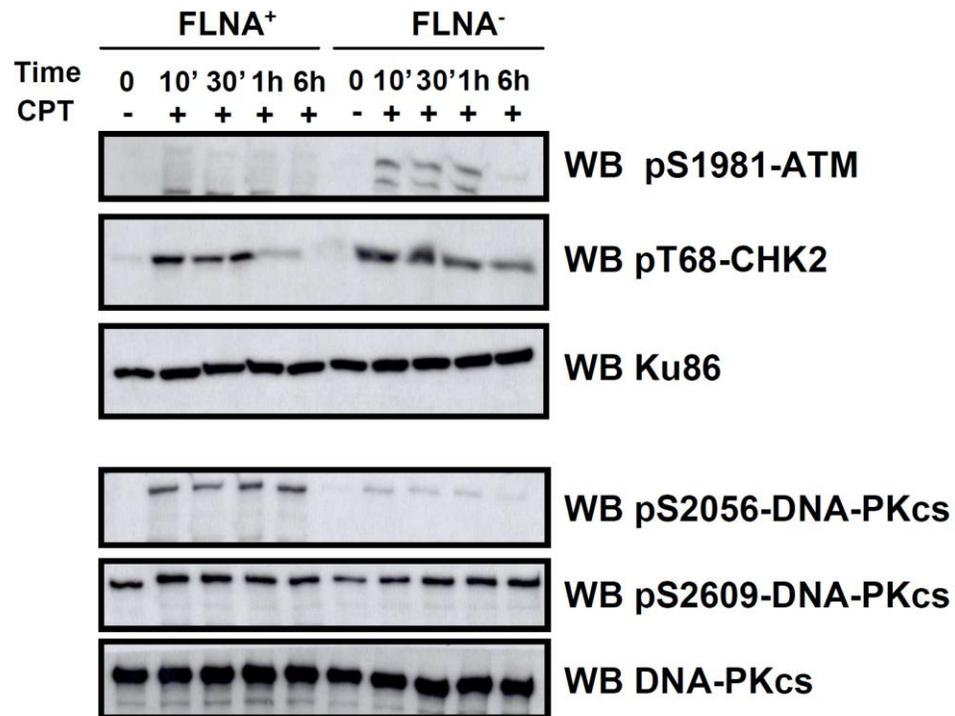
Figure 25. Effects of the CPT treatment on the DNA damaging signaling in FLNA-deficient and proficient cell line-ATM, CHK2 and DNA-PKcs activation.
Note increased phosphorylation levels of S1981 in ATM in earlier time points found in FLNA⁻ cells after Camptothecin (CPT) treatment (first panel). FLNA⁻ cells also displayed retention of phosphorylation of T68 in CHK2 even at 6h after drug removal (second panel). Levels of Ku86 were used as loading control (third panel panel). FLNA-deficient cells also display diminished levels of DNA-PKcs S2056 phosphorylation (fourth panel) but not of S2609 (fifth panel). Total levels of DNA-PKcs were used as loading control (sixth panel).
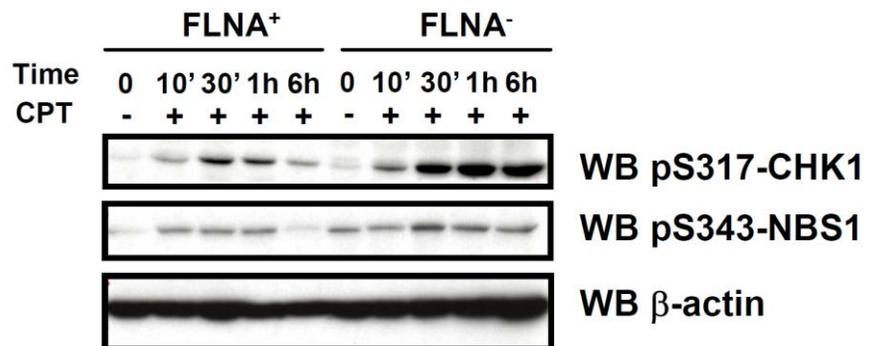
Figure 26. Effects of CPT treatment on the DNA damage response signaling in FLNA-deficient and proficient cell line-CHK1 and NBS1 activation. Levels of phosphoserine 317 in CHK1 progressively increase in FLNA⁻ remaining elevated even at 6h after drug removal (Top panel). Note also the retention of high levels of phosphorylation at S343 in NBS1 (middle panel). Levels of β-actin were used as loading control (bottom panel).

Figure 27. Effects of CPT treatment on the DNA damage response signaling in FLNA-deficient and proficient cell line-RPA phosphorylation. Deficiency in FLNA lead to increased and sustained phosphorylation of chromatin bound RPA as measured by the slower migrating band of RPA (top panel). Levels of Histone H2AX are used as loading controls (bottom panel).

