Distribution Agreement

In presenting this thesis or dissertation as a partial fulfillment of the requirements for an advanced degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis or dissertation in whole or in part in all forms of media, now or hereafter known, including display on the world wide web. I understand that I may select some access restrictions as part of the online submission of this thesis or dissertation. I retain all ownership rights to the copyright of the thesis or dissertation. I also retain the right to use in future works (such as articles or books) all or part of this thesis or dissertation.

Signature:

_____ _____

Elizabeth Virginia Minten Date

SIRT2 Stabilizes BRCA1 by Promoting Complex Formation with BARD1

By

Elizabeth Virginia Minten

Doctor of Philosophy
Graduate Division of Biological and Biomedical Sciences

Biochemistry, Cell, and Developmental Biology

_____
David S. Yu
Advisor

_____
William Dynan
Committee Member

_____
Lawrence Boise
Committee Member

_____
Christine Dunham
Committee Member

_____
Ya Wang
Committee Member

Accepted:

_____
Lisa A. Tedesco, Ph.D.
Dean of the James T. Laney School of Graduate Studies

_____
Date

SIRT2 Stabilizes BRCA1 by Promoting Complex Formation with BARD1

By

Elizabeth Virginia Minten
B.S., University of South Carolina, Honors College, 2015

Advisor: David S. Yu, M.D., Ph.D.

An abstract of
A dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
in Biochemistry, Cell, and Developmental Biology
2020

Abstract

SIRT2 Stabilizes BRCA1 by Promoting Complex Formation with BARD1
By Elizabeth Virginia Minten

Breast cancer type I susceptibility protein (BRCA1) and its major binding partner, BRCA1-associated RING domain protein I (BARD1) form a stable heterodimer that promotes genome integrity through a variety of pathways in the DNA damage response (DDR), including DNA double-strand break (DSB) repair through homologous recombination repair (HRR), apoptosis, and cell cycle regulation. Heterodimerization is necessary for the stabilization and nuclear retention of both proteins, as well as their role in tumor suppression. Perturbations in their interaction or function have been linked to a significant increase in the lifetime risk of developing certain cancers. In this work, we show using molecular analyses that the class III NAD^+-dependent sirtuin, SIRT2, promotes BRCA1-BARD1 heterodimerization in a deacetylase-dependent manner. A loss of SIRT2 or inhibition of SIRT2 enzymatic activity leads to decreased BRCA1 and BARD1 protein levels due to increased protein instability, as well as increased BRCA1/BARD1 cytoplasmic localization and a failure to form DNA damage foci. SIRT2 deficiency also leads to a loss of proper HRR function. We have found that SIRT2 deacetylates conserved lysines in the BARD1 N-terminus within the RING domain that interfaces with BRCA1, which promotes BRCA1-BARD1 interaction. Mutation of the lysines to nonacetyl-lysine mimics enhances BARD1 binding to BRCA1, while mutation to acetyl-lysine mimics impairs their interaction. Overall, this work provides a mechanism by which SIRT2 acts as an upstream regulator of BRCA1 and BARD1 heterodimerization and as a determinant of proper HRR function. These results aim to build a molecular foundation for better understanding the clinical significance of the interplay between SIRT2 and BRCA1-BARD1 in tumor suppression, cancer formation, and progression.

SIRT2 Stabilizes BRCA1 by Promoting Complex Formation with BARD1

By

Elizabeth Virginia Minten
B.S., University of South Carolina, Honors College, 2015

Advisor: David S. Yu, M.D., Ph.D.

A dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
in Biochemistry, Cell, and Developmental Biology
2020

Acknowledgements

I would like to first express my sincerest thanks to my PI, Dr. David Yu, for being my mentor and providing continuous support during my time in his lab. His mentorship, guidance, and feedback have been invaluable to my training as a scientist, and I am beyond thankful to have been lucky enough to conduct my PhD work under his mentorship. I have learned more than I could have ever imagined and am grateful for his patience and motivation to always aim for excellence.

I also am grateful to my committee for their advice and expertise. Thank you, Larry, Ya, Bill, and Christine for your time, encouragement, and invaluable feedback and suggestions. I'm also grateful to the BCDB program and Laney Graduate School at Emory for providing me many opportunities throughout my graduate career. I also am extremely lucky to have made so many great friends over the past 5 years who have helped me get through. Finally, I am beyond fortunate to have such an amazing family who has always been there for me. Thank you, mom and dad, for all that you've done – your endless love and support made this all possible.

Table of Contents

List of Figures

Abbreviations

53BP1	p53 Binding Protein
ATM	Ataxia-Telangiectasia Mutated
ATR	Ataxia-Telangiectasia and Rad3-Related Protein
BARD1	BRCA1-Associated RING Domain
BASC	BRCA1-Associated Genome Surveillance
BER	Base Excision Repair
BIR	Break-Induced Repair
BRAP2	BRCA1-Binding Protein 2
BRCA1/2	Breast Cancer Type 1/2 Susceptibility Protein
BRCT	BRCA1 C-Terminal
CNS	Conserved Noncoding Sequences
CRM1	Chromosome Region Maintenance 1
CTCF	CCCTC Binding Factor
CTSS	Cysteine Protease Cathepsin S
DDR	DNA Damage Response
DNA-PK	DNA-PKcs and Ku Heterodimer
DNA-PKcs	DNA-Dependent Protein Kinase Catalytic Subunit
DSB	Double-Strand Break
DUB	Deubiquitinating Enzyme
EMA	European Medical Agency
FA	Fanconi Anemia
FANC	Fanconi Anemia Subtype
FDA	Food and Drug Administration
HAT	Histone Acetylase
HDAC	Histone Deacetylase
HERC2	HECT and RLD Domain Containing E3 Ubiquitin Protein Ligase 2
HR(HRR)	Homologous Recombination Repair
HUWE1	HECT, Uba, and WWE Domain Containing E3 Ligase 1
ICL	Interstrand Crosslink
IP	Immunoprecipitation
IR	Ionizing Radiation
MLH1	MutL Protein Homolog 1
MMR	Mismatch Repair
MRN	MRE11-RAD50-NBS1
NBR2	Next To BRCA1 Gene 2
NER	Nucleotide Excision Repair
NHEJ	Non-Homologous End Joining
NLS	Nuclear Localization Sequence
PARP	Poly (ADP-Ribose) Polymerase
PARPi	PARP Inhibitor
PIKK	Phosphatidylinositol 3-Kinase (PI3K)-related Kinase
RFC	DNA Replication Factor C

RSR	Replication Stress Response
SCD	Serine Cluster Domain
SCFFBXO44	Skp1-Cul1-F-Box-Protein44
SDSA	Synthesis-Dependent Strand Annealing
SSB	Single Strand Break
ssDNA	Single-Strand DNA
Stat-1	Signal Transducer and Activator of Transcription
USP	Ubiquitin-Specific Protease
USP9X	Ubiquitin-Specific Peptidase 9X
UTR	Untranslated Region
UV	Ultraviolet

Chapter 1: DNA Repair: Translation to the Clinic

Published by the Journal of Clinical Oncology, May 2019.

1.1 Authors' Contributions

Elizabeth Minten was responsible for the literature research, the writing of the manuscript, manuscript preparation, and manuscript editing. Dr. David Yu is the guarantor of integrity and also helped with the manuscript preparation and manuscript editing.

1.2 Abstract

It has been well-established that an accumulation of mutations in DNA, whether caused by external sources (e.g., UV light, radioactivity) or internal sources (e.g., metabolic by-products such as reactive oxygen species), has the potential to cause a cell to undergo carcinogenesis and increase the risk for the development of cancer. Therefore, it is critically important for a cell to have the capacity to properly respond to and repair DNA damage as it occurs. The DNA damage response (DDR) describes a collection of DNA repair pathways that aid in the protection of genomic integrity by detecting myriad types of DNA damage and initiating the correct DNA repair pathway. In many instances, a deficiency in the DDR, whether inherited or spontaneously assumed, can increase the risk of carcinogenesis and ultimately tumorigenesis through the accumulation of mutations that fail to be properly repaired. Interestingly, while disruption of the DDR can lead to the initial genomic instability that can ultimately cause carcinogenesis, the DDR has also proven to be an invaluable target for anti-cancer drugs and therapies. Making matters more complicated, the DDR is also involved in the resistance to first-line cancer therapy. In this review, we will consider therapies already in use in the clinic and ongoing research into other avenues of treatment that target DNA repair pathways in cancer.

1.3 Conundrum: A double-edged sword of DNA repair perturbation

It is generally believed that the progression of a cell from a normal to a tumorigenic state occurs through a series of gene-altering steps that ultimately evade genomic stability-maintaining mechanisms and instead lead to genomic instability. Indeed, genomic instability is one of the hallmarks of cancer, reflecting the path from which the cancerous cells arose.[1] In normal cells, genomic integrity is protected by the DNA damage response (DDR), which describes the collection of pathways capable of detecting and repairing different types of DNA damage. Deficiencies in the DDR and its repair pathways, whether acquired or inherited, can accelerate the accumulation of mutations and help augment the loss of stability that ultimately gives rise to malignancy. Paradoxically, however, while deficiencies in DNA repair pathways can first lead to a loss of genomic integrity through the accumulation of mutations, the proteins involved in have proven to be viable and efficacious targets for cancer therapies, while at the same time also contributing to resistance to first-line cancer treatments. To reconcile these differences, it is important to recognize that cancerous cells arise from normal, nonmalignant cells, and thus share many of the same characteristics as their unaffected kin. A major goal in creating more effective cancer therapies is to find differences between cancerous and nonmalignant cells that allow treatments to more specifically target the unhealthy cells, therefore minimizing the toxicity cancer patients experience while undergoing therapy. An important clinical term regarding this notion of targeted toxicity is the therapeutic index or ratio, which is defined as the amount of tumor control that can be achieved for a given amount of healthy tissue toxicity.[2] The higher the ratio, the better the efficiency of the treatment in targeting cancerous as opposed to noncancerous cells, minimizing side effects to healthy tissues. One way to increase the therapeutic ratio is to use synthetic lethality.

Synthetic lethality is achieved when the simultaneous deficiency of two or more genes causes cell death, whereas a deficiency in only one is otherwise non-lethal.[3] Cancerous cells generally have higher incidences of DNA repair pathway deficiencies resulting in a higher dependence on any remaining intact DNA repair pathways to maintain genomic stability.[4] This dependency renders the cancerous cells vulnerable to agents that target the remaining intact pathways in a synthetic-lethal way that would otherwise be harmless to normal, healthy cells, and is a rapidly-expanding field of research in cancer treatments.[5, 6] A closely-related concept to synthetic lethality that also offers a promising new strategy for increasing the therapeutic ratio through the targeting of the DDR is 'acquired vulnerability,' otherwise known as 'collateral sensitivity.' This term describes the phenomenon in which acquired resistance to one drug confers hypersensitivity towards a different reagent, thereby offering the possibility of clinical exploitation should the vulnerability be identified.[7] Research has already begun to find that some of these acquired vulnerabilities are tied to the DDR, further underscoring the notion that the DDR is an invaluable target in the quest to find novel therapeutic approaches in the treatment of cancer.[8]

1.4 Types of DNA repair

The cell has a complex system of interconnected DNA repair pathways that allows it to respond to a variety of different types of damage and regulate the outcomes of the intended repair (Figure 1.1). Single-stranded DNA (ssDNA) damage can include a wide variety of insults, such as single-strand breaks (SSBs), deamination of bases, errors in base matching incurred during DNA replication, and the creation of bulky adducts. There are, in the broadest of terms, three main pathways that are responsible for the repair of ssDNA damage: base excision repair (BER), nucleotide excision repair (NER), and mismatch repair (MMR).[9] BER removes damaged bases

that do not significantly alter the overall structure of DNA, as opposed to NER, which can recognize and repair the bulky, structurally-altering ssDNA lesions.[10] Finally, MMR functions as a method of proofreading after DNA replication to catch any mismatched base pairs that were overlooked during DNA synthesis by DNA polymerases.[10] Mutations that impair any of these pathways can lead to disorders that substantially increase the lifetime risk of developing cancer, such as MUTYH-associated polyposis (BER), Xeroderma Pigmentosa (NER), and Lynch syndrome (MMR).[11] DNA can also be affected by interstrand crosslinks (ICLs), which form when two base pairs are covalently bound together.[12] ICLs are especially cytotoxic to cells as the covalent bond can block replication and/or transcription, and when left unrepaired, can lead to mutation and chromosomal breakage.[12] As with ssDNA repair pathways, defects in the DDR pathways responsible for repairing ICLs can lead to diseases that dramatically increase the chances of tumorigenesis: Fanconi anemia (FA) is a rare disease that is caused by mutation of the Fanconi anemia subtype (FANC) proteins. The FANC proteins function in the FA pathway, which is essential for the maintenance of genomic integrity through the repair of ICLs.[12]

In addition to ssDNA damage, cells can also encounter double-strand breaks (DSBs), the most deleterious type of DNA damage. DSBs are repaired by two main pathways: non-homologous end joining (NHEJ) and homologous recombination repair (HRR).[13] NHEJ is a form of repair template-independent repair and thus is an error-prone mechanism of repair active from G_1 through G_2. In some cases after a DSB has occurred, the ends of the broken strands will undergo resection, where degradation of overhangs will occur via exo- or endo-nuclease activity to stick the two ends broken DNA ends back together.[14] HRR, on the other hand, is considered to be an error-free method of DSB repair and occurs mainly in the S and G_2 phases of the cell cycle when there is a

sister chromatid available for use as a repair template.[15] More extensive end resection is done to the broken ends of the DSB as opposed to in NHEJ, where more minimal resection is needed. Of the two pathways, NHEJ is favored over HRR, even during the S and G_2 phases where HRR is more active, with some studies showing a 4:1 ratio of NHEJ to HRR in mammalian somatic cells.[14] Similar to ssDNA repair pathways, the impairment of NHEJ or HRR through the mutation of regulatory or involved proteins can lead to an increased risk of carcinogenesis and tumorigenesis due to accumulation of unresolved DSBs in the genome. For example, certain mutations in breast cancer 1/2 (BRCA1/2), proteins essential to proper HRR, are linked to an increased risk for developing a wide range of different cancers including breast, prostate, colon, ovarian, and pancreatic, with increased lifetime risks for cancer development as high as fifty-five percent.[16] In addition, patients with LIG4 syndrome have a predisposition to developing lymphoid malignancies due to a deleterious mutation in the gene encoding protein DNA Ligase IV, resulting in impaired NHEJ.[17]

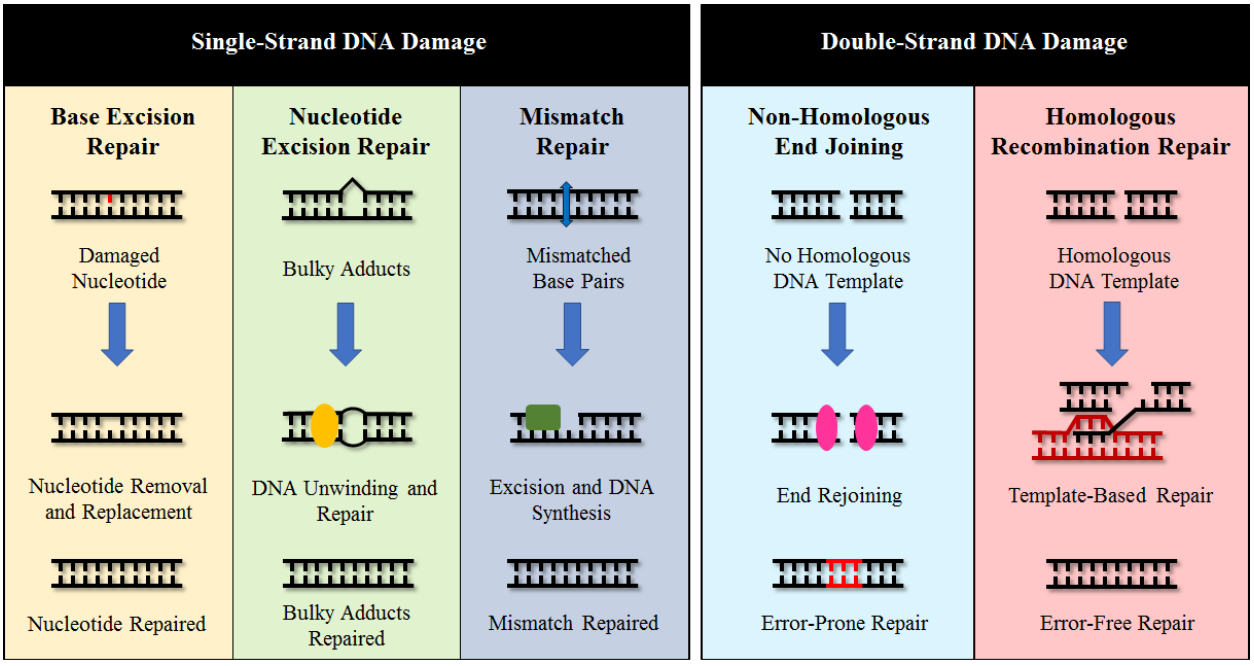

Figure 1.1. DNA Repair Pathways. DNA repair pathways of single-strand DNA (ssDNA) damage and DNA double-strand breaks (DSBs). From left to right for ssDNA repair: base excision repair (BER) is used to repair damage to nucleotides that do not significantly alter the DNA

structure. Nucleotide excision repair (NER) mends structurally altering DNA damage, such as bulky adducts. Mismatch repair (MMR) fixes pairs of nucleotides that were mismatched during DNA synthesis. Non-homologous end joining (NHEJ) repairs DNA DSBs without the use of a homologous DNA template by rejoining the broken DNA ends together and is prone to introducing errors. Homologous recombination repair (HRR) uses a homologous DNA template to repair DNA DSBs without error.

1.5 PARP Inhibitors

One of the most developed cancer therapies targeting DNA repair pathways that has risen to prominence within the last few years is the use of poly (ADP-ribose) polymerase (PARP) inhibitors (PARPi). PARPs are a class of 17 nuclear enzymes involved in multiple cellular functions.[18] These proteins, which can transfer either one or multiple (ADP-ribose) units from NAD^+ onto substrates to make poly (ADP-ribose) (PAR) chains, are found in all eukaryotes except yeast.[19] PARP1 has been found to play a crucial role in the DDR, including in ssDNA repair, NHEJ, and HRR.[20-22] PARP1 is recruited to sites of damage for various types of damage, aided by its DNA-binding domain, and has a number of functions, such as binding SSBs, recruiting downstream DNA repair proteins, and promoting HRR at stalled and/or collapsed replication forks.[22] Though PARP inhibitors (PARPi) were first found over 30 years ago, only in 2005 was it demonstrated through the work of two independent research groups that the use of PARPi is synthetically lethal in cells deficient in HRR, such as when there are mutations in the BRCA1/2 proteins.[21, 23] Indeed, PARPi have had enough success that as of October, 2018, four PARPi have been approved by the US Food and Drug Administration (FDA): olaparib (Lynparza), rucaparib (Rubraca), niraparib (Zejula), and the newest one, talazoparib (Talzenna).[24] These drugs have been approved for use in patients with *BRCA1/2* deficiencies in ovarian cancer and by the European Medical Agency (EMA) in patients who have responded to platinum-based chemotherapy with relapsed *BRCA1/2* mutant ovarian, fallopian tube, or primary peritoneal

cancers.[25] Olaparib, the first PARPi to be approved by the FDA in 2014, has also been approved for clinical use in patients with patients with *BRCA1/2* mutations and HER2-negative breast cancer.[24, 26, 27] These drugs have also shown promise in treating other types of HRR-deficient breast and prostate cancers. However, the exact mechanism describing this synthetic lethal relationship has not yet been fully elucidated.[28] Originally, it was hypothesized the synthetic lethality between PARP inhibition and BRCA1/2 mutation relied on the induction of persistent SSBs after PARPi inhibition. During replication, the replication fork would collapse when encountering the SSBs, and thus potentially create a DSB that was unable to be properly repaired by HRR.[29] In the absence of HRR, other DNA repair processes more prone to introducing deletions, mutations, and potentially genomic rearrangements, would take over, often times leading to cell death.[29] This model has changed with new evidence suggesting some of the PARPi "trap" PARP1 onto DNA, preventing its release and thus stalling repair.[29] However, as with many other types of cancer treatment, tumor resistance to PARPi is frequently seen and represents a major hurdle in long-term treatments.[30] The mechanism for acquired resistance has been suggested to fall into two broad main categories: One, secondary mutations restore necessary minimal HRR function, rendering the previously synthetic lethal phenotype ineffective.[29] Two, resistance can occur in an HRR-independent manner, such as through PARP protein expression loss, rendering PARPi ineffective.[29, 31] Research is already under way to establish what therapies can be used to prevent and/or counter PARPi resistance, taking advantage of the idea of acquired vulnerability, but more work needs to be done to make this goal a reality.[8]

1.6 Kinase inhibitors

Another route of targets that have seen moderate success in the cancer therapeutic field includes the class of DDR kinase inhibitors. As of January 2019, the FDA has approved of over 30 kinase inhibitors targeted at the treatment of cancers.[32] Phosphorylation plays a critical role in the regulation of many DDR pathways. Ataxia-telangiectasia mutated (ATM), which is a key player in the repair of DSBs through the HRR pathway and a serine/threonine kinase in the phosphatidylinositol 3-kinase (PI3K)-related kinase (PIKK) family, acts as an early signaling protein in the DDR and is responsible for the phosphorylation of hundreds of downstream targets.[33, 34] The protein is named after a rare autosomal recessive disorder, ataxia-telangiectasia, that results from mutations in the ATM gene. Patients who suffer from this disorder have symptoms such as radiosensitivity, immunodeficiencies, and an increased risk of cancer.[35] Studies have shown that ATM is synthetic lethal with PARP deficiencies, and that ATM inhibitors can sensitize cells to DSB-inducing reagents and IR.[36, 37] ATM inhibitors are currently being explored in a clinical setting: for example, the ATM inhibitor AZD0156 in conjunction with olaparib (a PARPi) or irinotecan (a topoisomerase inhibitor) is currently under review in an early-phase clinical I trial (clinical trial NCT02588105).[35] Ataxia telangiectasia and Rad3-related protein (ATR) shares many of the same characteristics as ATM. Another PIKK family member and serine/threonine kinase, ATR also functions as an early signaling kinase in the DDR response, primarily following replication stress. Preclinical studies have found that ATR has a synthetic lethal relationship with several DDR players, including XRCC1 and ATM, and is currently under clinical investigation for its potential as a target in cancer therapies.[38, 39] VX-970, or M6620, a potent ATR inhibitor is currently involved in phase II trials, used either as a single agent or in tumors with DNA repair deficiencies. A phase I trial in which VX-970 was used alone or in combination with carboplatin showed early evidence of potential efficacy.[40] Checkpoint kinase 1

(CHK1), a major downstream effector of ATR, and WEE1, are additional kinases of interest in the clinical setting. CHK1 is involved in a multitude of different functions in the cell and prevents cells with damaged or incompletely replicated DNA after exposure to IR or chemotherapeutic drugs from moving from G_2 onto mitosis. CHK1 inhibitors have already been used in NIH phase I clinical trials a monotherapy, such as LY2606368 (prexasertib) (clinical trial NCT02203513).[41] WEE1, which is involved in triggering the DDR after DNA damage has occurred, is also under clinical investigation as a target for inhibition in the treatment of certain cancers.[42] AZD1775, a WEE1 inhibitor, is currently being tested in phase I and II trials, both as a monotherapy and in combination therapies (e.g., clinical trial NCT02593019 and those listed in Table 1, respectively).

Attempts have also been made to inhibit the protein DNA-dependent protein kinase catalytic subunit (DNA-PKcs), another member of the PIKK group that serves a crucial role as a main regulator of the NHEJ pathway. DNA-PK, a complex made of the catalytic subunit DNA-PKcs and the Ku heterodimer, is itself a target of ATM and ATR and is dependent on the binding of DNA for activation.[43, 44]. Once bound to Ku and DNA, DNA-PKcs helps in the recruitment of other NHEJ proteins to begin DNA resection at the broken ends of the DSBs and later DNA ligating complex proteins to join the DNA ends. Interestingly, it has been found that in cell lines with defective DNA-PK function, whether through a lacking of Ku or DNA-PKcs, there exists a hypersensitivity to IR and chemical agents that cause DSBs.[44] On the flip side, upregulation of DNA-PK activity has been correlated with increased resistance to DNA damage in some cancers.[44] Therefore, DNA-PK is a promising target for anti-cancer therapies. One of the first DNA-PKcs inhibitors, wortmannin, was isolated from the fungi *Penicillium funiculosum* in 1957.[45] Wortmannin is a non-specific PI3K family inhibitor and has been found to be an effective

radiosensitizer.[46] However, wortmannin has proven to have limited clinical application, given the substance is poorly soluble in aqueous solutions, nonspecific, and toxic.[47] Subsequently, other general PI3K inhibitors have been developed with the aim of increasing clinical applicability while maintaining its sensitizing properties, such as LY294002 and its prodrug SF1126. Though LY294002 did not reach clinical trials due to similar issues as seen in wortmannin along with a quick metabolic clearance rate of 1 hour, SF1126 has so far successfully completed a phase I clinical trial that ended in 2011, which found the drug to be well-tolerated with promising results, and is undergoing a second phase I trial in patients with advanced hepatocellular carcinoma (NCT00907205 and NCT03059147, respectively).[44, 48, 49] NU7441, a DNA-PKcs specific inhibitor, is also being studied and has shown promising preclinical effects on certain types of cancers, such as non-small cell lung cancer.[50]

1.7 Radiation therapy: Combinatorial Approach

Radiation therapy, or the use of IR in the treatment of cancer, has been used for over a century and functions to exploit the genomic instability phenotype of cancerous cells, as a deficiency in the ability to repair DSBs frequently results in an increased sensitivity to IR.[51] Since its inception, radiation therapy has seen significant improvements that reduce toxicity to normal tissue, though this toxicity still remains a limiting factor.[4] One area of improvement that has been of great interest is the induction of ‘artificial synthetic lethality,’ where radiation therapy is combined with specific targets to DNA repair pathways redundant in nonmalignant cells, but crucial for cancerous cell survival. This combination of radiation therapy and inhibition of DNA repair pathways comes with the goal to increase the therapeutic index by conferring increased IR sensitivity only to cancerous cells.[52] Current radiotherapy techniques are used such that the dose is targeted to the

tumor to spare the surrounding healthy tissue from treatment, which also functions to limit the amount of damage normal tissues would receive after application of a systematic inhibitor of a DNA repair protein.[53] Together, the targeted radiation therapy and inhibited DNA repair in cancer cells would potentially lead to an increase in the therapeutic index, allowing for a reduction in the effective radiation dose while limiting toxic side effects.

There have been several agents targeting DNA repair pathway proteins that have been found in preclinical studies to be effective radiosensitizers, many of which are under current clinical investigation in phase I and II trials (Table 1). One protein that has been shown in preclinical studies to increase radiosensitivity is Artemis, an endo/exonuclease in the NHEJ pathway that is recruited and activated by DNA-PKcs to process broken DNA ends at DSBs.[54] Patients who have null mutations for *Artemis* show extreme radiosensitivity, making Artemis a target of interest for its projected efficiency in working synergistically with radiation therapy, as well as etoposide treatment (which, similar to IR, causes DSBs).[55] However, to date, no inhibitor of Artemis has been found, though compounds are currently being screened to identify potential inhibitors (NIH 5F31GM116569-03). NU7441, mentioned previously as a DNA-PKcs specific inhibitor, has also been shown to increase cell death after IR and etoposide treatment in different types of colon cancer cells in a DNA-PKcs dependent manner.[56] However, NU7441 has proven to be clinically unusable due to problems with bioavailability and solubility, and other DNA-PKcs inhibitors are currently being investigated in both preclinical and clinical phases (Table 1).[57] PARPi inhibitors, including the four FDA-approved inhibitors mentioned previously, are being heavily investigated in combination therapies with radiation in numerous types of cancers (Table 1). LY2606368, a CHK1 inhibitor, is also currently undergoing a phase I clinical trial in head and neck cancer in

conjunction with radiation, with an estimated completion date set for this year (Table 1, clinical trial NCT02555644). However, new CHK1 inhibitors are still being developed and tested under preclinical settings in combination with radiation, such as CCT244747, which when used on p53-deficient head and neck squamous cell carcinoma cells, increases radiosensitivity to paclitaxel-based chemoradiotherapy.[58] CHIR-124, SAR-020106, and SB-218078, three more CHK1 inhibitors, have also been shown in preclinical studies to increase radiosensitivity in cells with *p53* deficiencies or mutations.[59-61] WEE1 inhibitors, such as AZD11775 (adavosertib), have also made it to the clinic in ongoing phase I and II trials in combination with radiation (Table 1). Alongside Artemis, DNA-PK, ATM, CHK1, and WEE1, other DDR factors not described in this review are also being investigated clinically in conjunction with radiation (Table 1, last two trials). While all these proteins are promising future clinical targets, success in clinical trials will depend heavily on understanding the conditions under which these treatments will have a therapeutic gain.

Phase	Cancer	Status	Drug	Drug Target	Trial Identifier
I	Head and Neck	Completed	Olaparib	PARP	NCT01758731
I	Triple Negative Breast Cancer	Recruiting	Olaparib	PARP	NCT03109080
II	Inflammatory Breast Cancer	Recruiting	Olaparib	PARP	NCT03598257
I	Inoperable Breast Cancer	Recruiting	Olaparib	PARP	NCT02227082
I	Head and Neck	Recruiting	Olaparib	PARP	NCT02229656
I/II	Metastatic Castration-Resistant Prostate Cancer in Bone	Recruiting	Olaparib	PARP	NCT03317392
I	Soft-tissue Sarcoma	Recruiting	Olaparib	PARP	NCT02787642
I	Small Cell Lung Cancer	Recruiting	Olaparib	PARP	NCT03532880
I	Head and Neck	Recruiting	Olaparib	PARP	NCT02308072
I/II	Unresectable High Grade Glioma	Recruiting	Olaparib	PARP	NCT03212742
II	Glioblastoma	Ongoing	Olaparib	PARP	2014-001216-19
I/II	Diffuse Pontine Glioma	Completed	Veliparib	PARP	NCT01514201
II	Non-Small Cell Lung Cancer with Brain Metastases	Completed	Veliparib	PARP	NCT01657799
I	Peritoneal Carcinomatosis, Epithelial Ovarian, Fallopian, and Primary Peritoneal	Completed	Veliparib	PARP	NCT01264432
I	Brain Metastases	Completed	Veliparib	PARP	NCT00649207
I	Rectal Cancer	Completed	Veliparib	PARP	NCT01589419
I	Recurrent Breast Cancer	Completed	Veliparib	PARP	NCT01477489
II	Malignant Glioma without H3 K27M or BRAFV600E Mutations	Recruiting	Veliparib	PARP	NCT03581292
I	Pancreatic	Active	Veliparib	PARP	NCT01908478
I	Triple Negative Breast Cancer	Recruiting	Rucaparib	PARP	NCT03542175
I	Castrate Resistant Prostate Cancer	Recruiting	Niraparib	PARP	NCT03076203
I	Non- and Small Cell Lung Cancer, and Neuroendocrine	Recruiting	VX-970 (M6620)	ATR	NCT02589522
I	Oesophageal and Other	Not yet recruiting	VX-970 (M6620)	ATR	NCT03641547
I	HPV-Negative Head and Neck Squamous Cell Carcinoma	Recruiting	VX-970 (M6620)	ATR	NCT02567422
I	Brain Cancer	Recruiting	AZD1390	ATM	NCT03423628
I	Advanced solid tumors	Recruiting	M3814	DNA-PK	NCT03724890
I	Advanced Solid Tumors	Recruiting	MSC2490484A	DNA-PK	NCT02516813
I/II	Rectal Cancer	Not yet recruiting	M3814	DNA-PK	NCT03770689
I	Head and Neck	Recruiting	LY2606368 (Prexasertib)	CHK1/CHK2	NCT02555644
I	Head and Neck	Recruiting	AZD1775 (Adavosertib)	WEE1	NCT03028766
I	Cervical, Vaginal, and Uterine	Recruiting	AZD1775 (Adavosertib)	WEE1	NCT03345784
I	Intermediate/High Risk squamous head and neck	Recruiting	AZD1775 (Adavosertib)	WEE1	NCT02585973
I/II	Unresectable Adenocarcinoma of the Pancrease	Active	AZD1775 (Adavosertib)	WEE1	NCT02037230
I	Diffuse Intrinsic Pontine Glioma	Recruiting	AZD1775 (Adavosertib)	WEE1	NCT01922076
I	Glioblastoma	Recruiting	AZD1775 (Adavosertib)	WEE1	NCT01849146
I	Soft-Tissue Sarcoma	Recruiting	AMG-232	MDM2	NCT03217266
I/II	Non-Small Cell Lung Cancer	Completed	NFV	AKT	2006-001031-22

Table 1.1. A List of Clinical Trials. A list of clinical trials that have taken place or are currently ongoing in the US and Europe that combine radiation therapy with the listed DNA damage response (DDR) inhibitors. The phase of the trials, cancer types being investigated, current status as of submission, names of the drugs and the respective DDR targets, and trial identifier numbers have been listed. PARP, poly (ADP-ribose) polymerase; ATR, ataxia telangiectasia and Rad3-related protein; ATM, ataxia-telangiectasia mutated; DNA-PK, DNA protein kinase; CHK, checkpoint kinase. MDM2, Mouse double minute 2 homolog; AKT, Protein kinase B (AKT is more a historical name than something that actually stands for anything).

1.8 Future targets and Conclusion

In the past few years, there has been an explosion of new information about the DDR and its involvement in cancer, of which we have only just begun to understand how to use in a clinically-relevant setting. While there have been exciting steps in clinical advancements in the treatment of cancer, and there is a plethora of extensive preclinical data to support clinical application for many of these new therapies, researchers and clinicians alike have much to still uncover and learn, especially in the area of how the DDR contributes to carcinogenesis and tumorigenesis, as well as how DDR pathways can be exploited for better future treatments. As an example, though most current cancer therapeutic targets are DDR kinases, roughly only 4% of all DDR proteins are kinases, leaving a large field of novel, non-kinase targets that could be targeted. Indeed, new players in the DDR are continually being discovered, many of which are not kinases. In our own lab, we have found two new non-kinase players involved in the DDR: SIRT2 and SAMHD1. SIRT2, a class III histone deacetylase (HDAC), was found to be directly involved in the replication stress response acting as a DDR regulator that leads to ATR activation.[62-64] SAMHD1 is a dNTP triphosphohydrolase and HIV-1 restrictase known to have an association with cancer when mutated. Our lab discovered that SAMHD1 functions independently of its dNTPase activity in the HRR pathway and can be targeted for radiation and PARPi sensitization using virus like particles containing Vpx, a lentiviral accessory protein, that targets SAMHD1 for proteasomal degradation.[65] Continuing research into the DDR and its components, as well as the drugs that affect DNA repair function, will lead to further clinical advancements in the prevention of cancer and effective therapies.

Chapter 2: BRCA1 and BARD1

2.1 Discovery and Gene Structure of BRCA1

In 1990, Mary-Claire King and co published an article that described a genetic analysis done on families that showed a higher-than-normal susceptibility to developing early-onset breast cancer.[66] The analysis found a link between a region on the 17q21 chromosome to breast cancer susceptibility, and in 1994, the *Breast Cancer 1* (*BRCA1*) gene was identified and cloned for the first time.[66, 67] Since then, knowledge on the protein structure, function, and role in the cell has exploded. Nevertheless, the full extent of the role BRCA1 plays in normal cellular processes, cancer development, and cancer progression is still being investigated.