Figure 28. Effects of HU treatment on the DNA damage response signaling in FLNA-deficient and proficient cell line-CHK1, CHK2 and NBS1 activation. Note increased phosphorylation levels of T68 in CHK2 found in FLNA⁻ cells after treatment with Hydroxyurea (first panel). FLNA⁻ cells also displayed retention of phosphorylation of S317 in CHK1 even at 6h after drug removal (second panel). Levels of Ku86 were used as loading control (third panel). FLNA⁻ also displayed the retention of high levels of phosphorylation at S343 in NBS1 in late time points (fourth panel).
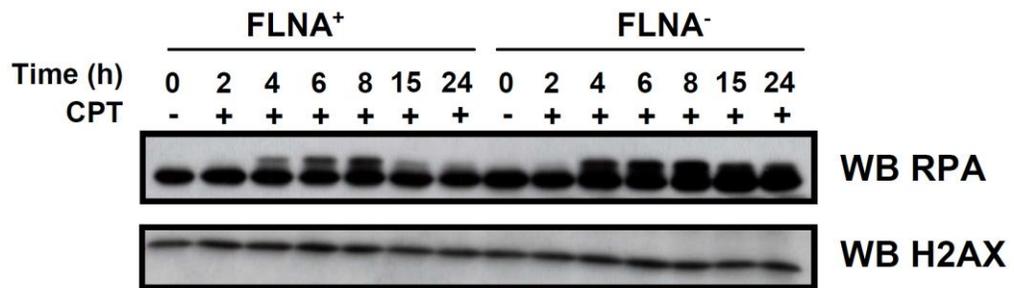
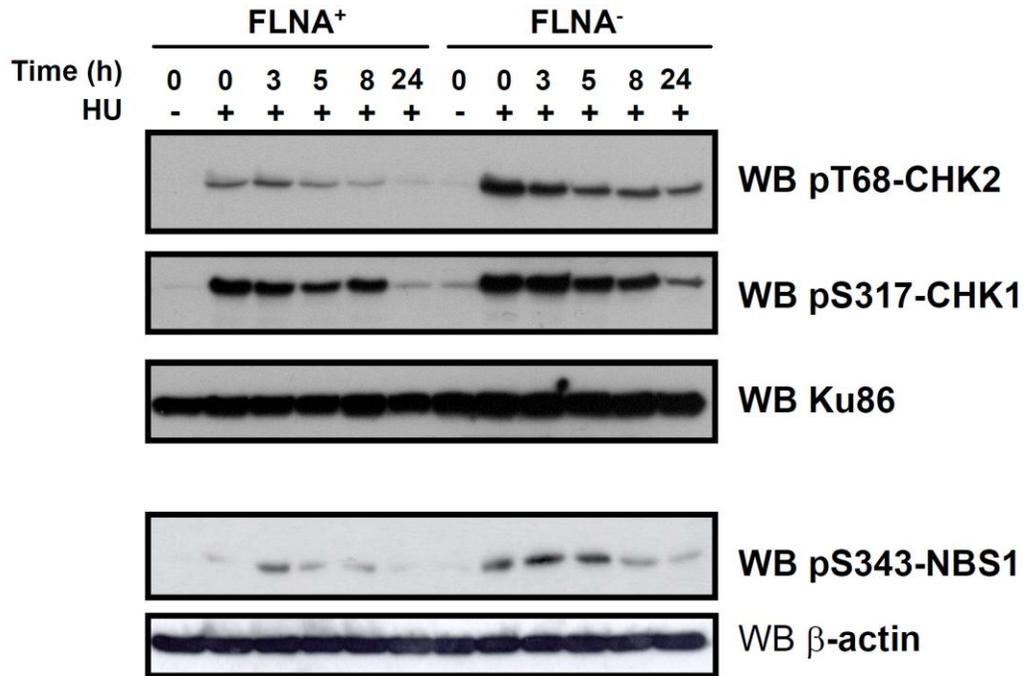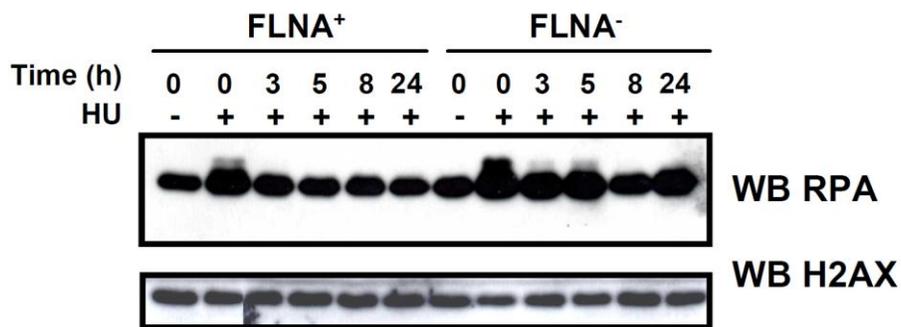**Figure 29. Effects of HU treatment on the DNA damage response signaling in FLNA-deficient and proficient cell line-RPA phosphorylation.** Deficiency in FLNA also lead to increased and sustained phosphorylation of chromatin bound RPA after Hydroxyurea treatment (top panel). Levels of Histone H2AX were used as loading controls (bottom panel).

FLNA Deficiency Impairs BRCA1 and Rad51 Foci Formation

To determine whether the deficiency in repair was due to the defective

recruitment of factors required for the DDR, we performed immunofluorescence analysis

in non-irradiated or irradiated cells at 1h and 24h time points after IR. The accumulation

of γ-H2AX and pS343-NBS1, early markers of DNA damage, was comparable in both

cell lines at 1h time point (Figure 30). In order to determine if there were small

differences, we quantified foci-positive cells (Figure 30, lower panel). The results were

comparable in both cell lines at 0 and 1 h after IR, but FLNA-negative cells showed the

increased number of foci-positive cells after 24h. Likewise, the recruitment of mediator

proteins MDC1 and 53BP1 was also comparable at 1h time point (Figure 31). Finally, the

repair factor RPA did not show any difference between the cell lines at 1h time point

(Figure 32). Consistent with our western blot results (Figures 21 and 24) where we

detected abnormally high levels of γ-H2AX, pT68-CHK2, and pS317-CHK1 at 24h time

point, we detected persistent foci of γ-H2AX, pS343-NBS1, and RPA at 24h time point

after irradiation only in FLNA-deficient cells (Figures 30, 31, and 32). Thus, the repair

defect in FLNA-deficient cells was not due to a failure into initiating the DNA damage

response.

Next, we investigated the ability of BRCA1 and Rad51 to form IR-induced foci.

The criteria for scoring cells as foci-positive and foci-negative are shown on Figure 34.

The detailed analysis showed that FLNA-deficient cells are unable to efficiently form

BRCA1 IR-induced foci as compared to FLNA-proficient cells (Figure 31, bottom panel).
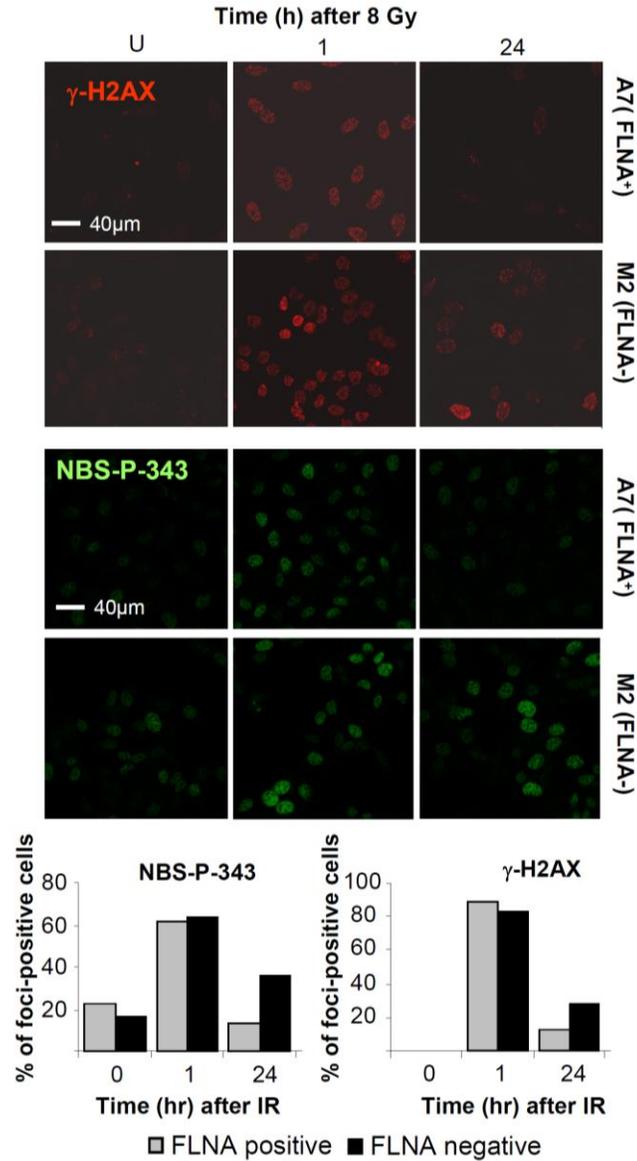
Figure 30. FLNA-null cells show no impairment in recruiting DNA damage response factors to IR-induced foci-**γ-H2AX and NBS1.** Early markers of DNA damage γ-H2AX (red) and phosphoserine 343 NBS1 (green) form foci irrespective of FLNA status. Note maintenance of foci after 24h only in FLNA⁻ cells. Lower panels show quantification of foci-positive cells (≥ 20 foci).