The *BRCA1* gene spans an 81-kb region on chromosome 17 and contains within its sequence 24 exons, 22 of which are coding.[67, 68] Exons vary in size, from 87 bp to 1338 bp, where exon 11 is the largest.[68] Transcription of the gene is controlled by two distinct promotors, α and β. Each promotor leads to transcription of a different exon 1, exons 1a and 1b, respectively – however, both code for the 5' UTR exons and contain the same translation start codon in exon 2.[69, 70] The remainder of the exons are labeled as exon 2 through 24, with no exon 4 being defined. The sequence originally reported as exon 4 was subsequently found to instead code for an intronic Alu element.[67, 71] While many splice variants of *BRCA1* have been found in both normal and tumorigenic tissues, there has yet to be a study looking at all of the different variants and their functionalities.[72] However, studies have suggested that there are four "predominant" *BRCA1* splice variants that are expressed at different levels in normal cells, which include full length *BRCA1*, Δ(11q), Δ(9, 10q), and Δ(9, 10, 11q).[73] Of the four, only full length BRCA1, Δ(11q)

(BRCA1a), and Δ(9, 10, 11q) (BRCA1b) have been found to be translated into proteins and play a variety of roles in different cellular processes.[74]

2.2 BRCA1 Protein Structure

The full-length BRCA1 protein contains a number of distinct regions that contribute to its function and interaction with other proteins and molecules. The N-terminus is of particular interest as it is the site of many of these functional domains, including a degron sequence that spans the first 167 residues of the protein.[75] The N-terminus also contains a RING domain coded by exons 2-6, located on amino acids 1-109, which is made up of a C3HC4 type RING finger capable of binding two zinc ions via eight required amino acids and two flanking alpha helices that help stabilize the domain.[76, 77] Within this same region, BRCA1 also contains two rev-type nuclear export sequences (NES) located from residues 22 to 30 (LECPICLEL) and 81 to 99 (QLVEELLKIICAFQLDTGL), the former of which has been shown to function via the chromosome region maintenance 1 (CRM1)/exportin pathway.[78, 79] Exons 11-13 code for a majority of the BRCA1 protein (~65%), spanning residues 224-1452 which contain two nuclear localizing sequences (NLS), interaction sites for different binding partners, a DNA-binding domain from amino acids 452-1079, a putative coiled-coil domain, and part of a serine cluster domain (SCD) located from amino acids 1280-1524.[80] The BRCA1 NLS sequences are located at residues 503-508 (NLS1) and 606-615 (NLS2) that are coded by exon 11.[81] Both sequences bind importin-α, suggesting BRCA1 is imported into the nucleus via the classical importin-α/β pathway.[81] However, NLS1 has been identified to be the more critical of the two sequences, as mutation of NSL1 completely impairs the ability of BRCA1 to interact with importin-α, but mutation of NSL2 does not completely demolish nuclear localization.[81] Within the SCD, a

significant amount of phosphorylation occurs, including on serines 1189, 1457, 1524, and 1542, which promote binding to other proteins and BRCA1 function in the DDR pathway. The C-terminus also contains two tandem BRCA1 C-terminal (BRCT) domains. The BRCT domain motif was initially identified in BRCA1 and has been found in a number of other different proteins, many of which are also involved in the DDR pathway.[82] In BRCA1, the two BRCT domains (BRCT1 and BRCT2) are found within residues 1642–1863, where BRCT1 and BRCT2 roughly span the residues 1642–1737and 1761–1863, respectively.[82-84] Phosphate-binding pockets, such as BRCT domains, are classified into two different classes: The first class recognizes and binds to phosphoserine (pS), while members of the second class can additionally recognize phosphothreonine (pT).[85] The BRCA1 BRCT domains recognize the motif pS-P-T-F, and thus are considered members of the first class.[86, 87]

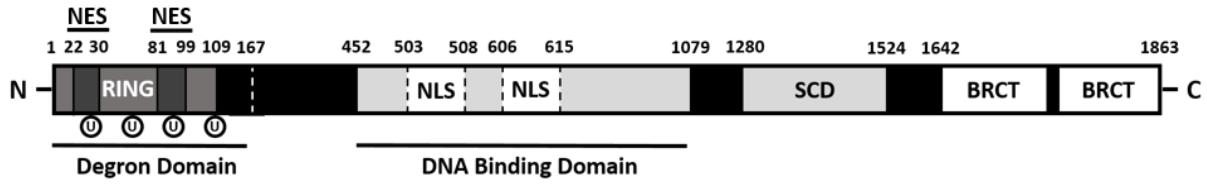

Figure 2.1. Primary Structure of BRCA1. Primary structure of the BRCA1 full-length protein indicating the location of the different functional domains.

2.3 Transcriptional Regulation of BRCA1

The *BRCA1* gene is part of a complex network of pathways that govern its regulation at both a transcriptional and translational level that is also regulated by the cell cycle. The 5' end of the *BRCA1* gene was identified to lie within a duplicated region, where about 30 kilobases upstream of *BRCA1* is a *BRCA1* pseudogene (*ψBRCA1*) that is a duplication of the region containing the first two exons of *BRCA1*.[88, 89] The human *BRCA1* gene is under the control of two promoters,

promoter α and promoter β as described in chapter 2.1. Promoter α, also called the minimal or proximal BRCA1 promoter, falls into a unique class of TATA-less bidirectional promoters where it controls transcription of the *BRCA1* gene and the *Next to BRCA1 gene 2* (*NBR2*) gene (located between *BRCA1* and *ψBRCA1*), whose start sites are separated by a 218 base pair intergenic region.[89, 90] Within the 218 base pairs, a segment spanning –202 to –166 (relative to the start site for the *BRCA1* 1a exon) has been shown to be required for BRCA1 promoter activities.[91] This region was found to contain two sub-elements: A 22-base pair RIBS* element from –204 to –182 which is activated by a GABPα/β complex, and a CRB/ATF1 element from –174 to –167 that is constitutively activated by binding of the transcription factor cAMP-response element binding protein (CREB).[92-94] [*RIBS element: Reiterative ihf BIME. Defined as a class of bacterial repetitive DNA elements which are found at the 3' end of transcription units. They were identified as a class of integration host factor (ihf) sites, where ifh is a histone-like heterodimeric protein in *E. coli*. BIME stands for bacterial interspersed mosaic element, which are sequences that contain repetitive extragenic palindromes (REP) sequences (small, imperfectly inverted repeats) arrange in groups of alternating copies.[95]]

In addition to a GABPα/β complex and CREB, other transcription factors or activating complexes have been found to play a role in the transcriptional regulation of BRCA1. Members of the E2F family of transcription factors in complex with RB have been found to regulate *BRCA1* expression in a variety of cell line models via binding to adjacent E2F binding sites, located between base pairs –39 to –32 (E2FA) and –18 to –11 (E2FB) upstream of the *BRCA1* promoter, with further studies suggesting correlation between E2F levels and breast cancer prognosis.[96-100] Mutations in either of the binding sites have been found in ovarian cancer cells lines to decrease BRCA1

promoter activity, suggesting that both are necessary for proper transcriptional activation.[101] A study done by Kanakkanthara *et al.* found that ZC3H18, a DNA-binding protein, is capable of binding only the E2FA site which promotes E2F4 association with the E2FB site, consequentially inhibiting E2F1 binding to either E2F sites.[101] p53, a tumor suppressor, downregulates BRCA1 expression at the transcriptional level.[102-104] p53 binding protein (53BP1), which contains two carboxyl terminal BRCT domains and is a tumor suppressor like *BRCA1*, positively regulates the minimal *BRCA1* promoter, binding to an element that overlaps E2FA between base pairs –40 and –25.[105] HMGA1b is one of two HMGA1 splice variants and binds within the –209 to –169 region of the human BRCA1 promoter to inhibit BRCA1 promoter activity.[106] Id4, a member of the Id family of dominant negative inhibitors of DNA binding, has been shown to negatively regulate BRCA1 expression when overexpressed in cells.[107, 108] c-Myc, a transcription factor involved in an array of cellular functions such as cell growth, apoptosis, and differentiation, binds two E boxes located –1292 to –1286 and –912 to –907 upstream of the exon 1a transcription start site.[109] Interestingly, *BRCA1* has also been reported to negatively regulate itself, repressing transcription when bound to its own promoter.[110]

Environmental factors such as hypoxia (which affects EF2 activity), estradiol (E2), and DNA damage, have also been shown to alter BRCA1 expression through modulation of *BRCA1* transcription factors.[97, 111, 112] Efforts to elucidate the mechanism of how estrogens, particularly E2, regulate *BRCA1* expression has proven to be complex. Initially, studies suggested E2 played an indirect role in promoting increased BRCA1 levels at the mRNA and protein level, though a 2005 study in Donato Romagnolo's lab demonstrated that estrogen receptor α (ERα) is recruited with its cofactor p300 to an activator protein 1 (AP-1) site located in the minimal BRCA1 promoter

(at positions –27 to –31 upstream of exon 1b) after E2 stimulation.[111] A subsequent study from the same lab expanded on this information, showing that *BRCA1* E2 stimulation was also dependent on the unliganded aromatic hydrocarbon receptor (AhR) binding to the *BRCA1* promoter.[113]

BRCA1 expression is also influenced by other variables in addition to transcription factors, including epigenetic modification and enhancers/repressors. In normal tissues, the region around promoter α and exon 1a is unmethylated, though methylation at CpG islands in the upstream region close to the transcription start site has been found in breast and ovarian carcinoma samples as well as in MCF-7 cells.[93] This methylation can inhibit transcription factor binding, including CREB. CpG methylation at position –173 (relative to exon 1a) lies within the CREB binding region and inhibits CREB binding to the BRCA1 promoter, consequentially suppressing *BRCA1* expression.[114] Two *in vivo* Specific protein 1 (Sp1) binding sites have been located within the BRCA1 gene, one at –355 and one at –1049.[115] The –355 site is unmethylated in normal tissue, though the –1049 site has been found to be methylated in normal tissue, suggesting Sp1 binding is also methylation insensitive in this region.[115] However, Sp1 binding has found to be methylation-dependent at the –355 site, though the methylation of a cytosine outside the central CpG on the antisense strand, which is unmethylated in normal tissues, has shown evidence of inhibiting Sp1 binding at the –1049 site.[115, 116] In contrast, the CCCTC binding factor (CTCF), which can bind the unmethylated BRCA1 promoter around the –430 region, is unable to bind when methylation occurs on the promoter.[116] Two putative cis-acting regulatory elements, conserved noncoding sequences (CNS) 1 and 2, have also been identified within intron 2, where CNS-1 acts as an potential enhancer and CNS-2 acts as a potential repressor.[117]

2.4 BRCA1 Protein Regulation and Stability

The BRCA1 protein is a phosphonuclear protein that is ubiquitously expressed in human tissues. However, BRCA1 levels vary across different tissues, and similar to how *BRCA1* is transcriptionally regulated, post-translational control of BRCA1 protein levels and compartment location in cells is complex.

The half-life of BRCA1 in asynchronous cells has been found to be relatively short – in HEK 293T, DU145, and HeLa cell lines, the half-life has been measured after cycloheximide treatment to be about 2-4 hours.[118, 119] While expression of BRCA1 is lowest in resting (G_0) cells and in early G_1 of cycling cells, levels begin to rise as the cell moves into late G_1, with levels peaking in S phase and remaining high into M phase.[120, 121] The increase of BRCA1 levels in S phase is preceded by a sharp increase in BRCA1 mRNA in late G_1 which persists into G_2, before decreasing as the cells again move back into G_1.[122, 123] Degradation of BRCA1 happens by ubiquitin-mediated proteolysis by the proteasome, which is also controlled by the cell cycle. Opposite to the pattern seen for BRCA1 levels, a detectable amount of BRCA1 ubiquitination occurs as the cells move from mitosis to G_1 and increases as cells move into S phase, before decreasing once more as the cell moves into G_2/M.[118] Though steady-state BRCA1 protein levels are highest in S phase, ubiquitination and degradation of BRCA1 is also most active in S phase, leading to a high amount of protein turnover.[118] Ubiquitination that leads to subsequent proteasomal degradation occurs in the N-terminal region of BRCA1 within the degron region. There are 14 lysines within the degron region within residues 1-167, all of which can serve as sites of ubiquitination to promote proteasomal degradation, and stabilization of the degron region can only be achieved when all 14

lysines are mutated.[75] One of the known few known proteins that ubiquitinates BRCA1 within the degron region is the E3 ligase HECT and RLD Domain Containing E3 Ubiquitin Protein Ligase 2 (HERC2), a huge 527 kDa protein that belongs to the HERC gene family.[124] BRCA1 co-immunoprecipitation with HERC2 has an inverse relationship with the BRCA1 steady-state levels, where HERC2 is more nuclear and has a higher rate of interaction with BRCA1 in S phase (when BRCA1 undergoes the highest levels of turnover) than in other phases of the cell cycle.[124] The Skp1-Cul1-F-box-protein44 (SCF^{FBXO44}) complex was suggested in 2004 to be involved in BRCA1 ubiquitination due to its role in the cell-cycle, which was later confirmed 8 years later in 2012 when it was found the complex is, like HERC2, able to ubiquitinate the BRCA1 degron and cause proteasomal degradation.[118, 125] Similarly, HECT, UBA, and WWE domain containing E3 ubiquitin protein ligase 1 (HUWE1), another E3 ligase, has also been found to bind BRCA1 in the degron region and promote proteasomal degradation.[126] The cysteine protease cathepsin S (CTSS) has also been found to regulate BRCA1 stability. Unlike HERC2 or the SCF^{FBXO44} complex, CTSS interacts with the BRCA1 C-terminus and cleaves the BRCT domains after radiation-induced damage and promotes BRCA1 ubiquitination in a mechanism that is independent of the degron domain, clearly indicating the necessity for further research into potential BRCA1 E3 ligases.[127] Ubiquitin-conjugating enzyme E2T (UBE2T) (an E2 ligase), has also been shown to mediate BRCA1 ubiquitination and degradation.[128] On the flip side, less is known about the deubiquitinating enzymes (DUBs) that remove ubiquitination from BRCA1 to promote protein stability and protect against degradation. So far, few have been identified; One includes ubiquitin-specific peptidase 9X (USP9X), a highly-conserved protein that belongs to the ubiquitin-specific protease (USP) family.[129] BRCA1 is also stabilized by its major binding

partner, BRCA1-associated RING Domain (BARD1), which will be discussed in further depth in a later section.

While BRCA1 has been found to mostly be a nuclear protein, BRCA1 shuttles back and forth between the cytoplasm and the nucleus. In the cytoplasm, BRCA1 has been found to bind to γ-tubulin at centrosomes during mitosis.[120, 121, 130, 131] BRCA1 has also been found in other cell compartments, including mitochondria in its phosphorylated form, and in the endoplasmic reticulum.[132, 133] BRCA1 is known to get into the nucleus via two distinct pathways. As mentioned previously, to get into the cytoplasm from the nucleus, BRCA1 is transferred across the nuclear membrane via the CRM1/exportin pathway through its NES sequence. BRCA1 is shuttled into the nucleus via the classical importin-α/β pathway, where importin-α/β receptors translocate NLS-cargo through the nuclear pore complex (NPC).[81] BRCA1 is also able to enter the nucleus through a second, NLS-independent pathway that involves BARD1 which will be further discussed later on. Cellular localization is also impacted through interactions with other proteins as well as mutations in BRCA1. Mutations in the C-terminal region have been shown to impede nuclear import, with the thought being that these mutations cause a global change in protein conformation.[134-136] BRCA1-binding protein 2 (BRAP2) binds to the NLS sequences in BRCA1, potentially only when the NLS sequences are flanked by phosphorylation, which impairs the interaction between BRCA1 and importin-α and sequesters BRCA1 in the cytoplasm.[137, 138] The serine/threonine protein kinase Bα, also known as ATK1, has also been found to retain BRCA1 in the cytoplasm.[139] The antiapoptotic protein BLC2 promotes BRCA1 localization in the mitochondria and the endoplasmic reticulum, possibly in relation to the role BRCA1 plays in

apoptosis while the ubiquitin ligase Ubc9 has also been suggested to promote the nuclear import of BRCA1.[133, 140]

2.5 Role of BRCA1 in HRR

The BRCA1 protein is involved in a number of different functions in the cell, most notably in DSB DNA repair. BRCA1 is an essential player in the HRR pathway, which is the mechanism by which DSBs are repaired free of errors during the S and G_2 phases of the cell cycle using sister chromatids as repair templates.[15]

When a DNA DSB forms and HRR is activated in humans, repair begins with resection that can be defined by two steps. The first step, called short-range resection, is initiated by the exonuclease MRE11 which cleaves the 5' broken DNA end with the help of RAD50 and NBS1 (these three proteins make up what is referred to as the MRN complex), all in conjunction with the protein CtBP-interacting protein (CtIP), to create a 3' ssDNA overhang.[141-143] This initial endonucleolytic cleavage helps promote the second step of end resection, where long-range resection enzymes elongate the 3' ssDNA overhangs.[143] RPA binds the 3' ssDNA overhangs to protect against degradation by nucleases and is replaced with the protein RAD51 in the canonical HRR pathway to create a nucleoprotein filament, also called a presynaptic filament.[144] The nucleofilament then begins searching for a homologous sequence (usually on the sister chromatid) to use as a template for repair.[145] The interaction between the RAD51 nucleoprotein filament with the template/donor dsDNA creates what is termed a synaptic complex, also referred to as a paranemic joint.[146] When a homologous sequence has been identified, an intermediate displacement loop (D-loop) is formed as the RAD51 nucleofilament invades the donor dsDNA, displacing one of the dsDNA strands in

what is called the postsynaptic stage. Most of the subsequent DNA synthesis is catalyzed by polymerase δ, with mixed evidence suggesting polymerase ε may or may not also be involved, to recreate the genetic information that was lost in the DSB and during end resection.[147, 148] At this stage, HRR can take one of three pathways to complete repair: canonical HRR, break-induced replication (BIR), or synthesis-dependent strand annealing (SDSA). In the canonical pathway, double Holliday junctions are formed which can lead to either reciprocal crossover or non-crossover between the invading at the template strands during resolution. In BIR, non-reciprocal crossover occurs when DNA synthesis continues to the end of the chromosome arm. Finally, in SDSA, the newly-synthesized DNA becomes ligated to the other end of the broken DNA molecule, leading to a non-crossover repair product (Figure 2.2).