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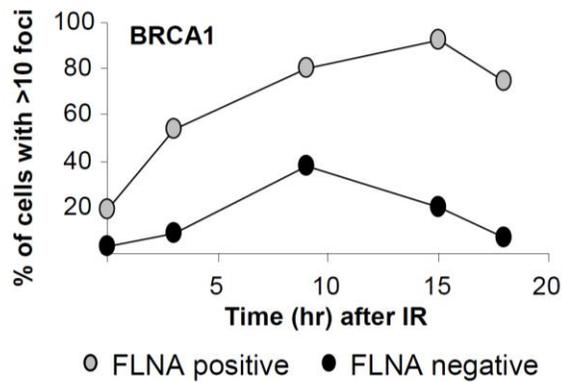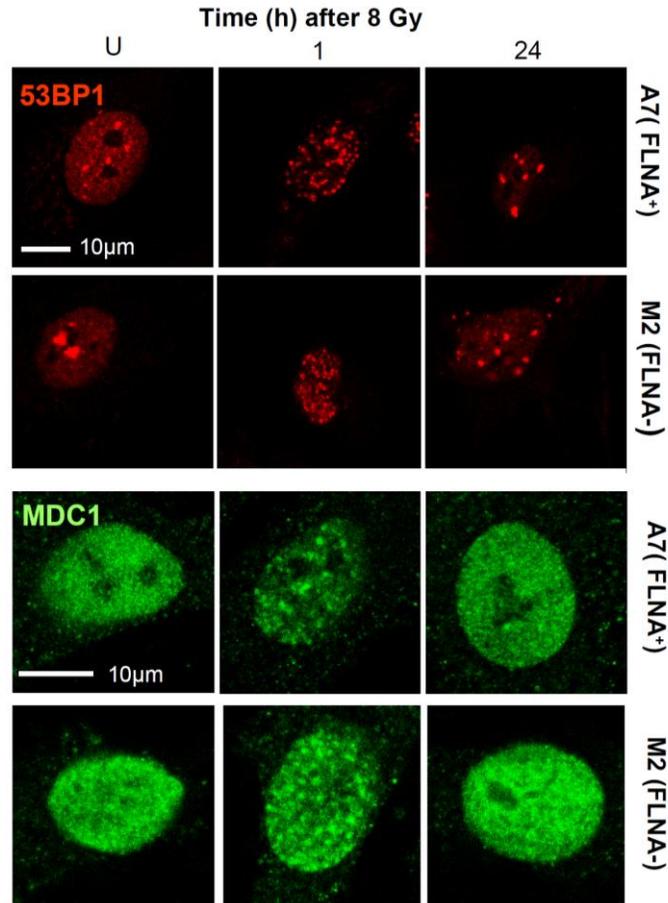
Figure 31. Recruitment of DNA damage response mediator proteins 53BP1 (red, top panel) and MDC1 (green, middle panel) and BRCA1 to foci. Recruitement of 53BP1 and MDC1 is comparable at early time points. BRCA1 foci formation was compromised in FLNA-deficient cells (lower panel).

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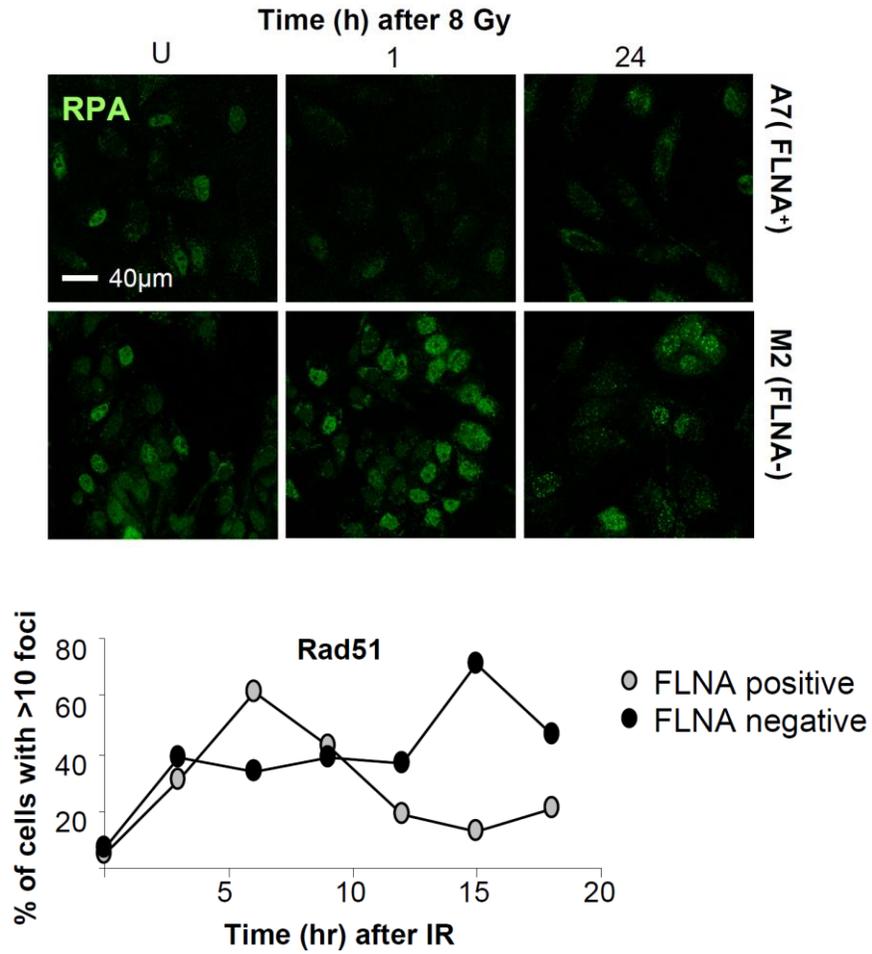
Figure 32. Recruitment of RPA and Rad51 to IR induced nuclear foci. Recruitment of repair factor p34 RPA (green, top panel) did not show any difference between the cell lines at the early time points. FLNA-deficient cells displayed a delayed kinetics of Rad51 foci formation (bottom panel).

Although Rad51 displayed a comparable initial response at 3h time point after IR, it failed to mount a response comparable to FLNA-proficient cells at 6h time point after IR. Rad51 presented a delayed kinetics of foci formation with the peak at 15h time point in FLNA-deficient cells (Figure 32, bottom panel). Taken together these data suggest that the compromised repair capacity in FLNA-deficient cells may, at least partially, be mechanistically tied to the inefficient HR.

The Lack of FLNA Leads to the Accumulation of ssDNA after the DNA Damage

During our analysis, we noted that RPA foci in FLNA-deficient cells were not only persistent 24h after the DNA damage, but also significantly larger (Figure 32). To determine whether the RPA foci were associated with chromatin, we pre-extracted cells with Triton X100 before fixation. This method has successfully been used to detect only tightly bound to chromatin fraction of RPA [235]. Interestingly, FLNA-deficient cells accumulate large chromatin-bound RPA foci, whereas FLNA$^+$ cells present fewer and smaller chromatin-bound RPA foci at 24h time point after IR (Figure 33). While most FLNA$^+$ cells have recovered from G2/M arrest and represent an asynchronous population at 24h time point, most FLNA$^-$ cells remain arrested in G2/M at 24h time point after IR [231]. Thus, these large tracts of ssDNA, found in FLNA$^-$ cells, are unlikely to be due to the replication foci.
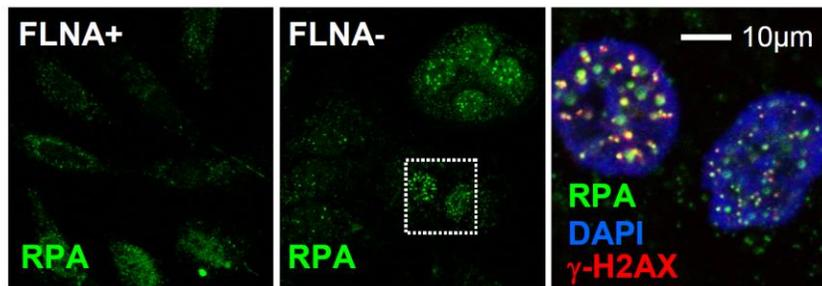
Figure 33. FLNA-deficient cells present with large chromatin-bound RPA foci at 24h after IR. Higher magnification of FLNA$^+$ and FLNA$^-$ cells after 24h post-IR. Left panel shows FLNA$^+$ cells stained for RPA (green). Middle panel shows FLNA$^-$ cells stained for RPA (green). Note that nuclear foci are significantly larger than in FLNA$^+$ cells. Right panel shows a blow up of the inset (white square in middle panel) with staining for DAPI (blue), RPA (green), and γ-H2AX (red).
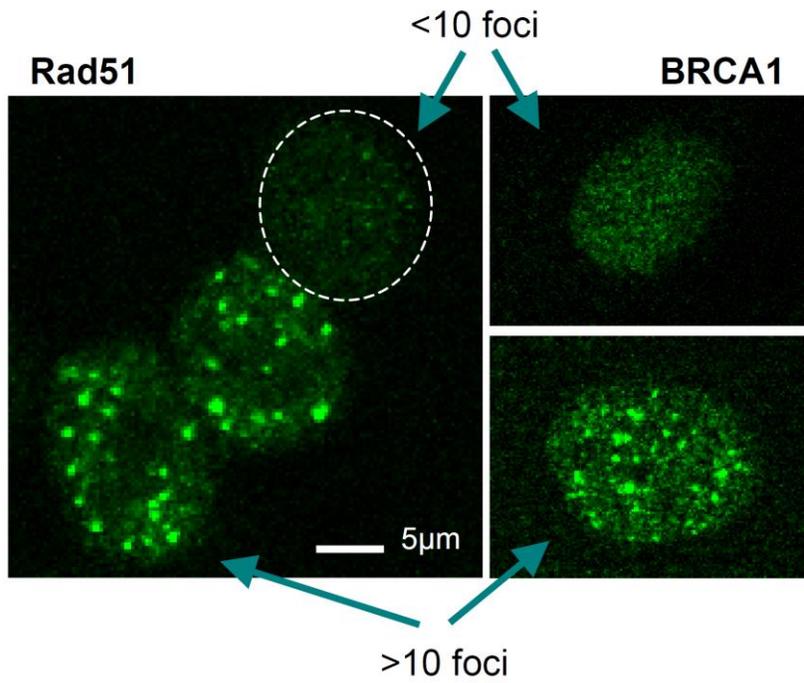
Figure 34. Criteria for scoring foci positive cells. Examples of foci-positive cells for BRCA1 and Rad51.

Analysis of the Expression of BRCA1-Interacting Fragment of FLNA and FLNA-Interacting Fragment of BRCA1 in FLNA Positive Cell Line

Expression of BRCA1-Interacting Fragment of FLNA Phenocopies the Loss of FLNA

To gain more insight into the mechanism by which FLNA participates in DNA repair, we transfected FLNA$^+$ and FLNA$^-$ cell lines with flag-tagged Filamin A aa 2477-2647 construct (BRCA1-interacting fragment). For the sake of simplicity, we will refer to this <u>FLNA</u> <u>B</u>RCA1-interacting <u>f</u>ragment as FLNA-Bf. At 24h post transfection, we irradiated cells with 8Gy IR and collected samples at different time points. The transfection of FLNA-Bf did not lead to the checkpoint recovery in FLNA$^-$ cells as measured by the phosphorylation of CDC2 Y15 (Figure 35). Interestingly, transfection of the same fragment in FLNA$^+$ cells led to a similar phenotype as that found for FLNA$^-$ cells as shown by phosphorylation of CDC2 Y15 and H2AX S139 (Figure 36). We also confirmed that expression of FLNA-Bf acts in a dominant negative fashion in a stable transfection context (Figure 37). We generated HCT116 cells stably expressing GFP-FLNA-Bf or GFP alone that were mock-treated and irradiated. Cells expressing GFP-FLNA-Bf retained high levels of phosphorylated H2AX up to 32h after damage while cells expressing GFP alone showed levels returning to unirradiated levels at 8h after damage (Figure 37).
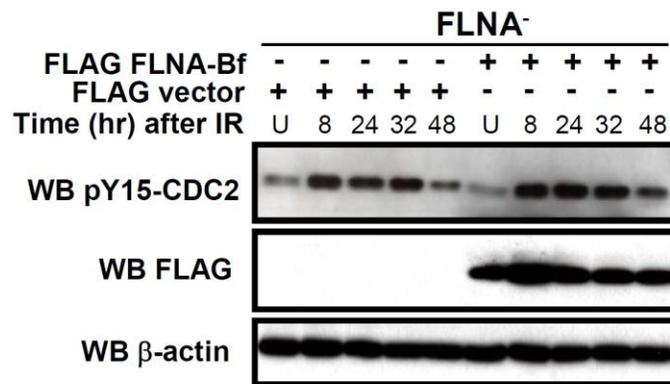
Figure 35. Expression of BRCA1-interacting fragment of FLNA in FLNA⁻ is unable to reverse the checkpoint recovery defect. FLNA⁻ cells were transfected with an empty FLAG vector or a FLAG FLNA-Bf constructs. Cells were mock-treated (U) or treated with 8 Gy IR and cells were collected at different time points. Expression of FLAG FLNA-Bf was unable to reverse the recovery defect.
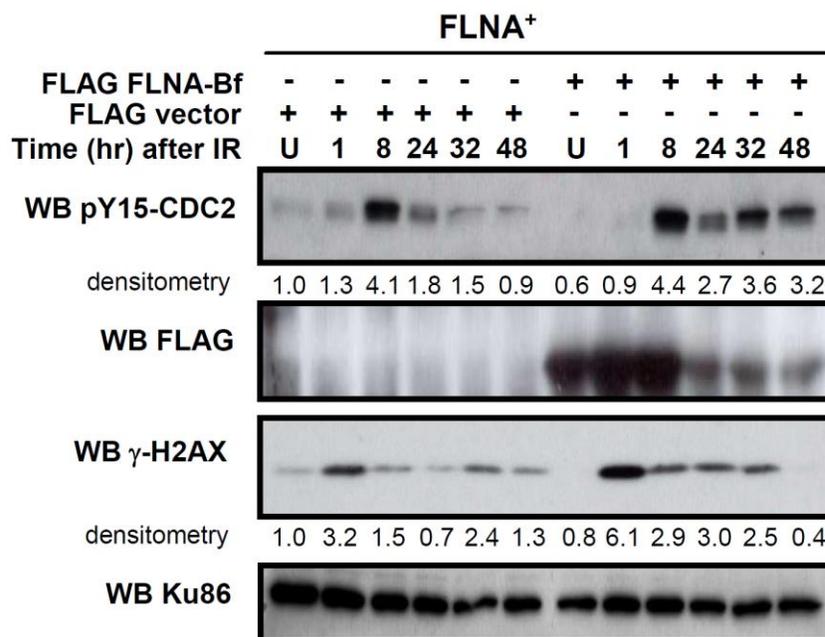
Figure 36. Expression of BRCA1-interacting fragment of FLNA phenocopies loss of FLNA. FLNA^{+} cells were transfected with empty FLAG vector or a FLAG FLNA-Bf constructs. Cells were mock-treated (U) or treated with 8 Gy IR and cells were collected at different time points. Cells expressing of FLAG FLNA-Bf displayed a phenotype similar to FLNA^{-} cells.
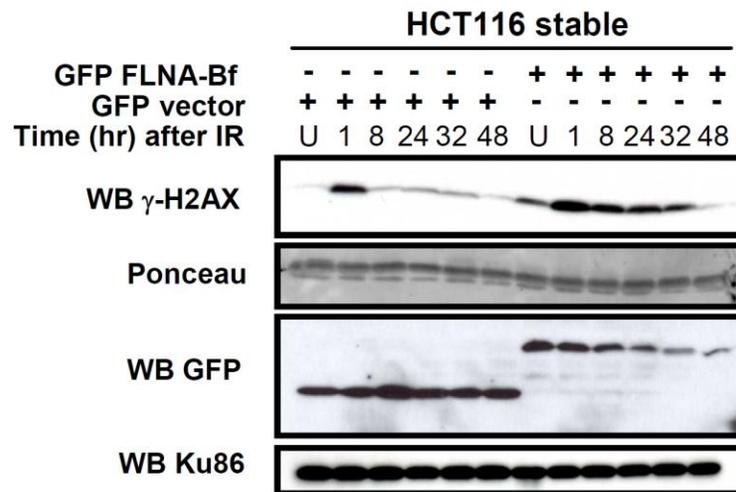
Figure 37. HCT166 cells stably expressing GFP FLNA-Bf display a phenotype similar to FLNA⁻ cells. . HCT116 cells were stably transfected with empty GFP vector or a GFP FLNA-Bf constructs. Cells were mock-treated (U) or treated with 8 Gy IR and cells were collected at different time points. Cells expressing of GFP FLNA-Bf displayed a phenotype similar to FLNA⁻ cells.