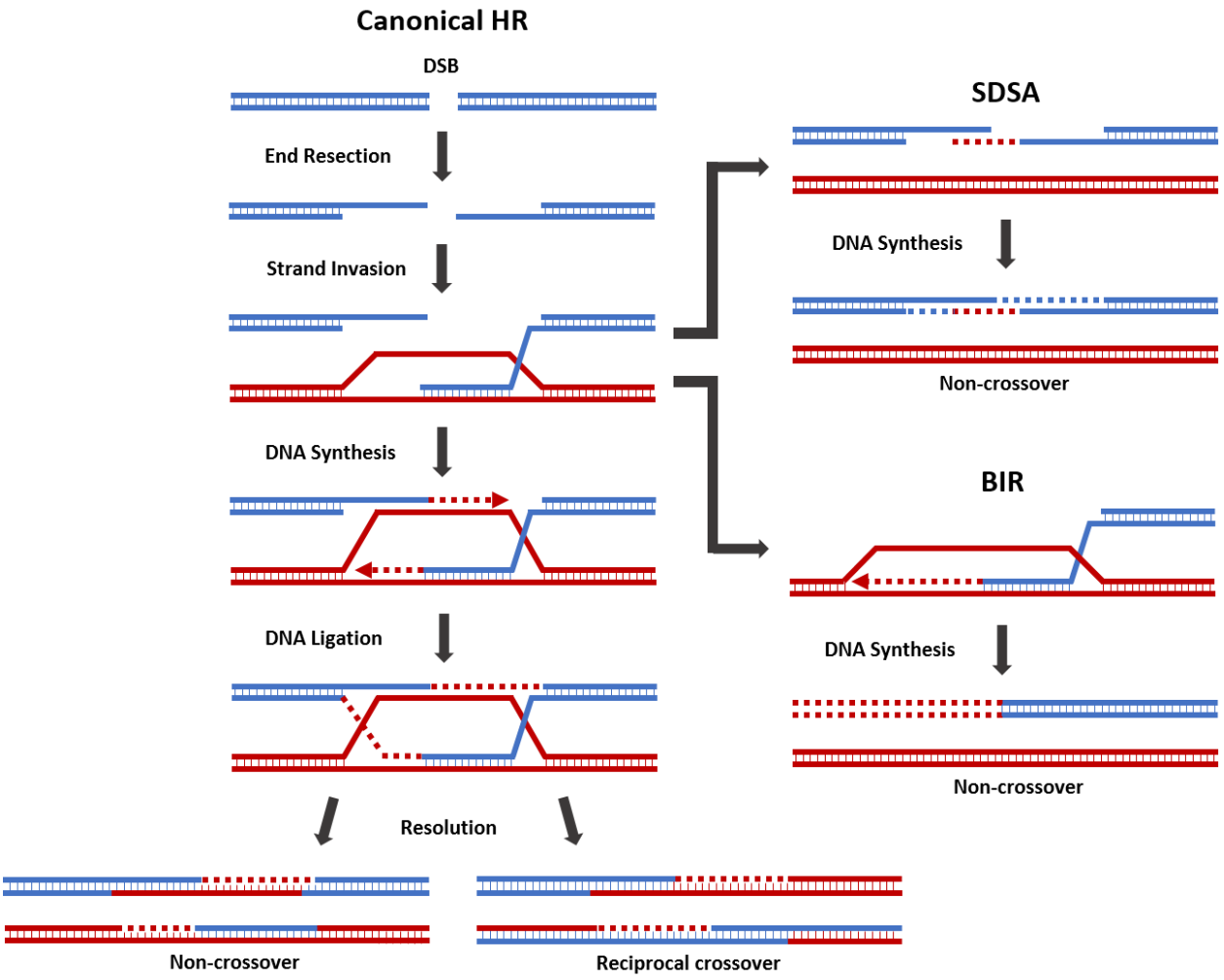

Figure 2.2. Flow Chart of HRR. Flow chart of the canonical HRR pathway, BIR, and SDSA.

BRCA1 is involved in HRR at varied points in the pathway in association with different proteins that form distinct complexes. Once a DSB has formed, a choice of pathway is made between HRR and NHEJ. BRCA1 plays a role in tipping the balance towards HRR by blocking the accumulation of 53BP1, which limits resection of DSBs and instead promotes NHEJ.[149, 150] During end resection, BRCA1 interacts with phosphorylated CtIP and RBBP8 to form the BRCA1-C complex through its C-terminal BRCT domains, though this interaction is not required for DNA resection, as CtIP is seemingly able to function independent of BRCA1 binding.[151-154] However, further

investigation into the BRCA1-CtIP interaction showed that while the interaction is nonessential for CtIP-mediated end resection, BRCA1 speeds up the process.[155] Interestingly, studies have shown the opposite where BRCA1 can inhibit end resection when in the BRCA1-A complex with the proteins Abraxas (also called FAM175A or CCDC98), MERIT40 (or NBA1), BRCC36, BRCC45, and RAP80.[156, 157] It is thought that interaction in these different complexes can help modulate BRCA1 function as related to DNA DSB repair pathway choice.[156, 158] Further downstream in HRR, BRCA1 also aids in RAD51 nucleofilament formation. BRCA2 and partner and localizer of BRCA1 (PALB2) are responsible for assisting in the removal of RPA and the loading of RAD51 on the ssDNA.[159, 160] The coiled-coiled domain in BRCA1 binds to and recruits PALB2, which in turn recruits BRCA2, to sites of damage to aid in RAD51 loading.[161] When BRCA1 binding to PALB2 is abrogated, PALB2 and BRCA2 are no longer stably recruited to damage sites, thus diminishing the ability of PALB2, BRCA2, and RAD51 to localize to sites of DNA damage. BRCA1 also interacts with BARD1 during HRR, though this will be further discussed later.

The recruitment of BRCA1 to sites of damage has been explained by a few possible theories of mechanism. One line of thought is that after damage has occurred, the E3 ubiquitin ligase RNF8 binds to the scaffolding protein mediator of DNA damage checkpoint protein 1 (MDC1). RNF8 and Ubc13, an associated E2 ubiquitin-conjugating enzyme, ubiquitinate MDC1 at lysine 1977 which binds RAP80. RAP80, which is part of the BRCA1-A complex, then recruits BRCA1 through Abraxis, which directly interacts with the BRCA1 BRCT domain.[162-165] In a second line of thinking, RNF8 and RNF168, another ubiquitin ligase, mediates polyubiquitination of H2AX

or H2A histones with Ubc13, which recruits RAP80, leading to subsequent BRCA1-A complex recruitment.[166-168] BARD1 additionally helps recruit BRCA1 to sites of damage.

2.6 Other Cellular BRCA1 Functions

In addition to HRR, BRCA1 is involved in other pathways in the DDR, including checkpoint signaling, RSR, NER, NHEJ, transcriptional activation, and apoptosis. After IR, ATM phosphorylates BRCA1 on serines 1387 and 1423 (the kinase ATR is also able to phosphorylate serine 1423) which are required for proper activation of the S and G_2/M checkpoints, respectively.[169, 170] The BRCA1-B complex, made up of BRCA1, TopBP1, and BRCA1 interacting protein C-terminal helicase 1 (BRIP1, also known as FANCJ or BACH1), contribute to HRR and S-phase cell cycle arrest. The mechanism by which the complex contributes to these activities has yet to be fully understood; as a helicase, BRIP1 may be involved in end resection, while TopBP1 is known to be required for S-phase checkpoint and ATR activation.[157, 171] BRCA1 seems to additionally affect the G_2/M checkpoint through the regulation of G_2/M checkpoint proteins, including CHK1, WEE1, and the 14-3-3- family of proteins.[172-174] The BRCA1-A and C complexes have also been implicated in G_2/M checkpoint arrest in response to DNA damage.[175]

BRCA1 additionally takes part in the RSR, which functions to protect and repair stalled replication forks during DNA synthesis. In conjunction with BRCA2, BRCA1 protects the DNA at stalled replication forks from MRE11 degradation by loading RAD51 onto the exposed ssDNA.[176-178] Similar to HRR, BRCA1 has an antagonist relation with 53BP1 in determining how stalled replication forks will ultimately be repaired.[179] The BRCA1-B complex has also been implicated in being involved in the RSR.[180] The role of BRCA1 in NHEJ has also been investigated, both in

the canonical pathway (C-NHEJ) and the alternative pathway (A-NHEJ; also referred to as microhomology-mediated end-joining or MMEJ).[181] As previously mentioned, the BRCA1-C complex promotes HRR, competing with the pro-C-NHEJ factors 53BP1 and RIF1 for space at DSB sites.[182, 183] While there has been conflicting evidence as to whether BRCA1 is indeed required for proper C-NHEJ function after a pathway choice has been made in favor of C-NHEJ, it has been suggested that BRCA1 does positively regulate the pathway at a later step.[184] Phosphorylation by Chk2 at serine 988 and by ATM (or ATR) at serines 1423 and 1524 have been implicated in promoting precise end-joining.[185-187] Studies have also suggested that the interaction of BRCA1 with the C-NHEJ protein Ku80 is required for proper C-NHEJ function through stabilization of the Ku heterodimer at DSBs in G_1.[188] In contrast, there is some evidence to suggest that BRCA1 acts against A-NHEJ, as loss of BRCA1 has been reported to increase the frequency of DNA mutagenesis and A-NHEJ.[189] Furthermore, disrupting the interaction between BRCA1 and BRIP1 seems to compromise C-NHEJ and promote A-NHEJ.[190] However, as is the case with C-NHEJ, the role of BRCA1 in A-NHEJ is still unclear with conflicting results from different studies. This may be the result of one or more causes, such as the use of different cell types or the use of varied assays. In addition to the DNA DSB repair pathways, BRCA1 seems to be involved in detecting damage in the NER pathway through the BRCA1-associated genome surveillance (BASC) complex, which is made up of the MRN complex, ATM, MutL protein homolog 1 (MLH1), BLM (a helicase), DNA replication factor C (RFC), MutS protein homolog 2 (Msh2), and Msh6.[191] In addition to repairing bulky lesions in DNA, NER is also used to repair actively-transcribed DNA in a process called transcription-coupled repair.[191] It is thought that in conjunction with BARD1, BRCA1 promotes degradation of RNA Polymerase II through E3 ligase activity (discussed further in a later section) to allow the repair machinery to the sites of damage

as well as to potentially prohibit transcription after DNA damage to prohibit transcription of affected genes.[192, 193] BRCA1 may also upregulate transcription of the proteins DDB2 and xeroderma pigmentosum C, both of which function in the NER pathway, as well as the BER-related proteins OGG1, NTH1 and REF1 (APE1).[194-196] The pathways involved in the DDR are complex, and as such, it is no surprise that the field still has more work to do to have a better understanding of the many roles of BRCA1 in the DDR.

BRCA1 also regulates the transcriptional activity of many genes that are involved in an array of different cellular pathways in the DDR to help maintain genomic stability. BRCA1 helps regulate genes, with evidence suggesting that BRCA1 may act as an activation domain contributor. After phosphorylation by ATM and ATR at serines 1423 and 1524, BRCA1 promotes ATM-mediated p53 activation, subsequently leading to an increase in p21 (a CDK inhibitor involved regulation of the G_1/S checkpoint) transcription.[197] In an interesting show of nuance, modulation of transcriptional activation through p53 is selective: BRCA1 promotes the transcription of genes related to DNA repair and cell-cycle arrest but not the pro-apoptotic genes regulated by p53.[198, 199] Intriguingly, p53 also seems to regulate BRCA1 function by directly binding to BRCA1 to mediate nuclear export after DNA damage has occurred, which leads to increased cellular susceptibility to IR.[200] BRCA1 is also involved at different points in the estrogen response signaling pathway. In this pathway, estrogens will bind to and stimulate ERα, which leads to activation via dimerization and binding to estrogen response elements to promote transcription of certain genes, typically those associated with proliferation, such as cyclins E and D, vascular endothelial growth factor, and epithelial growth factor.[157, 201] BRCA1 binds ERα and inhibit downstream signaling, lowers the expression of ERα-regulated genes, and binds to COBRA1,

which is part of a complex that inhibits ERα-mediated transcription.[202-205] BRCA1 also binds to chromatin remodelers such as the histone deacetylases HDAC1 and 2 and BRG1 and BRD7, which are part of the SWI/SNF chromatin remodeling complex.[206-208] Oct-1 recruits BRCA1 to a range of different gene promoters to promote transcription of different proteins including ERα, GADD45 (which is pro-apoptotic and a DNA damage-induced cell cycle inhibitor), and as previously mentioned, the BER pathway proteins OGG1, NTH1, and REF1 (APE1), and the NER pathway proteins DDS and xeroderma pigmentosum C.[194, 196, 209-211] Interestingly, BRCA1 has also been reported to repress GADD45 under normal cellular conditions in a complex with CtIP.[152, 212] Both proteins also interact with ZBRK1 in a complex that represses GADD45 as well as HMGA2, angioproietin-1 (ANG1), and RFC1, where BRCA1 is necessary for repression to occur via ZBRK1.[213, 214] BRCA1 furthermore acts as a transcriptional corepressor with c-Myc and n-myc-interacting protein (NMI) to downregulate cancer-associated genes like psoriasin and hTERT.[215, 216] After DNA damage and subsequent phosphorylation at serine 1423, BRCA1 is additionally recruited to a complex involving the proteins BCLAF1, U2AF35, U2AF65, Prp8, and SF3B1 to promote efficient splicing of HRR-related genes, including ATRIP, BACH1, and EXO1.[217] Hypophosphorylated RB, which interacts with E2F and prevents transcription of specific genes to inhibit cell proliferation, also binds to BRCA1. This interaction may keep RB in a hypophosphorylated state and promote cell growth inhibition.[218]

As mentioned in previously BRCA1 seems to play a role in the apoptotic pathway in another function to act as a tumor suppressor.[219] Interestingly, BRCA1 seems to have conflicting roles in that BRCA1 can both inhibit and promote apoptosis, suggesting it acts as a gatekeeper for determining the fate of a cell depending on the extent of DNA damage that has occurred: repair or

death. Early after its initial discovery, BRCA1 was linked to the promotion of apoptosis.[219] Later on, it was found that BRCA1 acts as a co-activator with signal transducer and activator of transcription protein (Stat-1) to upregulate the pro-apoptotic genes ISG54, OAS, MxA, and IRF7 after interferon-γ induction. BRCA1 has also been linked to caspase-3 activity after UV damage: In SNU251 cells, XIAP, which binds to and inhibits caspase-9 from activating caspase-3 during apoptosis, also complexes with BRCA1.[220] The interaction between BRCA1 and XIAP is disrupted after UV-induced BRCA1 phosphorylation on serines 1423 and 1524, which in turn allows caspase-9 to dissociate from XIAP and activate caspase-3 to promote apoptosis.[220] It is thought that BRCA1 acts as a scaffold between XIAP and casepase-9, and dissociation from BRCA1 is necessary for dissolution of the XIAP-caspase-9 complex.[220] Phosphorylated BRCA1 has also been found in mitochondria and in complex with BCL2 in the endoplasmic reticulum, which decreases HRR and has been speculated to increase apoptosis.[133] On the contrary, BRCA1 loss has been shown to increase apoptosis after cisplatin treatment.[221] Furthermore, after IR damage, BRCA1 and p65 recruit p50 to promoters to activate transcription of anti-apoptotic NF-kB targets, including BCL2 and XIAP.[222]

BRCA1 has also been implicated in telomere regulation to maintain genomic stability. Telomeres, which cap the ends of chromosomes and are made up of hexameric DNA repeats (TTAGGG), are related to DNA repair and DSBs in a few different ways. First, DNA damage can shorten telomeres and increase cell senescence and chromosomal instability. Second, telomerase gets recruited to DSBs, raising the possibility of generating telomeres in the middle of a chromosome. Third, telomeres protect ends of chromosomes from being recognized as DSBs. Six proteins form the shelterin complex that recognizes telomeric DNA and protects telomeres from being recognized

by DSB repair pathway proteins by helping form the telomere loop at the end of the chromosome. BRCA1 inhibits telomerase via transcriptional repression of telomerase reverse transcriptase (TERT), which is the catalytic subunit of telomerase, by inhibiting c-Myc from stimulating TERT expression at the TERT proximal promoter.[223] A later study showed that BRCA1 is present on telomeres and interacts in a DNA-dependent manner with the shelterin proteins TRF1 and TRF2.[224] It was found that BRCA1 is recruited by the MRN complex to telomeres where they both regulate the length of the telomeric overhang that promotes telomere extension and stability.[224]

2.7 Discovery, Structure, and Regulation of BARD1

Shortly after BRCA1 had been identified, BRCA1–associated RING domain 1 (BARD1) was discovered in 1996 as a binding partner of BRCA1 in the N-terminal region.[225] While BARD1 has been studied mostly as an accessory to BRCA1, there has recently been more attention paid to BARD1 in the role the protein plays in BRCA1 regulation, cancer development and progression, and genome maintenance.

The group of researchers who discovered BARD1 mapped the human BARD1 gene to chromosome 2, close to the telomeres at 2q34 and 2q35.[225] BARD1 is coded by 11 exons which produce a protein made up of 777 amino acids.[225] Similar to BRCA1, BARD1 contains a conserved RING finger domain at the N-terminus that encompasses residues 46 to 90. In a unique combination, BARD1 also contains four highly-conserved ankyrin (ANK) repeats to make an ankyrin repeat domain (ARD) from residues 425-555 (with the last being slightly truncated), and two BRCT domains in the C-terminus from residues 568-777.[225-227] Unlike BRCA1, the BRCT

domains in BARD1 recognize a pSer-Asp/Glu-Asp/Glu-Glu motif, indicating BARD1 selectivity differs from that of the BRCT domains in BRCA1.[228] BARD1 contains an NES from residues 102-120 as well as six predicted NLS sequences, two close to the RING domain and NES, two near the ARD, and two within the BRCT domains, though NLS2 and NLS6 seem to have little impact on BARD1 nuclear localization, while NLS3 has strong nuclear localization activity.[229, 230]

Figure 2.3. Primary Structure of BARD1. Primary structure of the BARD1 full-length protein indicating the location of the different functional domains.

Like BRCA1, BARD1 is predominantly nuclear and is regulated by the cell cycle, where levels are lowest during G_1 and rise through S, peaking in G_2 and M.[230] A number of different isoforms of BARD1 besides the full-length version have been identified, which are called α, β, κ, γ, δ, φ, ε, η, π, and ω, though all lack part or all of their RING and/or ANK repeat domains and have been to be linked to tumor-permissive phenotypes rather than normal cellular functions.[231] Relative to BRCA1, there is less much information on the regulation of BARD1 at both the transcriptional and molecular level; A 2002 study found that E2F4 was enriched on the *BARD1* gene, while a later 2007 study found that ERα interacts with three estrogen response elements (ERE) half sites within the ninth intron of the BARD1 gene.[232, 233] The same 2007 study showed that treatment of MCF-7 cells with 17β-estradiol (E2) leads to an upregulation of BARD1 at the mRNA and protein level, where E2 binds to ERα to promote interaction and upregulation of the BARD1 gene, thus providing a potential mechanistic explanation for the hormone-linked association between BRCA1/BARD1 deficiencies and increased breast/ovarian cancer risk.[233] Regulation at the

transcriptional level has also been investigated with regard to long non-coding RNAs and microRNAs, which may play a role in the increased expression of tumor-promoting BARD1 isoforms and inhibition of expression of full-length BARD1. The BARD1 promoter upstream of exon 1 controls expression of the full-length protein as well as the aforementioned isoforms. The majority of the full-length BARD1 transcripts and some of the isoform transcripts have a long 3'UTR region (BARD1β, BARD1δ, and BARD1γ seem to have shorter 3'UTR regions), with the longest 3'UTR spanning about 3030 nucleotides, where some regions have a high degree of conservation.[234] An alternative promoter has been observed in intron 9 that controls the production of the putative isoform BARD1 9'S, of which the first six amino acids are unique with the remainder corresponding to residues 636-777 of full-length BARD1, and the non-coding long RNA BARD1 9'L, which contains a longer portion of the intron 9 fused to exon 10.[234] BARD1 9'L has been shown to protect BARD1 mRNA from repression by miR-203 and miR-101, which reduce expression of full-length BARD1, BARD1β, BARD1δ, and BARD1γ mRNAs.[234] In conjunction with the putative RNA binding sites in the 3'UTR region of full-length BARD1 and BARD1 isoforms, it stands to reason BARD1 9'L may act as a regulator of BARD1 expression.[234] MiR-19a and miR-19b have also been reported to down-regulate the BARD1ω

At the protein level, BARD1, again similar to BRCA1, undergoes phosphorylation that varies with the cell cycle, where hyperphosphorylation occurs during M phase.[235] Seven potential sites of phosphorylation have been identified by mass spectrometry, where T299 has been validated as a phosphorylation site during mitosis.[235] Furthermore, a different study showed that the N-terminus of BARD1 is targeted by CDK2-cyclin A_1/E_1 which decreases BARD1 stability through impairment of heterodimer formation with BRCA1.[236] BARD1 stability is also affected by the

anaphase promoting complex (APC/C), an E3 ubiquitin ligase, which targets certain proteins (including BARD1) for degradation by the 26S proteasome in a cell-cycle dependent manner to properly regulate mitotic spindles formation.[237] BARD1 is also cleaved during apoptosis by the calpain protease.[238]

2.8 BARD1 Cellular Functions

Though BARD1 has been mostly studied in the context of BRCA1, BARD1 has been suggested to have functions separate from BRCA1. The first evidence of their distinct roles came from observations that after embryonic day 11 in mice, BARD1 and BRCA1 are differentially expressed in certain tissues.[239, 240] Indeed, since then, it has been shown that BARD1 has independent roles in different cellular pathways. BARD1 levels increase in response to different stimuli, such as genotoxic stress and hypoxia, and induces apoptosis by binding to and stabilizing p53.[241-243] BARD1 has also been linked to S-phase progression, growth, and genetic stability. At later stages of mitosis, BARD1 dissociates from BRCA1 to interact instead with BRCA2 and Aurora kinase B to promote the completion of cytokinesis.[244] BARD1 has also been found to bind to mRNA polyadenylation factor CstF-50, a complex involved in 3' end cleavage ad polyadenylation of pre-mRNA, after DNA damage *in vitro* and *in vivo*.[245] The CstF-50 binding domain on BARD1 falls within the region containing the ANK repeats and the BRCT domains: The BARD1 mutation Q564H, which has been linked to breast and ovarian cancers, reduces BARD1 binding to CstF-50, while BARD1 phosphorylation by ATM at T714 after DNA damage promotes interaction with and inhibition of CstF-50 to prevent mRNA maturation.[245, 246] These observations may suggest a mechanism by which loss of proper BARD1 function leads to increased mRNA production and

promotes a cell-growth permissive phenotype rather than proper inhibition of the cell cycle after DNA damage.

BARD1 has been linked to the induction of apoptosis in a manner independent of BRCA1. After DNA damage, BARD1 translocates to mitochondria to induce apoptosis, potentially through the tumor suppressor p53 which shows a significant translocation to the mitochondria after DNA damage. P53 competes with CstF-50 to bind to BARD1 within the ARD and the region adjacent to the BRCT domains, and BARD1 binding to p53 has been shown to stabilize p53 and promote apoptosis.[242, 247] Though these observations have been shown to not depend on BRCA1, evidence suggests BRCA1 interferes with BARD1-mediated apoptosis.[241, 243] As mentioned previously, BRCA1 has also been shown to interact with p53, suggesting an interesting and complex dynamic between BRCA1, BARD1, and p53.[200]

Interactions with other proteins have given further evidence that BARD1, like BRCA1, is a tumor suppressor. It has been established that cervical cancer can be caused by certain human papillomavirus (HPV) strains mediated by the major oncogenic viral protein HPV E6 which binds to and sequesters p53. BARD1 has been found in cervical cells to bind to HPV E6 and repress HPV E6 action in cervical cancer cells, underscoring the tumor suppressor functions of BARD1 independent of BRCA1.[248] Furthermore, BARD1 has been linked to Ewing's sarcoma (EWS) gene product (an RNA-binding protein), NF-κB, and PAR signaling after DNA damage.[249-251] A vast majority of Ewin family tumors shows a fusion between the N-terminus of EWS and the DNA-binding domain of the transcription factor FLI1, where BARD1 interacts with EWS and the EWS-FLI1 fusion, suggesting a potential link with a tumor suppressor pathway.[249] NF-κB, which

binds in vitro to the C-terminus of BARD1, has also been linked to cancer, while protein poly-ADP-ribosylation by PARP1 at sites of DNA damage recruits the BRCA1-BARD1 complex through interaction with the BARD1 BRCT domains.[250, 251]

2.9 BARD1 with BRCA1

While BRCA1 and BARD1 have separate functions, many of their roles are dependent on their interaction with the other. Each are able to form homodimers via their RING domains, but they preferentially create a more stable obligate heterodimer involving residues 1-109 of BRCA1 and residues 26-119 of BARD1, to the point where most BRCA1 is in complex with BARD1 through their respective RING domains.[76, 252, 253] BRCA1 and BARD1 are necessary for their mutual stabilization, where BARD1 helps cover the BRCA1 degron domain and protect against HERC2 ubiquitination and subsequent proteasome-mediated degradation.[124, 254] BRCA1 and BARD1 also promote mutual nuclear retention, as heterodimerization masks the NES of both proteins.[230, 255, 256] Furthermore, BARD1 plays a chaperone role for BRCA1 translocation into and retention in the nucleus independent of the BRCA1 NLS, where BRCA1 similarly aids in BARD1 nuclear retention.[229, 230, 242, 253, 255, 257, 258] The primary NES that spans residues 81-99 on BRCA1 is located in one of the two α-helices that flank the RING domain and contributes to the interaction interface with BARD1.[258] Given these observations, it has been suggested that the binding of BARD1 to BRCA1 covers the BRCA1 NES and prevents association with CRM1, leading to a retention of BRCA1 in the nucleus.[229, 255] Reciprocally, BARD1 has an NES at a similar position in the BRCA1-BARD1 interaction region BRCA1 also masks the BARD1 NES to promote BARD1 nuclear retention.[229] However, despite their propensity to form a complex, an NMR structure of their interaction shows the heterodimer is not tightly packed at their RING motifs in contrast to

the close helical interface between the two protein, suggesting the potential for flexibility in the RING motifs.[258] The NMR data also suggested evidence for certain contacts between BRCA1 and BARD1 residues, namely BRCA1 residues R7, E10, E85, and D96, and BARD1 residues D117, K110, R43, and H36, respectively.[258] A pathogenic mutant in BRCA1, C61G, which disrupts the interaction between the two proteins, underscores clinical significance of the heterodimerization BRCA1 and BARD1, as the mutation which leads to embryonic lethality, decreased DNA repair efficiency, and an increased risk of tumor formation.[259]

When in a complex together, BRCA1 and BARD1 have E3 ubiquitin ligase activity.[253, 260] BRCA1 and BARD1 occurs through interaction with the E2 ubiquitin-conjugating UbcH5 and other E2 enzymes, which bind BRCA1 on the opposite side of the BARD1-biding region.[254] This activity has been found to be influenced by different factors: Phosphorylation of BARD1 by CDK1 or CDK2 in its N-terminus inhibits E3 ligase activity as does the de-ubiquitinating enzyme (DUB) BAP1.[236, 261] The synthetic mutation I26A in BRCA1 leads to a loss of E3 ligase function without disrupting complex formation with BARD1, though interestingly, this mutant does not seem to occur naturally.[157, 254] However, there has been some question as to how essential the ligase function is for cell survival and HRR, potentially due to the fact that the extent to which BRCA1-BARD1 E3 ligase activity plays a role in different cellular functions has yet to be extensively investigated.[262, 263] Mice with the I26A BRCA1 mutation form tumors at a similar frequency to mice with wild-type BRCA1, suggesting the E3 ligase activity of the heterodimer may be dispensable for tumor suppression.[263] Nevertheless, a number of putative ubiquitination substrates have been identified *in vitro*, including topoisomerase II α, ERα, RNA polymerase II, the histones H2A, H2AX, H2B, H3, and H4, NPM1, CtIP, RPB8, TFIIE, and BRCA1-BARD1

itself, and studies have shown BRCA1-BARD1 E3 ligase activity is important for a number of different cellular functions, including proper DNA repair, in human cells. The BRCA1-BARD1 heterodimer adds ubiquitin through a K6 linkage on proteins, though this ubiquitination seems to have functions not linked to degradation, where autoubiquitination even increases BRCA1-BARD1 stability and E3 ligase activity, as well as the cellular response to DNA damage.[260, 264-267] Furthermore, through this enzymatic activity, the heterodimer regulates the enzymatic activity of topoisomerase II α and RNA Pol II, where ubiquitination of topoisomerase II α by the BRCA1-BARD1 heterodimer occurs after hypoxia and downregulates topoisomerase II α activity, whereas ubiquitination occurs on phosphorylated RNA polymerase II after DNA damage has occurred to promote RNA polymerase II degradation.[192, 193, 241, 268, 269] At the beginning of mitosis, BRCA1 is involved in centrosome duplication, though localization to the centrosome is BARD1- and Ran GTPase-dependent.[270] Later on in mitosis, BRCA1-BARD1 has also been found to regulate centrosome number via ubiquitination of γ-tubulin, which plays an important role in nucleation of microtubule polymerization, and promote ubiquitin-mediated degradation of the Aurora B kinase in cytokinesis.[244, 271] BRCA1-BARD1 ubiquitination may also control ERα and progesterone receptor levels.[272, 273]

The E3 ligase activity of the heterodimer has also been shown to play a role in DNA repair and maintaining genomic stability. Interestingly, cells that express a BRCA1-BARD1 heterodimer that lacks ligase activity are sensitive to DNA-damaging agents such as IR, etoposide, olaparib, and camptothecin, though at the same time a resistance to replication-stressing agents such as aphidicolin and hydroxyurea.[274] Furthermore, RPA and RAD51 foci formation during S-phase after IR was reduced, the resection length on ssDNA was shortened, and 53BP1 was found to co-

localize with BRCA1 at foci.[275] Normally, BRCA1 is required to push 53BP1 to foci periphery in order to promote HRR, but the co-localization of 53BP1 with BRCA1 in the center of foci suggests that rather than BRCA1 physically impeding 53BP1 from sites of damage, the ligase activity is the driver for 53BP1 repositioning.[275] The E3 ligase activity of the heterodimer may also be critical for chromatin remodeling, as the complex is able to monoubiquitinate H2A, H2B, and H2AX in vitro.[260, 264, 265, 276] It is thought that BRCA1-BARD1 modify H2A at lysines K125 (H2K125), K127 (H2K127) and K129 (H2K129) after DNA damage to aid in promoting end resection and HRR in a SWI/SNF-Related, Matrix-Associated Actin-Dependent Regulator Of Chromatin, Subfamily A, Containing DEAD/H Box 1 (SMARCAD1)-dependent manner.[277, 278] BRCA1-BARD1-mediated ubiquitination of H2A in heterochromatin was also reported to repress satellite DNA, which in turn promotes genomic stability.[279] The BRCA1-BARD1-mediated ubiquitination and degradation of RNA polymerase II after DNA damage has also been thought to act as a mechanism to allow access to sites of damage by repair machinery as well as repress transcription on a genome-wide level while at the same time promoting upregulation of genes involved in DNA repair.[192, 193] Furthermore, reports have indicated that BRCA1 recruitment to sites of damage independent of γ-H2AX is dependent on the BRCT-domains on BARD1, and the BRCA1-BARD1 complex is required for phosphorylation of p53 to induce G_1/S arrest after DNA damage caused by IR.[197, 251]

2.10 Clinical Implications of BRCA1 and BARD1

Mutations or deficiencies in either BRCA1 or BARD1 have been well-established to have clinical impacts. Knockout of *BRCA1* in mice is embryonic lethal, while mice with *BRCA1* knockdown using a Cre recombination system develop breast carcinomas similar to triple negative breast

cancers seen in humans.[280, 281] Similarly, knockout of *BARD1* in mice is embryonic lethal, where the embryos die around day E8.[282] Furthermore, inactivation of the *BARD1* gene using a Cre recombination in mice leads to the development of breast carcinomas similar to those seen in mice with BRCA1 inactivation using the same Cre construct.[281] Interestingly, mice form tumors twice as quickly when BARD1 or BARD1/BRCA1 are knocked down than BRCA1 alone, suggesting BARD1 may have a dominant role.[281]

A number of germline and somatic mutations have been identified across the BRCA1 and BARD1 proteins that occur in ovarian, breast, and uterine cancers. Pierre Paul Broca, a 19th century French physician widely known for his discovery of Broca's area, also took an interest in the causes of cancer and speculated from observations of families with a high rate of cancer diagnoses that there might be an inheritable factor in the development of cancer.[283] However, it wouldn't be until the late 20th century when Hall *et al.* identified the region on chromosome 17 that contains the *BRCA1* gene.[66, 67] It is thought that while only a small percentage of breast cancer cases are inherited (~5% to 10%), those who inherit pathogenic *BRCA1* mutations have a much greater lifetime risk of developing cancer: Though estimates vary between different studies, it has been estimated that those with BRCA1 mutations have a 55% to 80% chance of developing breast cancer a 16% to 68% of developing ovarian cancer by the age of 70.[284, 285] There have been over 1700 unique *BRCA1* mutations identified that have been reported to the Breast Cancer Information Database, where roughly half have been validated as clinically significant, which indicates an increased risk of developing cancer over one's lifetime for those with the mutations. Within BRCA1, there are three regions that show a high frequency of mutation, including the RING domain, exons 11-13, and the BRCT domains.[286] Mutations within the BRCA1 RING domain have been found to

disrupt the E3 ubiquitin ligase activity of the BRCA1/BARD1 heterodimer, which seems to contribute to a predisposition to breast and ovarian cancers.[253, 256, 287] Mutations in exons 11-13, which make up the majority of the protein-coding region of BRCA1, affect the binding of BRCA1 to its different substrates. These substrates, which include RB, PALB2, c-Myc, Rad50, and Rad51, are involved in a wide range of critical cellular pathways such as DNA repair, cell cycle progression, and transcriptional regulation.[286] Mutations within this region can also affect the two NLSs.[81] Interestingly, associations have been found between cancer cells and the mislocalization of BRCA1 to the cytoplasm, though it comes as little surprise given BRCA1 can only properly function in DNA repair when in the nucleus. Mislocalization could be due to either mutation of the NLSs, or mutations in the RING domain that decrease BRCA1 binding to BARD1.[288] Mutations in the C-terminus have also been implicated in contributing to BRCA1 cytoplasmic localization, potentially through inducing altered protein folding.[134] Other C-terminus mutations within the BRCT domains impact binding to other BRCA1-binding proteins, including p53.[286] Interestingly, patients with inherited BRCA1 mutations tend to form breast cancers that are classed as triple-negative due to no expression of ER, PR, and ERB2/HER2, and express basal-cell markers.[289-293]

While advancements in technology continue to identify new BARD1 mutations associated with an increased predisposition towards cancer, the frequency of mutations that have been found in the protein coding exons of BARD1 is much lower than BRCA1 (with an estimated mutation rate ranging from 2.8% to 6.1% in inherited breast/ovarian cancer patients, while 16% to 40% of these patients show BRCA1/BRCA2 mutations), suggesting that many BARD1 mutations may be embryonic lethal.[231, 294] Furthermore, unlike BRCA1, there seem to be no “hot-spot” regions of

mutation. Nevertheless, several mutations have been identified that establish BARD1 as an important tumor suppressor. In 1998, the first missense mutations linked to an increased cancer risk were identified as Q564H, V695L, and S761N.[295, 296] Other missense mutations potentially linked to breast, ovarian, and uterine cancers have similarly been found since then in cancer patients, including P24S, V85L, S241C, N295S, K312R, R378S, Q406R, N470S, V507M, I509T, and C557S, though conflicting studies suggest that more work must be done before any conclusions can definitively be made between the association of these mutations with an increased cancer risk.[295-301] However, the discrepancies in findings may be explained by the sample population used in the studies as well as the possibility that other genetic factors may influence the pathogenicity of certain *BARD1* mutations.[302-310] Other BARD1 variants have also been detected, including silent mutations/polymorphisms (A502, H506, 1203T>C), mutations leading to splicing variants (1315-2A>G, 1977A>G), duplications (E652Vfs*69), deletions (L359_P365del), and truncations (Q564*).[297, 301, 302, 311, 312] Other BARD1 variations have also been described that have suggested to be linked to other cancers.[231]