Expression of BRCA1-Interacting Fragment of FLNA Phenocopies Loss of FLNA

Next we asked whether expression of a GST-tagged BRCA1 FLNA-interacting -
fragment (BRCA1-Ff) could also lead to a dominant negative phenotype (Figure 38). In
order to verify the specificity of the interaction we transfected a mutated BRCA1-Ff
carrying the Y179C mutation and determined whether it lead to a dominant negative
phenotype. Introduction of the Y179C mutation (Figure 19) significantly reduced the
BRCA1-FLNA interaction. The wild type BRCA1-FLNA-Ff led to increased and
sustained phosphorylation of CDC2 Y15 and H2AX (Figure 38) while the BRCA1-Ff
Y179C (Figure 38) displayed a dominant negative effect that is intermediate between
vector control (Figure 36) and the wild type construct (BRCA1-Ff; Figure 38). This
intermediate effect could be due to the residual binding of BRCA1-Y179C mutant to
FLNA. Alternatively, this could also be due to the inability of the mutant to disrupt the
binding of FLNA to other regions of endogenous BRCA1 that participate in the
interaction (Figure 16).
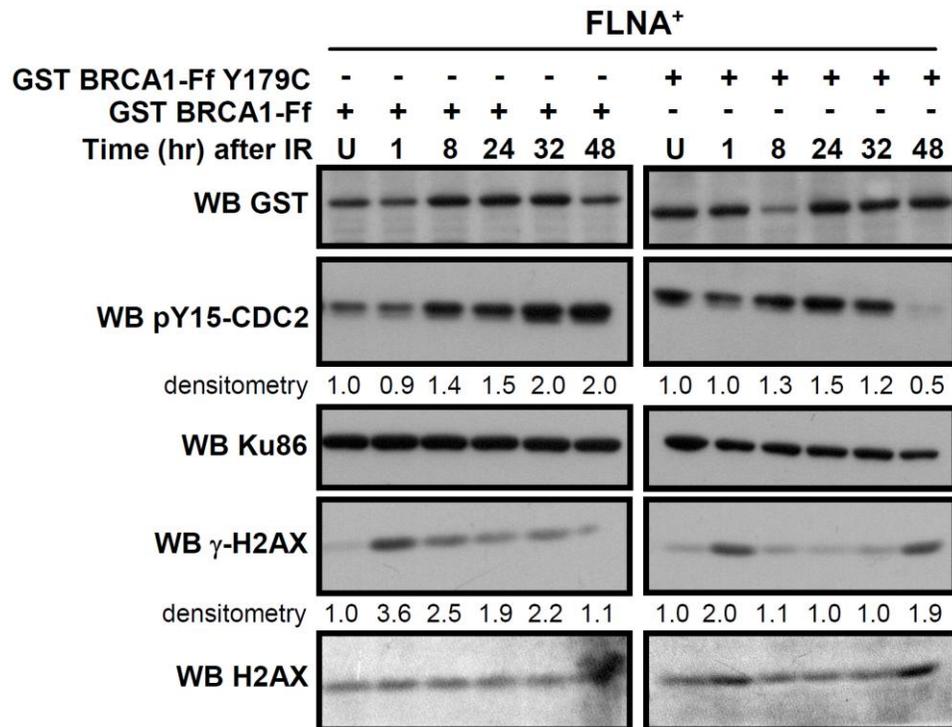
Figure 38. Expression of FLNA-interaction fragment of BRCA1 Y179C mutant does not phenocopy loss of FLNA. FLNA^{+} cells were transfected with a GST BRCA1-Ff or a GST BRCA1-Ff Y179C. Cells were mock-treated (U) or treated with 8 Gy IR and cells were collected at different time points. Only cells expressing of GST BRCA1-Ff but not GST BRCA1-Ff Y179C displayed a phenotype similar to FLNA^{-} cells, confirming that the effect is specific.

Analysis of the Effect of FLNA on DNA-PKcs and Ku86 Interaction

FLNA is Required for Efficient Interactions Between DNA-PKcs and Ku86

Our previous experiments demonstrated that FLNA$^-$ cells display defective DNA repair, showed signs of compromised HR, and accumulated large tracts of ssDNA. Because mammalian cells also repair DSBs using non homologous end joining (NHEJ) we hypothesized that lack of FLNA also had an impact on the NHEJ pathway.

First, we tested whether FLAG-FLNA aa 2477-2647 interacted with NHEJ factors. FLAG-FLNA aa 2477-2647 immunoprecipitated DNA-PKcs in 293FT cells independent of DNA damage (Figure 39). To determine whether FLNA was required for the stability of the Ku86/DNA-PKcs complex we performed immunoprecipitation experiments in FLNA$^+$ and FLNA$^-$ cell lines in the presence or absence of irradiation (Figure 40). Interestingly, in FLNA$^-$ cells Ku86 and DNA-PKcs complex formation was compromised in IR-treated and untreated cells (Figure 40).

Next we tested whether FLNA was required for Ku86 loading onto chromatin after DNA damage. Ku86 was efficiently recruited to chromatin upon DNA damage in the presence and absence of FLNA (Figure 41) while we detected DNA-PKcs in chromatin only in the presence of FLNA (Figure 23). Interestingly, loading of Ku86 onto chromatin persisted longer and with consistently higher levels in FLNA$^-$ than in FLNA$^+$ cells (Figure 41).

Finally, we tested whether BRCA1 was required to stabilize the interaction between DNA-PKcs and Ku86. As a model for studying BRCA1 functions in mammalian cells often is used HCC 1937 cell line. HCC 1937 cell line was established from 24-year-old breast cancer patient, who is a carrier of BRCA1 5382insC mutation [236] and does not express p53 due to acquired p53 mutation [236]. We examined *BRCA1*-deficient HCC1937 cell line [236] and a HCC1937 derivative reconstituted with full length BRCA1 (gift from J. Chen). Complex formation between Ku86 and DNA-PKcs was not dependent on BRCA1 under IR-treated or untreated conditions (Figure 42).
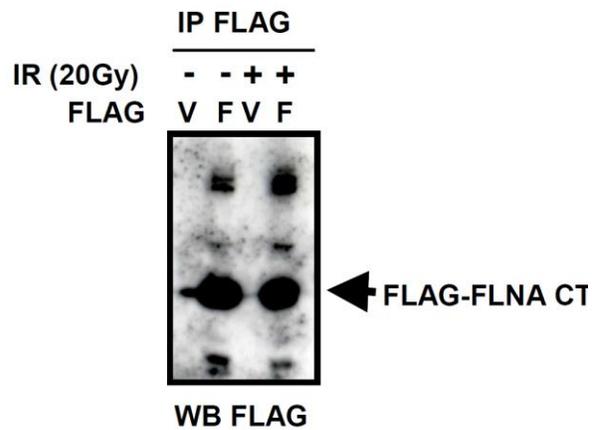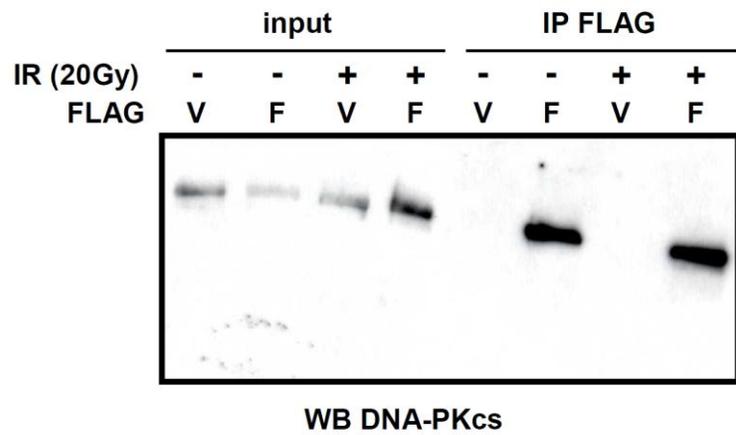
Figure 39. FLNA interacts in vivo with DNA-PKcs. *Upper pannel,* 293FT cells were transfected with FLAG-FLNA aa 2477-2647 (F) or empty FLAG vector (V) and mock-treated or irradiated with 20 Gy. Cells were collected after 1h and immunoprecipitated using α-FLAG antibody. FLAG-FLNA aa 2477-2647 co-immunoprecipitates DNA-PKcs in the presence and absence of IR. *Lower panel,* control for expression and the efficiency of the immunoprecipitation.
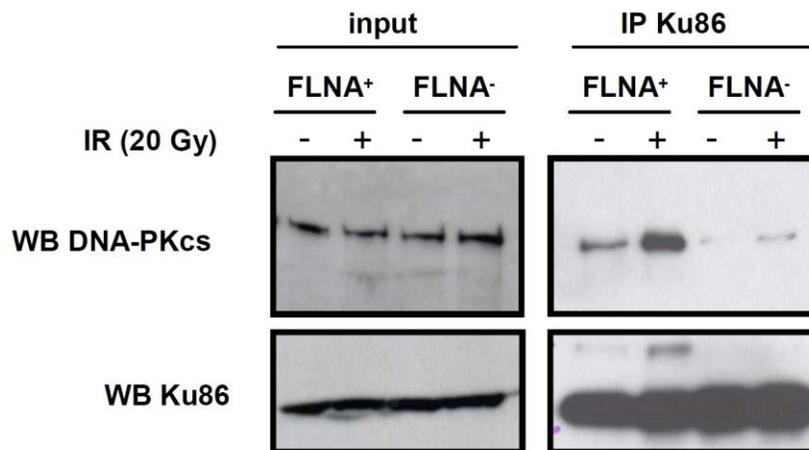
Figure 40. FLNA mediates DNA-PKcs interaction to Ku86. The interaction between DNA-PKcs and Ku86 is compromised in cells lacking FLNA. *Left panel* shows that levels of DNA-PKcs and Ku86 are similar in both cell lines and in the presence and absence of irradiation. *Right panel* shows that Ku86 and DNA-PKcs interact in FLNA^{+} cells in the absence of damage and complex formation is significantly increased in the presence of irradiation. Complex formation in the presence and absence of IR is severely compromised in FLNA^{-} cells.
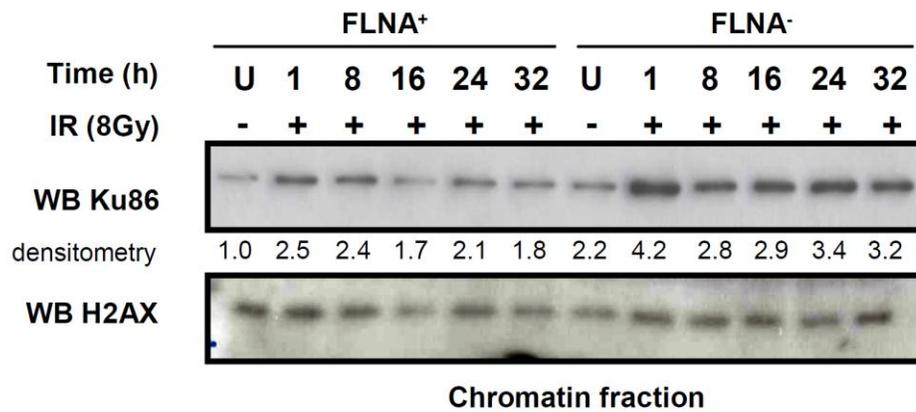
Figure 41. Loading of Ku86 onto chromatin after DNA damage is increased in FLNA⁻ cells. FLNA⁺ and FLNA⁻ cells were mock-treated (U) or treated with 8 Gy IR and cells were collected at different time points. FLNA⁻ cells show increased amounts of Ku86 after IR. Histone H2AX levels are used as a loading control.
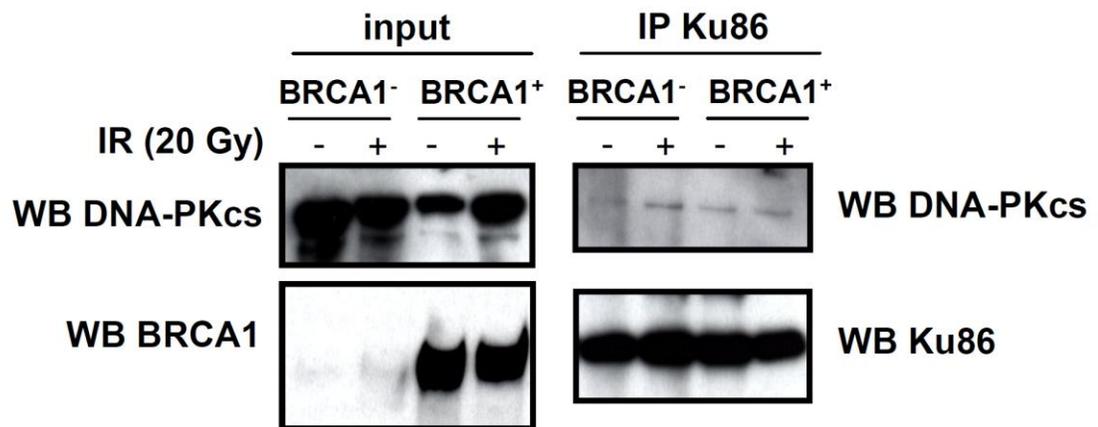
Figure 42. BRCA1 is not required for the stabilization of the interaction between DNA-PKcs and Ku86. HCC1937 (BRCA1-) and HCC1937-BRCA1wt (BRCA1+) were mock-treated or treated with 20 Gy IR and cells were lyzed after 1h. DNA-PKcs co-immunoprecipitated with Ku86 independent of BRCA1 status.