The different splicing variants of BRCA1 and BARD1 have also been identified and seen to highly express in a variety of cancers, though interestingly, of the two, only BRCA1 promoter methylation has been found in cancer cases, whereas no methylation and subsequent inactivation has been seen with BARD1.[234, 240, 243, 298, 313-323] The expression of the BARD1 isoforms has been found to vary depending on the type of cancer, but have been correlated to a poor clinical outcome in breast, ovarian, lung, and colon cancer.[315, 318, 319] However, this comes as little surprise as all of the identified BARD1 isoforms are missing part of the RING domain and/or the ANK repeats, both of seem to be required for proper function for tumor suppression, which also explains why full-

length BARD1 is downregulated or not detectable in cancers. For example, BARD1β, which lacks exons 2 and 3 and has an alternative open reading frame in exon 1, lacks the RING finger domain and is thus unable to interact with BRCA1. This variant was found along with BARD1δ, an isoform lacking exons 3 to 6 and thus part of the RING domain and ARD, in a rat ovarian cancer cell line where both isoforms were highly expressed, while full-length BARD1 was undetectable at the mRNA or protein level.[240, 243] BARD1β has since been found to have a dominant negative effect and promote cell growth and an invasive phenotype by overriding the mitotic checkpoint. At the molecular level, BARD1β acts as a scaffold between Aurora B and BRCA2 during telophase and cytokinesis at the midbody, which prevents ubiquitination and degradation mediated by the E3 ligase activity of the wild-type BRCA1-BARD1 complex.[244] The long non-coding RNA BARD1 9'L has also been shown to have high expression in cancer cells and promote full-length BARD1 and BARD1 isoform upregulation, competing with mi-R-101 and miR-203 that reduce BARD1 expression.[234] Furthermore, the BARD1ω isoform, which has associated with acute myeloid leukemia, has been shown to be downregulated in the presence of miR-19a and miR-19b.[322]

2.11 SIRT2

The sirtuins, i.e. the Silent information regulator 2 proteins, are a family of nicotinamide adenine dinucleotide (NAD^+)-dependent class III deacetylases named after the first identified sirtuin protein, Silent Information Regulator (*SIR2*), that was characterized as a targeted gene transcription repressor in *Saccharomyces cerevisiae*.[324] Sirtuins have been found to be broadly conserved throughout the three kingdoms of life: Eukaryotes tend to have multiple, diverse family members, whereas bacteria and archaea typically have one or two sirtuins that play a role in metabolism and DNA regulation.[325, 326] All sirtuins contain a highly-conserved catalytic core domain that catalyzes lysine deacetylation through the transfer of the acetyl group to NAD^+. The

reaction is thought to begin via a nucleophilic attack by the carbonyl oxygen on the acetyl-lysine on the C1' of the nicotinamide ribose of NAD^+ which leads to the release of nicotinamide and the formation a positively charged O-alkyl-amidate intermediate. Nicotinamide acts as a noncompetitive inhibitor of the sirtuins by reacting with this intermediate of the reaction and reversing the reaction back to where it began. In absence of inhibition, the intermediate undergoes an internal attack of its 2' OH that is dependent on a conserved histidine in the sirtuin enzyme that ultimately leads to deacetylation of the lysine and the formation of 2'-O-acetyl-ADP-ribose.[327] *SIR2* piqued particular scientific interest when it was found that the gene played a role in extending the replicative lifespan of yeast, whereas SIRT2 deletion resulted in a decreased lifespan.[328, 329] Since then, *SIR2* homologues orthologs in different species, including humans, have been the focus of a multitude of investigations for their putative role in aging, health, and longevity.

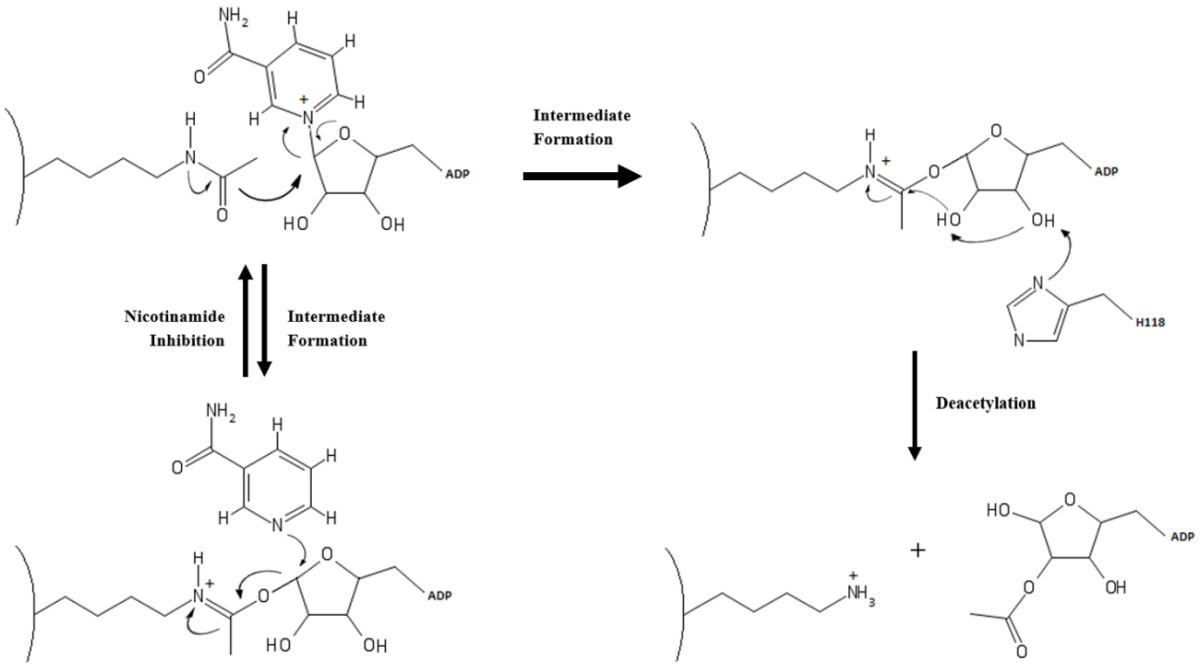

Figure 2.4. Lysine deacetylation by sirtuins and nicotinamide inhibition.

There are seven mammalian sirtuins that are homologues of the yeast *SIR2*, SIRT1-7.[330] While all of the members contain a core deacetylase domain, each varies in their N- and C-termini and have diverse functions and localizations in the cell.[331] SIRT1 and SIRT6 are localized to the nucleus where they play a role in epigenetic regulation through histone deacetylation, though SIRT1 has been found to transiently localize to the cytoplasm, while SIRT7 is primarily found in the nucleolus.[332, 333] SIRT2 is found mostly in the cytoplasm but also localizes to the nucleus in a cell-cycle dependent manner.[334-337] SIRT3, SIRT4, and SIRT5 are mitochondrial proteins that aid in the regulation of the metabolism and response to oxidative stress.[338] Some of the sirtuins have also been demonstrated to have other enzymatic activities besides lysine deacetylation including deacylation of fatty acyl groups, desuccinylation, demalonylation, and mono-ADP-ribosylation. SIRT1, 2, 3, and 5 have been reported to have high deacetylase activity on histone H4, while SIRT4, SIRT6, and SIRT7 seem to have very low levels of deacetylase activity.[339] SIRT1, SIRT4, and SIRT6 additionally demonstrate ADP-ribosyltransferase activity.[339] Reports have shown that SIRT5, while having deacetylase activity, preferentially targets acylcarboxyl moieties for removal and can remove glutaryl, succinyl, and malonyl groups from its substrates.[340-343] SIRT6 also has defatty-acylation activity and hydrolyzes different long-chain fatty acyl groups from its substrates.[344, 345] SIRT7 has also demonstrated desuccinylase activity.[346]

Sirtuin	Localization	Enzymatic Activity
SIRT1	Nucleus, cytoplasm	Deacetylation ADP-Ribosylation
SIRT2	Cytoplasm, nucleus	Deacetylation
SIRT3	Mitochondria	Deacetylation
SIRT4	Mitochondria	Deacetylation ADP-Ribosylation
SIRT5	Mitochondria	Deacetylation, Demalonylation Desuccinylation Deglutarylation Deacylcarboxylation
SIRT6	Nucleus	Deacetylation ADP-Ribosylation Deacylation
SIRT7	Nucleolus	Deacetylation Desuccinylation

Table 2.1. A table showing the cellular localizations and enzymatic activities of the mammalian sirtuins

Of the seven mammalian sirtuins, only SIRT2 is primarily located in the cytoplasm. Initially, SIRT2 function was characterized to function in the cytoplasm where it was found to colocalize with microtubules in the cytoplasm but was later shown to move into the nucleus in certain circumstances.[337] SIRT2 enzymatic activity in the cytoplasm is well established where it influences the cytoplasmic metabolism in response to NAD levels and targets a wide array of cytoplasmic substrates involved in metabolic regulation, such as aldolase and phosphoglycerate kinase.[347, 348] SIRT2 also targets for deacetylation a number of other cytoplasmic proteins including alpha-tubulin, p65, FOX01, nuclear factor κB (NF-κB) signaling, and microtubular

dynamics.[348] The microtubule network impacts many aspects of normal cellular functioning, such as cell morphology, cell motility, cell polarity, and subcellular transport. Formation of microtubules, which are made up for α- and β-tubulin heterodimers, is highly conserved, thus necessitating a nuanced control through post-translation modification.[349] Acetylation occurs at lysine 40 on α-tubulin, which is controlled by the opposing enzymatic activities of deacetylases such as histone deacetylase 6 (HDAC6) and SIRT2, and acetyltransferases, including MEC-17 and αTAT1.[337, 350-352] During different stages of mitosis, SIRT2 colocalizes with different mitotic structures that involve microtubules: SIRT2 associates with the centrosome in prophase and the mitotic spindle in metaphase, then with the midbody via Aurora B during cytokinesis.[353]

Beginning in late S phase and to the G_2/S checkpoint, SIRT2 is shuttled into the nucleus from the cytoplasm where it localizes to chromatin.[353] The mechanism of import of SIRT2 into the nucleus is still under investigation; passive entry into the nucleus through the nuclear pore complex is limited by protein size, where proteins larger than about 40 kDa are excluded and those under 40 kDa are thought to freely diffuse between the nucleus and cytoplasm. Proteins above this size limit typically contain an NLS sequence that allows recognition by importin receptors for entry into the nucleus. SIRT2, which has a molecular mass of about 43 kDa, is right at this upper size limit but contains no known canonical NLS sequence, which raises the question of whether or not SIRT2 freely diffuses into the nucleus or is imported by a different mechanism. One study using a proteomics-based approach has reported that SIRT2 interacts with nuclear importin proteins, including KPNA2, IPO7, and TNPO1, which is negatively regulated by the C-terminus.[354] On the other hand, nuclear export of SIRT2 has been better established. SIRT2 contains a NES from

residues 18-74 sequence which is CRM1-dependent, though experiments have suggested there are alternative SIRT2 nuclear export pathways.[353]

Like SIRT1, SIRT2 targets histone 4 at lysine 16 (H4K16) for deacetylation when in the nucleus to promote chromatin condensation during mitosis.[335, 355] Deacetylation of H4K16 by SIRT2 also allows for methylation of H4K20 by N-lysine methyltransferase KMT5A, which further condenses chromatin.[336] SIRT2 also targets the nuclear substrates HOXA10, p300, CBP, and p53.[356-358]

SIRT2, like the other sirtuins, has been implicated in a number of different cellular functions such as checkpoint regulation, the DDR, and metabolism. Interestingly, despite being a mostly cytoplasmic sirtuin, SIRT2 has been linked to mitochondrial regulation where SIRT2 deficiency leads to an increase in acetylation of mitochondrial proteins, oxidative stress, lowered ATP production, and a lack of mitophagy, which is an important process for removing damaged mitochondria.[359-361]

SIRT2 has also been implicated as a player in the DDR and has been found to play a role as one of the proteins involved in the RSR after replication fork stalling. ATM and ATR are two DDR-related kinases that are key regulators in the proper maintenance of genomic integrity.[362] The proteins ATM and ATR are early responders to different types of DNA damage and promote the activation of cell cycle checkpoint pathways that lead to DNA repair and cell cycle arrest.[363] ATR-interacting protein (ATRIP) binds to ATR and is required for proper ATR function.[364] SIRT2 has been found to be an important regulator of the ATR checkpoint pathway through ATRIP: After replication stress has occurred, SIRT2 deacetylates ATRIP at lysine 32 which

promotes ATR autophosphorylation at threonine 1989 and recruitment of the ATR-ATRIP complex to sites of DNA damage.[64] SIRT2 also plays a role in the RSR through cyclin dependent kinase 9 (CDK9), which interacts with ATR and other checkpoint signaling proteins. SIRT2 deacetylates CDK9 at lysine 48 which leads to CDK9 kinase activity and proper activation of the RSR.[62] SIRT2 has also been found to promote NER.[365]

The murine protein Sirt2 has already been established as a tumor suppressor, but in humans, the role of SIRT2 in cancer has yet to be fully understood due to the seemingly complicated nature of SIRT2 function and regulation. Like SIRT1, SIRT2 shows opposing roles as a tumor promoter and a tumor suppressor. *Sirt2* knockout mice develop normally, though exhibit abnormalities stemming from potentially increased genomic stability such as impaired mitotic function, aneuploidy, reduced cell proliferation, and the formation of sex-specific tumors.[366] SIRT2 deficient females develop tumors at about 10 months of age primarily in mammary glands, with a 60% incidence rate by 24 months, while SIRT2 deficient males begin by 8 months to develop cancers in multiple organs, including the pancreas, stomach, liver, prostate, duodenum, and lungs.[366] Evidence supporting SIRT2 as a tumor promoter comes from the studies showing the role of SIRT2 in the RSR and checkpoint activation to prevent hyperploid cell formation through regulation of mitotic integrity. A deficiency in SIRT2 has been found in a slew of cancers, including hepatocellular carcinoma, prostate cancer, non-small cell lung cancer, breast cancer, glioma, and neck squamous cell carcinoma.[355, 366-370] Inhibition of SIRT2 has demonstrated a decrease in E-cadherin which plays a role in endothelial mesenchymal transition (EMT) (where cancerous cells can gain increased motility to promote tumor metastasis), whereas SIRT2 normally inhibits WNT signaling through a direction interaction with B-catenin that increase after IR or

oxidative stress. The WNT signaling pathway involves target genes such a *c-myc*, *cyclin D1*, and *survivin*, and overactivation of the pathway has been linked to breast cancer, prostate cancer, ovarian cancer, leukemia, melanoma, and gastrointestinal cancers, suggesting that SIRT2 is crucial for proper regulation of this pathway.[371, 372] However, the opposing roles of SIRT2 in tumor formation and progression may be linked to timing. In breast cancer, low SIRT2 expression correlates early on with a poor prognosis, while in later stages, SIRT2 is highly expressed correlates with more aggressive tumor types, potentially through the promotion of DNA repair and dysregulation of the cell cycle.[369] Similarly, high SIRT2 levels have been found in leukemia, neuroblastoma, HCC, and pancreatic cancers, where it was shown SIRT2 promotes increased vascularization and cell growth, likely through deacetylation of KRAS and/or enhancement of N-MYC/c-MYC.[373, 374]

2.12 Establishing SIRT2 in the BRCA1-BARD1 Axis

The sirtuins, BRCA1, and BARD1 are involved in similar pathways, such as apoptosis, cell cycle checkpoint regulation, and DNA repair. In many instances, the sirtuins seem to act at points early on in many of the pathways, especially in DDR pathways, such as in the case of ATR activation by SIRT2 in the RSR or chromatin remodeling induced by SIRT1 after DNA damage. These observations suggest that the field has yet to fully uncover the extent of the intertwined and complex role sirtuins play in maintaining cellular homeostasis, protecting against genomic insult, and role in preventing and/or promoting tumorigenesis and cell proliferation.

In addition to being part of the DDR response, SIRT1 has been directly linked to BRCA1 function, while there is evidence that SIRT2 may similarly be involved in the regulation of BRCA1 and

BARD1, directly or indirectly. SIRT1, which targets known DNA repair proteins, has been shown to deacetylate BRCA1 at K830 which leads to a change in regulation of the intra-S checkpoint.[375] In contrast, the connection between SIRT2 and BRCA1/BARD1 has been less clear, though there are indications that they may be more closely intertwined than what is currently understood. For instance, the APC/C complex, which is responsible for ubiquitinating and promoting BARD1 degradation, is deacetylated and stabilized by SIRT2, suggesting SIRT2 may be a part of the mechanisms that regulate BARD1, and consequently, BRCA1, protein levels.[366] In addition, BRCA1-BARD1 and SIRT2 both interact with the kinase Aurora B. SIRT2 associates with Aurora B via the midbody during cytokinesis, while BRCA1-BARD1 E3 ligase function promotes ubiquitin-mediated degradation of Aurora B in cytokinesis.[244, 271, 353] The BARD1β isoform also acts as a scaffold between Aurora B and BRCA1 during telophase and cytokinesis at the midbody in competition with the BRCA1-BARD1 complex.[244] Even more, BRCA1 is a substrate of ATR, which is deacetylated and activated by SIRT2.[64, 376] It is also of interest to note that female mice deficient in *sirt2* develop mammary tumors, which is reminiscent of the cancers that are typically seen in patients with inherited BRCA1 and/or BARD1 mutations and serves as further evidence for a potential link between SIRT2, BRCA1, and BARD1.

Given all of these observations, and the role our lab has found for SIRT2 in the RSR pathway, we questioned whether SIRT2 has a role in regulation of the HRR pathway. In the following chapter, we provide evidence that BRCA1 and BARD1 are substrates of SIRT2. Moreover, BARD1 deacetylation by SIRT2 promotes BRCA1-BARD1 heterodimerization, which facilitates BRCA1 and BARD1 stability, nuclear retention, recruitment to DNA damage sites, and function in HRR. Excitingly, these data extend our knowledge of the involvement of the SIRT2 in the DDR by

showing SIRT2 has a role in HRR in addition to RSR while also identifying an upstream mechanism for the regulation of the BRCA1-BRCA1 heterodimer. A deeper understanding of the regulatory mechanisms that govern the molecular pathways associated with carcinogenesis, tumorigenesis, cell death, and cell proliferation also provides the opportunity to identify new risk factors associated with developing cancer and developing therapeutic approaches for the prevention and treatment of cancers.

Chapter 3: SIRT2 Promotes BRCA1-BARD1 Heterodimerization Through Deacetylation

This work is currently under revision and will be re-submitted to Cell Reports.

3.1 Author Contributions and Declaration of Interests

The list of authors is Elizabeth V. Minten, Chunyang Li, Hui Zhang, Priya Kapoor-Vazirani, and David S. Yu. E.V.M., C.L, H.Z., and D.S.Y. conceived and designed the study. E.V.M, C.L., and P.K. performed the experiments. E.V.M. and D.S.Y. wrote the manuscript with input from all authors. The authors declare no competing interests.