Lack of FLNA Leads to an Increase in End Joining Activity

Given the inability of DNA-PKcs to interact with Ku86 and thus to be efficiently loaded on chromatin we decided to assess in vivo end joining activity. In order to measure end joining we used the pGL2 plasmid that has a luciferase reporter gene driven by a constitutive promoter. First, we transfected FLNA$^-$ and FLNA$^+$ cells with undigested pGL2 plasmid or digested with either *Hind*III, which cuts the plasmid between the CMV promoter and the reporter gene, or *Eco*R1, which cuts the plasmid within the luciferase gene. Luciferase activity obtained by transfecting with the digested plasmids was measured as a percentage of the activity obtained in cells transfected with the undigested plasmid. Luciferase activity obtained from the *Hind*III-digested plasmid reflects total end-joining activity, while that obtained from *Eco*R1-digested plasmid reflects precise end-joining activity. Intriguingly, FLNA$^-$ displayed up to 4-fold higher total and precise end joining activity compared to FLNA$^+$ cells (Figure 43). In summary, lack of FLNA leads to an increase in end joining activity on an episomal template.
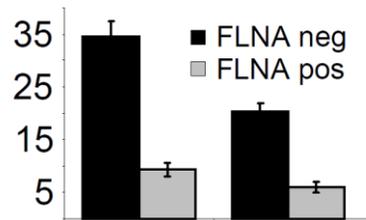
Figure 43. Lack of Filamin A leads to an increase in end joining activity. FLNA[-] and FLNA^{+} cells were co-transfected with a digested or undigested luciferase reporter plasmid and an internal *Renilla* luciferase control. The reporter plasmid was either cut between the promoter and the reporter gene or inside the coding region for luciferase and recovered luciferase activity is a measure of total (left) or precise (right) end joining, respectively. On the X axis the relative liciferase units are depicted.

DISCUSSION

In this work we shed light on the mechanism by which Filamin A (FLNA) is required for efficient DNA repair. Our data indicates that lack of FLNA impacts on both homologous recombination and non homologous end joining. FLNA is an actin-binding protein and its inactivation leads to an array of disorders such as otopalatodigital spectrum disorder, Melnick-Needles syndrome, and periventricular heterotopia [230]. Although of unclear significance, at least two families carrying germline mutations in *BRCA1* have been shown to manifest ventricular heterotopia [237, 238]. FLNA interacts with a variety of proteins, including BRCA2 [233] and deficiency in FLNA leads to sensitivity to DNA damage and a defect in the recovery from G2 arrest [231]. Thus, we investigated further its role in the DNA damage response.

FLNA binds BRCA1 using its extreme C-terminus which contains its dimerization domain. BRCA1 interaction with FLNA is mediated by a 30 amino acid region in the N-terminus of BRCA1 which contains a conserved domain called Motif 2 [145]. Introduction of the Y179C mutation in Motif 2 significantly decreases the interaction. Analyses by the Align GV-GD method or by a yeast-based recombination assay suggest that Y179C may act as a deleterious mutation [87, 239]. On the other hand, this variant has been found co-occurring in *trans* with a known deleterious mutation,

which indicates that it is unlikely to have severe effects [90]. Thus, the Y179C may constitute a hypomorphic mutation with moderate effects on breast cancer predisposition. Of note, Motif 2 is close to the region that has been implicated in binding of BRCA1 to Ku86 [218].

In order to dissect the molecular role of FLNA in the DDR we took advantage of a well-characterized genetically-defined system. A melanoma cell line lacking FLNA was isolated and subsequently reconstituted with FLNA yielding a pair of cell lines in which the only difference is the presence or absence of FLNA [227]. When we irradiated FLNA$^-$ and FLNA$^+$ cells, we noticed that FLNA$^-$ took much longer to resolve DSBs (Figures 21, 22). To elucidate the mechanism underlying the repair defect we systematically investigated the proficiency of damage signaling in FLNA$^-$ cells.

Initially we investigated the recruitment and activation kinetics of the upstream kinases, as well as their downstream substrates after DNA damage. We found that FLNA deficiency led to the hyperactivation of ATM as judged by phosphorylation of ATM S1981 and CHK2 T68, surrogate markers of ATM activation [240-242]. Similarly, lack of FLNA also led to a hyperactivation of ATR, as measured by CHK1 S317 phosphorylation, a marker for ATR activation [114]. Moreover, we also found sustained levels of phosphorylation of NBS1 S343 to be higher in FLNA$^-$ cells. Although the role of NBS1 phosphorylation in the DNA damage signaling is poorly understood, it is generally thought to reflect ATM and ATR activation [199, 243]. We also determined that major mediator proteins BRCA1, MDC1, and 53BP1 formed IR-induced foci irrespective of FLNA status. However, BRCA1 foci formation was significantly impaired

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in FLNA-deficient cells. In addition, Rad51 foci formation displayed a delayed kinetics in cells lacking FLNA. These data indicate that FLNA-deficient cells have impaired homologous recombination. Indeed, Yue et al. showed that FLNA-deficient cells have a reduced ability to repair I-SceI-induced DSBs [232].