3.2 Summary

The breast cancer type I susceptibility protein (BRCA1) and BRCA1-associated RING domain protein I (BARD1) heterodimer promotes genome integrity through pleiotropic functions, including DNA double-strand break (DSB) repair by homologous recombination repair (HRR). BRCA1-BARD1 heterodimerization is required for their mutual stability, HRR function, and role in tumor suppression; however, the upstream signaling events governing BRCA1-BARD1 heterodimerization are unclear. Here, we show that SIRT2, a sirtuin deacetylase and breast tumor suppressor, promotes BRCA1-BARD1 heterodimerization through deacetylation. SIRT2 complexes with BRCA1-BARD1 and deacetylates conserved lysines in the BARD1 RING domain, interfacing BRCA1, which promotes BRCA1-BARD1 heterodimerization and consequently BRCA1-BARD1 stability, nuclear retention, and recruitment to DNA damage sites, as well as efficient HRR. Our findings define a mechanism for regulation of BRCA1-BARD1 heterodimerization through SIRT2 deacetylation, elucidating a critical upstream signaling event

directing BRCA1-BARD1 heterodimerization, which facilitates HRR and tumor suppression, and delineating a role for SIRT2 in directing DSB repair by HRR.

3.3 Introduction

Inherited mutations in *BRCA1* predispose to breast and ovarian cancers.[377-381] BRCA1 promotes genome integrity through pleiotropic functions, including DSB repair by HRR, protection of stalled replication forks from nucleolytic degradation, cell cycle checkpoint activation, mRNA splicing and microRNA biogenesis, and avoidance of replication-transcription conflicts.[169, 172, 177, 217, 245, 246, 382-389] In particular, BRCA1's role in multiple steps in the error-free HRR pathway, which protects against DSBs and stalled replication forks, is thought to be important for its tumor suppressor function.[380, 390-393]

BRCA1 forms a stable heterodimeric complex with BARD1 through the association of their amino (N)-terminal RING domains, which is important for their mutual stability, nuclear localization, recruitment to DNA damage sites, and ubiquitin E3 ligase activity.[76, 225, 229, 252, 253, 255, 260, 265, 287] Mice deficient in *Brca1* or *Bard1* develop indistinguishable basal-like mammary carcinomas, suggesting that the tumor suppressor functions of BRCA1 and BARD1 are likely mediated through their heterodimerization.[281] Indeed, the RING domain of BRCA1 is essential for tumor suppression in a conditional mouse model for *BRCA1*-associated breast cancer carrying *C61G*, a common pathogenic missense variant that disrupts BRCA1-BARD1 heterodimerization.[259] Furthermore, a number of additional germline mutations in the RING domains of *BRCA1* and *BARD1* have been found in patients with hereditary breast and ovarian cancers, highlighting the potential significance of BRCA1-BARD1 heterodimerization in tumor suppression.(ClinVar;

BRCAExchange)[256, 287, 394, 395] Mutational analyses based on the nuclear magnetic resonance (NMR) structure of the BRCA1-BARD1 RING domain heterodimer complex, which consists of a pair of RING finger motifs and flanking anti-parallel α-helices (which we will henceforth refer to as the RING domain), have shown that the BRCA1-BARD1 interface is mediated by residues important for hydrophobic interactions or that contribute to structural stabilization; however, while suggested, the contribution of charged interactions has not been demonstrated.[76, 254, 258, 265, 396, 397]

The interaction of BRCA1 and BARD1 is critical for their mutual stability, likely by masking BRCA1's degron domain, which is located in its first 167 amino acids, and is ubiquitinated by HERC2, HUWE1, and FBXO44 and deubiquitinated by USP9X.[75, 124-126, 129, 252, 253] CTSS, a cysteine protease, has also been shown to promote BRCA1 ubiquitination and subsequent degradation after cleaving its C-terminal BRCT domains.[127] BARD1 is also degraded in a ubiquitination-mediated process via the APC/C complex.[237] Furthermore, BRCA1-BARD1 stability is impacted by their interaction with UBE2T, GUARDIN, TACC3, and TUSC4, suggesting tight regulation of BRCA1-BARD1 stability.[128, 398-400]

Sirtuin 2 (SIRT2) is a sirtuin family NAD^+ dependent deacetylase, which regulates multiple biological processes, including genome maintenance, aging, tumorigenesis, and metabolism.[339, 401-403] Significantly, mice deficient in *Sirt2* develop breast and other cancers, suggesting that SIRT2 functions in tumor suppression.[336, 366] We previously defined a role for SIRT2 in directing the replication stress response (RSR), a subset of the DNA damage response (DDR) through the acetylation status of ATRIP and CDK9 and furthermore, showed that somatic cancer-associated *SIRT2* mutations impair the activity of SIRT2 in maintaining genome integrity; however, SIRT2's

role in downstream DNA repair is less well-established.[62, 63, 403, 404] SIRT2 was recently reported to promote nucleotide excision repair (NER), but its role in promoting DSB repair by HRR is unclear.[365] Furthermore, BRCA1 function in the intra-S checkpoint is activated by K830 acetylation via a pCAF/SIRT1 axis; however, the role of SIRT2 deacetylation, or more generally of upstream signaling events, in governing BRCA1-BARD1 heterodimerization are not known.[375]

In this study, we show that BARD1 deacetylation by SIRT2 at conserved lysine sites in its RING domain promotes BRCA1-BARD1 heterodimerization, thereby facilitating BRCA1-BARD1 stability, nuclear retention, recruitment to DNA damage sites, and function in HRR. Our findings define a mechanism for regulation of BRCA1-BARD1 heterodimerization through SIRT2 deacetylation, elucidating a critical upstream signaling event directing BRCA1-BARD1 heterodimerization and delineating a role for SIRT2 in promoting DSB repair by HRR.

3.4 Results

SIRT2 Interacts with the BRCA1-BARD1 Complex

To determine if SIRT2 interacts in a complex with BRCA1 and BARD1, we performed co-immunoprecipitation (co-IP) analyses. Co-IP of FLAG-SIRT2 expressed in human embryonic kidney (HEK) 293T cells pulled down HA-BRCA1 and endogenous BARD1 (Figure 3.1A). In a reciprocal co-IP, HA-BRCA1 pulled down GFP-SIRT2 and endogenous BARD1, and endogenous BARD1 pulled down FLAG-SIRT2 and endogenous BRCA1 (Figures 3.1B and 3.1C). The endogenous interaction of SIRT2 with BRCA1 and BARD1 was validated by co-IP in HeLa cervical and HCT116 colorectal cancer cells (Figures 3.1D and E), suggesting that the interaction of SIRT2 with BRCA1 and BARD1 is physiologic and not cell type specific.

SIRT2 Deacetylates BRCA1 and BARD1

To determine if SIRT2 deacetylates BRCA1, we performed an *in vitro* deacetylation assay with purified acetylated HA-BRCA1, FLAG-SIRT2, and NAD^+ with or without nicotinamide, a sirtuin inhibitor. SIRT2 deacetylated HA-BRCA1 in an NAD^+-dependent manner, where deacetylation was inhibited by nicotinamide (Figure 3.1F). These findings were validated in cells where we transfected HEK293T cells with FLAG-SIRT2 wild-type (WT) or H187Y, a deacetylase-inactive mutant.[337] FLAG-SIRT2 WT but not H187Y deacetylated HA-BRCA1 expressed in cells (Figure 3.1G). Similar results were obtained for BARD1, where FLAG-SIRT2 WT but not H187Y deacetylated acetylated GFP-BARD1 *in vitro* in a NAD^+-dependent manner that was inhibited by nicotinamide (Figure 3.1H), and FLAG-SIRT2 deacetylated GFP-BARD1 expressed in HEK293T cells (Figure 3.4E).

SIRT2 Deacetylase Activity Promotes BRCA1-BARD1 Stability

To determine the functional significance of this interaction, BRCA1 protein levels were measured by western blot analysis in HCT116 cells after SIRT2 knockdown. BRCA1 levels were decreased following SIRT2 knockdown (Figure 3.2A), which was also observed in U2OS cells (Figures 3.S1A and 3.S1B). Since BRCA1 and BARD1 stabilize each other when in complex, decreased BARD1 levels were also reduced after SIRT2 knockdown (Figures 3.2B and 3.S1A).[252, 253] Treatment with AGK2, a SIRT2-specific inhibitor, produced similar results, indicating these results were dependent on SIRT2 deacetylase activity (Figures 3.2D and 3.E).[405] To determine if SIRT2 regulates BRCA1 and BARD1 at the transcriptional level, we performed quantitative RT-PCR following SIRT2 depletion or AGK2 treatment. No corresponding significant decrease in

BRCA1 or BARD1 mRNA levels was observed (Figures 3.2C and 3.2F), suggesting regulation occurs at the post-transcriptional level. To validate these findings, HEK293T cells were treated with cycloheximide, an inhibitor of translation, alone or with AGK2. A significantly greater decrease in BRCA1 protein levels was observed following AGK2 treatment (Figure 3.2G). We observed no significant difference in BARD1 protein levels at this time point, consistent with prior reports of BARD1's significantly longer half-life, and combined AGK2 and cycloheximide treatment times beyond the 8 hours required to detect noticeable BARD1 degradation resulted in significant toxicity (data not shown).[118] The decrease in BRCA1 protein levels following SIRT2 knockdown or AGK2 treatment was alleviated by proteasomal inhibition with MG132 (Figures 3.2H and 3.2I). Given that we have previously shown that SIRT2 knockdown in HCT116 and U2OS cells does not result in a significant change in cell cycling, collectively, our data suggest that SIRT2 deacetylase activity promotes BRCA1-BARD1 stability by impairing its degradation.[62, 64]

SIRT2 Deacetylase Activity Promotes BRCA1-BARD1 Nuclear Retention, Recruitment to DNA Damage Sites, and Homologous Recombination

The BRCA1-BARD1 interaction has been reported to mask each other's nuclear export signals (NES) thereby promoting their nuclear retention.[229, 255] We thus hypothesized that SIRT2 deficiency might result in increased cytoplasmic localization. Indeed, a significantly greater increase in BRCA1 and BARD1 cytoplasmic localization was observed in U2OS cells depleted for SIRT2 or treated with AGK2 (Figures 3.3A-F). To determine if SIRT2 deficiency furthermore impairs BRCA1-BARD1 recruitment to DNA damage sites, we examined U2OS cells treated with IR following SIRT2 depletion. A significant decrease in BRCA1 and BARD1, but not γH2AX,

foci was observed following SIRT2 deficiency (Figures 3.3 G-J), suggesting that SIRT2 promotes BRCA1-BARD1 recruitment to DNA damage sites and that SIRT2 may direct BRCA1-BARD1 function in DSB repair. To determine directly if SIRT2 functions in HRR, we examined SIRT2 depletion in U2OS cells integrated with a direct repeat (DR)-GFP reporter substrate in which expression of the I-SceI endonuclease generates a DSB that when repaired by HRR restores GFP expression. SIRT2 depletion caused a significant impairment in HRR, which could be rescued by FLAG-SIRT2 WT but not H187Y (Figures 3.3K, 3.3L and 3.S2A). Moreover, in epistasis studies, combined SIRT2 and BRCA1 depletion caused no significant further impairment in HRR compared with BRCA1 depletion alone (Figures 3.3M, 3.3N, and 3.S2B), implying that SIRT2 functions with BRCA1 in promoting HRR. Interestingly, BRCA1 knockdown also caused an increase in SIRT2 protein levels (Figure 3.3N), suggesting that BRCA1 may be involved in a feedback loop to regulate SIRT2. Collectively, our results suggest that SIRT2 deacetylation promotes BRCA1-BARD1 nuclear localization and recruitment to DNA damage sites, and thus facilitating HRR.

BARD1 RING Domain Deacetylation by SIRT2 Promotes BRCA1-BARD1 Heterodimerization

The stability and nuclear retention of BRCA1-BARD1 is dependent on heterodimerization of its RING motifs and flanking α-helices (RING domain) located in their respective N-termini.[252, 253, 255] Moreover, the BRCA1 degron has also been mapped to its N-terminus.[75] To provide insight into the mechanism by which SIRT2 may regulate BRCA1-BARD1 stability, we mapped the region of BRCA1 interacting with SIRT2 by co-IP of overlapping FLAG-HA-NLS BRCA1 fragments spanning full-length (FL) BRCA1 and GFP-SIRT2 expressed in HEK293T cells.[75]

FLAG-HA-NLS BRCA1 (1-324) but not BRCA1 (263-551) or more carboxyl-terminal BRCA1 fragments co-IP'd with GFP-BARD1 (Figure 3.4A). In further mapping experiments, His-SIRT2 co-IP'd with BRCA1 (1-167) (Figure 3.S3A), suggesting that BRCA1 amino acids 1-167, where its degron is located, are sufficient for interaction with SIRT2. Interestingly, the BARD1-binding domain is also located in this region, and BARD1 has been reported to stabilize BRCA1 by protecting it from ubiquitination.[118, 225] To determine whether SIRT2 regulates the interaction of BRCA1 and BARD1, we performed a co-IP of GFP-BARD1 in HEK293T cells treated with a short course of AGK2 to minimize significant degradation of BRCA1 and BARD1, and found that GFP-BARD1 pulled down a lower amount of endogenous BRCA1 following AGK2 treatment compared to a control (Figure 3.4B). Similarly, a reciprocal co-IP of HA-BRCA1 pulled down a decreased amount of GFP-BARD1 following AGK2 treatment (Figure 3.4C), suggesting that SIRT2 deacetylase activity promotes the interaction of BRCA1 and BARD1.

To identify the specific lysine targets of SIRT2 deacetylation, we analyzed the NMR structure of the BRCA1-BARD1 heterodimer complex.[258] (Figures 3.4D and 3.S3B). Of note, the positive charge of BARD1 lysine 96 (K96) forms a potential salt bridge with the negative charge of BRCA1 aspartic acid 40 (D40) measuring 2.2 angstroms (Å), and to a lesser extent, potential electrostatic interactions measuring about 5 Å are formed between BARD1 K46 and BRCA1 glutamic acid 85 (E85) and BARD1 K100 and BRCA1 E10 (Figure 3.4D). All three BARD1 lysine sites are evolutionarily conserved (Figure 3.S3C), and acetylation at these sites may lead to a loss of electrostatic attraction with the corresponding negative charged residue on BRCA1. We found no evidence that BRCA1 is acetylated at its N-terminus (1-167), which contains its RING domain, by IP of FLAG-HA-NLS BRCA1 (1-167) and western blot with an anti-acetyl K antibody (Figure

3.S3D) or reciprocally, IP with an anti-acetyl K antibody and western blot for FLAG-HA-NLS BRCA1 (1-167) (Figures 3.S3E and 3.SF). However, mutation of BARD1 K46, K96, and K110 to arginines (R) (3KR), a non-acetylated lysine mimic, caused a significant decrease in acetylation of BARD1 to a similar extent as overexpression of FLAG-SIRT2, though expression of FLAG-SIRT2 did not further deacetylate BARD1 3KR (Figure 3.4E). These findings suggest that BARD1 is primarily acetylated at its RING motif and flanking α-helices amongst K46, K96, and K110 and that SIRT2 only deacetylates BARD1 at these sites.

We then analyzed the interaction of endogenous BRCA1 with GFP-BARD1 WT, 3KR, or a mutant in which K46, K96, and K110 were replaced by glutamine (Q) to mimic an acetylated state (3KQ). Strikingly, co-IP of GFP-BARD1 3KR pulled down a significantly greater amount of endogenous BRCA1 than GFP-BARD1 WT (Figure 3.4F) while co-IP of GFP-BARD 3KQ pulled down a significantly decreased amount of endogenous BRCA1 than GFP-BARD1 WT (Figure 3.4G), suggesting that BARD1 acetylation at its RING domain impairs interaction with BRCA1. To determine if SIRT2 deacetylation of BRCA1 could also be contributing to BRCA1-BARD1 heterodimerization and to narrow down the region SIRT2 targets, a 14KR HA-BRCA1 mutant was created where all lysines in the first 167 residues of BRCA1 were mutated to arginines. However, the 14KR mutant did not show any difference in BRCA1 binding to BARD1 compared to BRCA1 WT (Figure 3.SG). Additional evidence further supported the hypothesis that BARD1 is the major target of SIRT2 in BRCA1-BARD1 complex stabilization: While the RING-containing region of BARD1 was able to pull down GFP-SIRT2 via IP, a pathogenic C61G mutant of BRCA1, which is unable to bind BARD1, and a RING-less fragment of BARD1 (Δ34-126) were unable to IP SIRT2-FLAG (Figure 3.SH and 3.SI). We next examined BRCA1 protein levels in HEK293T cells

expressing GFP-BARD1 3KR and treated with or without cycloheximide and/or AGK2. Four hours after cycloheximide treatment, a greater amount of endogenous BRCA1 was observed in cells expressing GFP-BARD1 3KR compared with GFP-BARD1 WT (Figure 3.4H and J). Moreover, expression of GFP-BARD1 3KR rescued the degradation of BRCA1 caused by AGK2 treatment (Figure 3.4I and K), implying that SIRT2 deacetylation of the BARD1 RING domain promotes BRCA1 stability. Overall, these results indicate that SIRT2 most likely binds the BRCA1-BARD1 complex via BARD1, where BARD1 deacetylation promotes BRCA1-BARD1 heterodimerization (Figure 3.5).

3.5 Discussion

Our findings reveal a critical upstream regulatory mechanism governing BRCA1-BARD1 heterodimerization through SIRT2 deacetylation and provide important insights into the interplay between the SIRT2 and BRCA1-BARD1 breast tumor suppressor proteins, whereby BARD1 deacetylation by SIRT2 at conserved lysine sites in its RING domain, critical for interfacing with BRCA1 through charged interactions, promotes BRCA1-BARD1 heterodimerization, thereby facilitating their mutual stability, nuclear retention, recruitment to DNA damage sites, and function in HRR. Furthermore, these findings identify BRCA1-BARD1 as an interacting partner and substrate for SIRT2, establish SIRT2 as a positive regulator of DSB repair by HRR, and further our understanding of how *Sirt2* deficiency results in genomic instability and carcinogenesis.