During the course of our experiments we noticed a consistent increase in the number of FLNA^{-} cells displaying IR-induced RPA foci. These foci progressively increased in size at later time points after IR. RPA is a ssDNA binding protein and participates in DNA metabolism processes where there is generation of ssDNA such as replication, repair, and recombination [244, 245]. Phosphorylation leads to inability of RPA to associate with the replication centers and leads to the association with DNA damage-induced foci instead [246]. In addition, we also verified the retention of phosphorylated RPA in the chromatin-bound fraction of FLNA^{-} cells after treatment with CPT or HU. Consistently, we found more hyper-phosphorylated forms of RPA associated with chromatin in FLNA[-] (Figures 27, 29). In addition, the hyper-phosphorylation of RPA persisted longer in FLNA[-] (Figures 27, 29). Interestingly, lack of NHEJ proteins DNA-PKcs and Ku86, which together with Ku70 form the active DNA-PK complex, leads to accumulation of ssDNA in S phase [247]. Thus, we further investigated how the lack of FLNA impacted on DNA-PK complex formation.

Remarkably, Ku86 failed to interact with DNA-PKcs in the absence of FLNA. The reduced stability of the interaction is not due to Ku86 failure to load onto chromatin, as FLNA^{-} cells displayed sustained higher levels of chromatin-bound Ku86 than FLNA^{+} cells after damage. Ku86 is one of the first molecules to bind DNA ends after DSBs

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[214] and recruits DNA-PKcs via its C-terminus [113]. Taken together these results establish that lack of FLNA results in an unstable association of Ku86 and DNA-PKcs impairing the function of the complex. This impaired DNA-PK activity leads to a continuous build up of ssDNA and Ku86 on chromatin.

Over 16 phosphorylation sites have been identified in DNA-PKcs although their role is still poorly understood. Nevertheless, DNA-PKcs phosphorylation status is thought to influence its activity [215]. DNA-PKcs interacts with Ku86 and free ends of DNA in an unphosphorylated form [216], and autophosphorylation is required for NHEJ progression [217]. Thus, we investigated the status of the two major phosphorylation clusters in DNA-PKcs, namely the 2056 and 2609 clusters. Cluster 2609 was consistently phosphorylated upon treatment with IR, CPT and HU irrespective of the FLNA status. However, phosphorylation of the 2056 cluster was significantly reduced in FLNA⁻ cells in CPT but not in IR or HU treatment indicating that there are damage-specific effects of DNA-PKcs phosphorylation. Nevertheless, the fact that DNA-PKcs is phosphorylated upon damage in the absence of FLNA suggests that DNA-PKcs is interacting with the Ku86/DNA complex albeit transiently. Alternatively, it is possible that phosphorylation of DNA-PKcs is not mediated by autophosphorylation at the synaptic complex but rather via hyperactive ATM and ATR in FLNA-deficient cells.

We showed that FLNA and BRCA1 interact and that FLNA deficiency leads to a marked decrease in BRCA1 foci formation after damage. To investigate further the role of BRCA1 we tested whether expression of the BRCA1 FLNA-interacting fragment in FLNA-proficient cells could also act in a dominant negative fashion leading to a

phenotype similar to FLNA-deficient cells. Strikingly, expression of the BRCA1-Ff lead

to a defect in DNA repair as judged by CDC2 pY15 and γ-H2AX markers. This effect is

specific because expression of BRCA1-Ff containing a mutation that disrupts

FLNA/BRCA1 interaction does not lead to the same phenotype. Taken together, these

data establish that BRCA1 participates in the FLNA-dependent regulation of the DNA

damage response.

Our data shows that absence of FLNA leads to defective DSB repair. The defect

is a combined result of compromised HR and NHEJ processes. At this stage we cannot

distinguish whether FLNA-deficiency leads to a defective step that is common to both

pathways or, alternatively, it impacts different steps in these pathways. In fact, the

interplay between these two arms of the DNA repair process is not fully understood

[178], in particular after IR, which generates an array of different DNA modifications.

The observed phenotype is consistent with a model in which Ku86 recognizes and binds

free ends of DNA, but in the absence of FLNA, fails to make a stable complex with

DNA-PKcs. We propose that unstable Ku86/DNA-PKcs interaction results in impaired

end processing, accumulation of ssDNA, and hyperactivation of DNA damage signaling.

Repair via NHEJ can be arbitrarily divided into early (damage recognition and

processing of non-ligatable ends) and late (ligation) stages [214]. DNA-PK has multiple

functions and is essential for early and late stages of classical NHEJ in mammalian cells

[214]. To determine whether the lack of FLNA also impacted on the late stages of NHEJ

we transfected cells with cleaved plasmids, which are thought not to require extensive

processing, to monitor precise and total end joining activity. Cells lacking FLNA

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displayed a significantly increased end joining activity, indicating that FLNA is not required for the late stages of end joining. The increase in end joining activity could be due to the fact that deficient DNA-PK activity may lead to an unrepressed DNA ligase IV-independent alternative end joining pathway [248-250]. Alternatively, the failure of FLNA-deficient cells to promote stable Ku86/DNA-PKcs complex on chromatin may increase the availability of free DNA-PKcs to act on the plasmid template. Further research will be needed to discriminate between these possibilities.

In addition, in FLNA-deficient cells BRCA1 displays impaired foci formation suggesting that FLNA also plays a role in stabilizing BRCA1 at the DSBs. BRCA1 colocalizes with Rad50/Mre11/NBS1 complex at IR-induced foci [120, 212] and inhibits Mre11 exonuclease activity [148].Thus, the diminished amounts of BRCA1 at IR-foci may lead to an unregulated Mre11 exonuclease activity with formation of the observed extended tracts of RPA-coated ssDNA in FLNA-deficient cells (Figures 31, 32, and 33). BRCA1 has also been implicated in the regulation of Rad51 [251, 252], although the mechanism by which it happens is obscure [253]. The kinetics of Rad51 foci formation in FLNA-deficient cells suggests that there is no problem in the initial recruitment to foci (see Figure 32, bottom panel, 3h time point). The extended plateau observed in Rad51 foci (from 3 to 12h after IR) may indicate an accumulation of DSBs that do not fulfill the end processing requirements for efficient Rad51 loading. Although further research will be needed to test this proposed model, it provides a tractable system to dissect the interplay between different processes involved in DNA repair.

It is possible that FLNA provides a framework for the assembly of factors in the synaptic complex. While unrepaired DNA in yeast (which lacks recognizable DNA-PKcs and FLNA orthologs) migrates to so-called DNA repair centers [254], the picture is different in mammalian cells where broken chromosome ends are essentially immobile [255, 256]. It will be interesting to determine whether lack of FLNA affects the mobility of broken ends.

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