BRCA1-BARD1 heterodimerization is critical for its functions in HRR and tumor suppression.[380, 391-393] Indeed, a number of germline mutations in the RING domains of *BRCA1* and *BARD1* have been found in patients with hereditary breast and ovarian cancers.[259] Previous mutational analyses

have reported that the BRCA1-BARD1 interface is mediated by residues important for hydrophobic interactions or that contribute to structural stabilization.[76, 254, 265, 396, 397] Given that BARD1 3KR has increased interactions with BRCA1 and BARD1 3KQ has impaired interactions with BRCA1, our data support a model whereby BARD1 acetylation at its RING domain (K46, K96, and/or K110) leads to loss of electrostatic interaction with the corresponding negatively charged residues in BRCA1, thereby impairing BRCA1-BARD1 heterodimerization (Figure 3.5). This leads to increased access to the BRCA1 N-terminal degron for ubiquitination and proteasomal degradation, which in turn destabilizes BARD1. Impairment of BRCA1-BARD1 heterodimerization also leads to unmasking of the BRCA1 and BARD1 NESs, leading to cytoplasmic accumulation, impaired recruitment to DNA damage sites, and impaired HRR. BARD1 deacetylation by SIRT2 at its RING domain promotes charged interactions leading to BRCA1-BARD1 heterodimerization, thereby facilitating BRCA1-BARD1 stability, nuclear retention, recruitment to DNA damage sites, and function in HRR.

Given that SIRT2 deficiency and BARD1 3KQ does not fully abolish interaction with BRCA1, it is likely that there are additional mechanisms contributing to BRCA1-BARD1 heterodimerization. Indeed, while we have found no evidence that the N-terminus of BRCA1 is acetylated, K50 has been reported to be acetylated in a high throughput mass spectrometry analysis as reported in PhosphoSite Plus, and furthermore, we have shown that SIRT2 deacetylates BRCA1 *in vitro* and in cells. Thus, it is possible that BRCA1 deacetylation by SIRT2 at its RING domain may also contribute to BRCA1-BARD1 heterodimerization. Alternatively, it may be possible that SIRT2 deacetylates BRCA1 at a site outside of this region that contributes to its functions in controlling BRCA1 not related to BRCA1-BARD1 heterodimerization or stability, as BARD1 3KR can

stabilize the AGK2-induced degradation of BRCA1. In addition, other factors such as GUARDIN and TACC3 may also contribute to BRCA1-BARD1 heterodimerization; however, their precise mechanisms have not yet been fully elucidated.[398, 399]

We have previously shown that SIRT2 directs the RSR at least in part through deacetylation of ATRIP and CDK9.[62, 64] Our finding that SIRT2 promotes DSB by HRR provides further evidence that SIRT2 also has a role in downstream DNA repair in addition to its previously defined function in upstream checkpoint signaling. Furthermore, the identification of BARD1 as a binding partner and substrate of SIRT2 adds to the growing number of SIRT2 substrates that function in promoting genome integrity, providing support for SIRT2 in regulating a network of proteins involved in the DDR.[403] Our finding that SIRT2 directs BRCA1-BARD1 function in HRR provides an additional layer of insight into how SIRT2 dysregulation leads to genomic instability and carcinogenesis. In this regard, it is noteworthy that similar to BRCA1 and BARD1, SIRT2 also functions in breast tumor suppression.[336, 366] Dissecting BRCA1-BARD1's precise contributions to SIRT2's breast tumor suppressor function will be of significant future clinical interest.

3.6 STAR★METHODS

Transfections: Transfections were done on 5 million cells in 60 mm plates using Lipofectamine 2000 or 3000 (Invitrogen) and performed per the manufacturer's instructions. 5 to 10 μg of the indicated plasmids were used. Cells were split after 16 hours of incubation and allowed to recover for a further 24-48 hours post-transfection before harvest.

Immunoprecipitation: Briefly, cells were harvested and washed once with PBS. Cells were then lysed for 30 minutes on ice with CHAPS buffer (10% (vol/vol) glycerol, 150 mM NaCl, 50 mM Tris pH 7.5, .75% CHAPS) with the usual protease inhibitors added fresh. 1 μM TSA and 20 μM of nicotinamide were added with the usual protease inhibitors when probing for acetylation. The cells were then spun down for 15 minutes at 4 ^{o}C and the resulting pellet discarded. An equal volume of minus CHAPS buffer (10% (vol/vol) glycerol, 150 mM NaCl, 50 mM Tris pH 7.5) was added to the supernatant to dilute the CHAPS concentration to .375%. The lysate was precleared via incubation for an hour with 30 μL of either protein G agarose beads (Invitrogen) or protein A agarose beads (Invitrogen). Protein G agarose beads were used when the IP antibody was mouse, while protein A agarose beads were used when the IP antibody was rabbit. The lysate was then added to 30 μL of preconjugated beads overnight on a rotator at 4 ^{o}C. FLAG-tagged proteins were IP'd using FLAG M2 affinity beads (Sigma) while HA-tagged proteins were IP'ed using HA agarose beads (a2095; Sigma). Endogenous IPs were done with the indicated antibody. Negative controls consisted of IP with lysate using IgG rabbit or mouse for endogenous IPs and lysate not expressing tagged proteins for non-endogenous IPs. The beads were then washed three times with the .375% CHAPS buffer. The beads were then resuspended in 15 μL of .375% CHAPS buffer and 5 μL of 4x SDS before being boiled for 5 minutes at 100 ^{o}C before being run on an SDS-PAGE. Immunoprecipitation experiments were each performed at least 4 times total.

DR-GFP Assay: To measure efficiency of HRR-mediated DSB repair, 3 million U2OS cells stably expressing a DR-GFP reporter gene, described previously, were transfected with 60 nM of siRNA using Lipofectamine RNAiMax (Invitrogen) as per the manufacturer's instructions.[406] The next day, media was removed, and cells were transfected with 5 μg I-SceI. 72 hours after I-SceI

transfection, cells were harvested for flow cytometry and the data analyzed to measure HRR efficiency based on GFP expression. Experiments were repeated at least three times and tested for significance using a paired one-tailed t-test.

Immunoblot: Cells were harvested and washed once with PBS. Cells were then lysed for 30 minutes on ice using a 1% Nonidet P-40 (NP-40), 250 mM NaCl, 50 mM HEPES pH 7.9, 1 mM $MgCl_2$, 1 mM DTT, and .5 mM EDTA buffer with freshly added protease inhibitors. Samples were then resolved by SDS-PAGE and probed with the antibodies indicated in the figure. Signal detection was done with a Li-Cor Odyssey system. All western blot experiments were done at least 4 times.

Antibodies and reagents: The following antibodies were used: BRCA1 (ab16780, Abcam: 1/1000 for western and 1/200 for IF) and (sc-6954, Santa Cruz Biotechnology: 1/300 for western). BARD1 (A300-263A; Bethyl, 1/1000 for western), (E-11; Santa Cruz Biotechnology, 1/1000 for western), (ab226854; Abcam, 1/800 for IF), and (Antiserum 59P; a generous gift from Dr. Richard Baer we gratefully thank him for, 1/50 for IP). H2AX (s139; Cell Signaling Technology, 1/200 for IF) and (05-636 clone JB2301; MilliporeSigma, 1/4000 for IF). GAPDH (sc-47224; Santa Cruz Biotechnology, 1/1000 for western). Acetyl lysine (ICP0380; Immunechem, 1/500 for western). SIRT2 (09-843; Millipore, 1/500 for western), and (custom-made; ThermoFisher, 1/1000 for western). α-tubulin (T6074; Sigma-Aldrich, 1/1000 for western). Acetyl α-tubulin (ab179484; Abcam, 1/1000 for western). FLAG (2368S; Cell Signaling Technology, 1/1000 for western) and (sc-51590; Santa Cruz Biotechnology, 1/1000 for western). HA (H9658 clone HA-7; Sigma-Aldrich, 1/1000 for western) and (c29F4; Cell Signaling Technology, 1/1000 for western). GFP (ab290; Abcam, 1/1000 for western and 1 μL antibody per 2 mg lysate for IP) and (sc-996; Santa Cruz Biotechnology, 1/1000

for western). IgG (10500C; Invitrogen) and (NI03; MilliporeSigma). MG132 was used at a 5 μM dose for 6 hours while AGK2 was used at a 32 μM or at the indicated concentration.

In vitro deacetylation assay: To purify FLAG-SIRT2 from cells, HEK293T cells were transfected with 5 μg of FLAG-SIRT2. 48 hours post-transfection, cells were lysed using an in vitro deacetylase buffer (180 mM KCl, 20 mM HEPES pH 7.4, 1.5 mM $MgCl_2$, .2 mM EGTA, 20% glycerol, and 1% NP-40) supplemented with fresh protease inhibitors. A FLAG IP was performed overnight after an hour of preclearing with CL-4B sepharose beads. After three washes with TBS buffer (50 mM Tris pH 7.5, 150 mM NaCl), the beads were resuspended in TBS buffer (50 mM Tris pH 7.5, 150 mM NaCl)where FLAG-SIRT2 was eluted over the course of an hour on a cold rotator using 5 μL of FLAG peptide (Sigma) per 100 μL TBS. The supernatant was then collected and stored at -80^{o}C for future use. To determine enzyme concentration, a sample of the supernatant was run on a western along with a BSA standard. For the deacetylation assay, HEK293T cells were transfected with the indicated plasmid. After 36 hours, cells were treated with 10 mM of nicotinamide and .5 μM of trichostatin A (TSA), a class I and II deacetylase inhibitor, for an additional 12 hours. Cells were then harvested using the in vitro deacetylase buffer supplemented with protease inhibitors and 20 mM of nicotinamide and 1 μM of TSA. An IP was then done on the tag of the transfected plasmid. The beads were then washed three times with and resuspended in deacetylation buffer not containing nicotinamide or TSA. The beads were then split evenly between the different experimental groups. FLAG-SIRT2 and the other indicated components were then added to their respective tube. The final amounts and concentrations per condition used were: 1 μg of FLAG-SIRT2 (WT or H187Y) per 24 μL of total volume, 1 μM TSA (all conditions), 25 mM nicotinamide, 625 μM of $MgCl_2$ (all conditions), and 10 mM of NAD^+ (all conditions). Tubes containing the beads and the indicated reagents were then added to a 30^{o}C water bath where

the beads were gently agitated every 15 minutes to maintain proper mixing. After 3 hours, 4x SDS was added to each sample and then run on a western blot for analysis. For BRCA1 (*in vitro* and in cells), 3 μg of the histone acetylases (HATs) p300, pCAF, and CBP were transfected with BRCA1 to increase the acetylation signal.

Immunofluorescence: After the indicated treatment, U2OS cells were seeded on coverslips and allowed the indicated recovery time. Cells were then permeabilized in CSK buffer with .5% triton-X for 5 minutes then fixed in 4% PFA for 10 minutes. Cells were blocked in PBS with 15% FBS for one hour then immunostained for one hour or overnight with the indicated primary antibodies in PBS with 15% FBS. Cells were then incubated with secondary antibody (Alexa Fluor 488 or 555 mouse or rabbit, Invitrogen) for one hour before being mounted onto slides using DAPI Fluoromount-G® (SouthernBiotech). The percentage of cells showing cytoplasmic BRCA1 localization was counted from 100 cells per replica with three replicas and significance between groups calculated using a paired one-tailed t test. All images were captured on a Zeiss Observer Z1 microscope using AxioVision Rel 4.8 software at 63x magnification.

RT-qPCR: Briefly, cells were first lysed with TRIzol. 200 μL of chloroform was added per mL of TRIzol then set on a rocker for 15 minutes at room temperature. The samples were centrifuged for 15 minutes at 12k rpm at room temperature and the aqueous phase collected. A 1:1 volume of isopropanol was added to the aqueous phase then allowed to incubate for 2 hours on ice. The samples were again centrifuged for 15 minutes at 12k rpm to pellet the RNA. The RNA was washed once with 75% ethanol and allowed to air-dry before being resuspended in nuclease-free water. cDNA was then created using an OligoT kit (18080-051; Invitrogen) using a C1000©

Thermal Cycler (Bio-Rad). Taqman probes to each DNA sequence of interest were then used for qPCR. The probes used were: BRCA1 (TaqMan Hs01556193_m1; ThermoFisher), BARD1 (TaqMan Hs00957646_m1; ThermoFisher), and GAPDH (TaqMan Hs99999905_m1; ThermoFisher). Each experiment was done in triplicate with four internal replicates per sample. qPCR was performed on a 7500 Fast Real-Time PCR system (ThermoFisher). Results were analyzed using a paired two-tailed t test.

Knock down: Knockdowns were done using RNAi Max reagent (Invitrogen) and performed per the manufacturer's instructions. Cells would be split after 24 hours of incubation and allowed to recover for a further 48 hours. The following siRNAs were used: BRCA1 (M-003461-02; Dharmacon). SIRT2-1 (D-004826-05; Dharmacon) and SIRT2-2 (s105116657; QIAGEN).

Plasmids: The following plasmids were used: HA-BRCA1 is 1xMyc-3xHA-BRCA1 in the pcDNA3.1 backbone. GFP-BARD1 was kindly provided by Dr. Xiaochun Yu and made has been described previously.[251] BRCA1 fragments, including 167 BRCA1 and 14KR, were generously provided by Dr. Yanfen Hu and are as previously described.[75] His-SIRT2 was a kind gift from Dr. Michael Tainsky's lab and has been previously described.[334] FLAG-SIRT2 WT and H187Y are set in a pcDNA3.1 backbone.

3.7 Acknowledgements

We thank members of the Yu lab for their helpful discussion and technical expertise. This work was supported by: The National Institute of General Medical Sciences of the National Institute of Health (NIH) [5T32GM008367-29]; the National Cancer Institute of the NIH [F31CA225124 to

E.V.M.; R01CA178999 to D.S.Y.]; Department of Defense [OC160540 to D.S.Y.], Basser Center for BRCA [32356 to D.S.Y.], and Winship Cancer Institute/Brenda Nease Breast Cancer Research Fund [53237 to D.S.Y.]. Research reported in this publication was supported in part by the Emory Integrated Genomics Core (EIGC) Shared Resource of Winship Cancer Institute of Emory University and NIH/NCI under award number P30CA138292. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

3.8 Figures

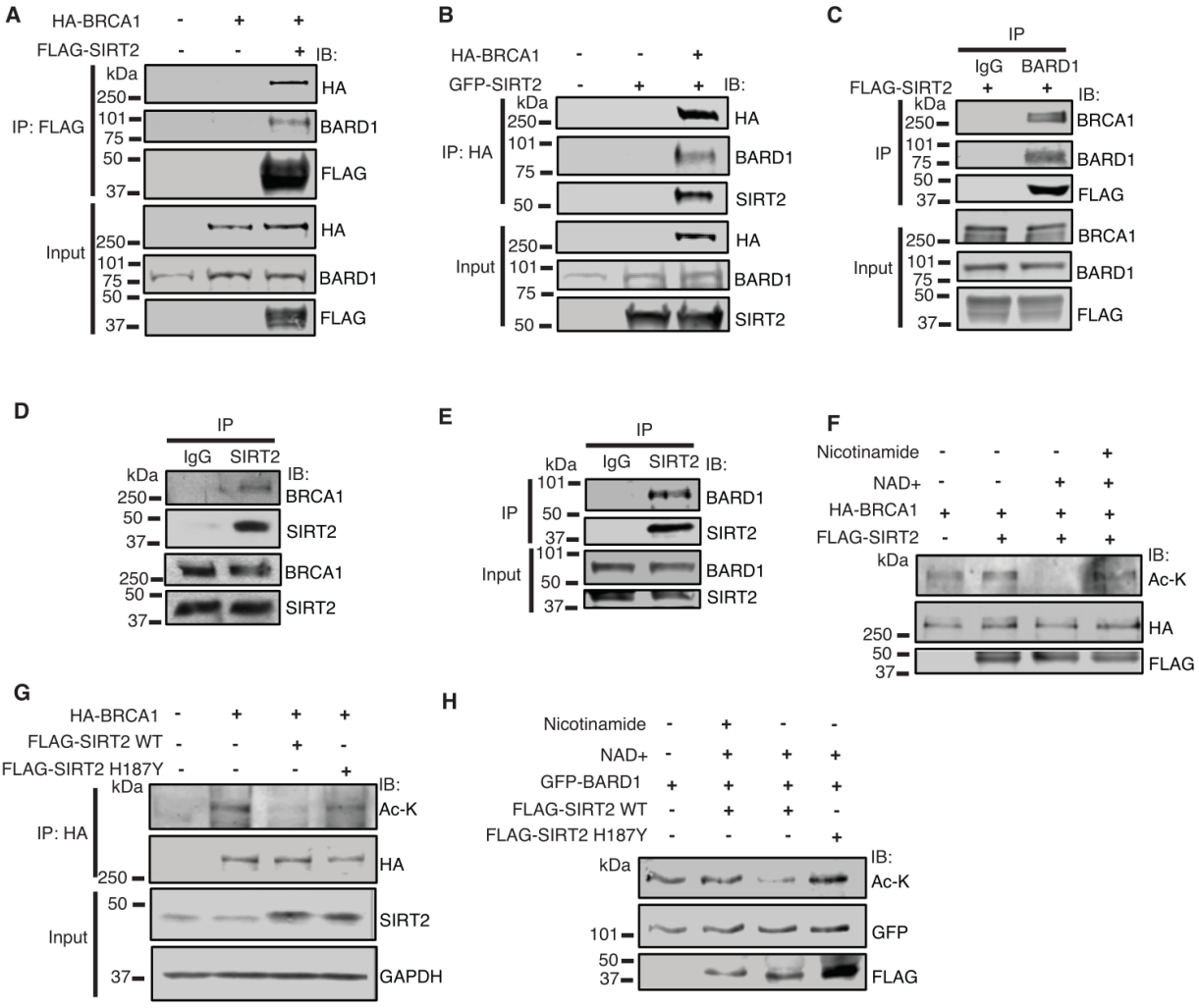
A
HA-BRCA1 - + +
FLAG-SIRT2 - - + IB:
kDa
IP: FLAG
250
101
75
50
37
HA
BARD1
FLAG
Input
HA
BARD1
FLAG
B
HA-BRCA1 - - +
GFP-SIRT2 - + + IB:
IP: HA
HA
BARD1
SIRT2
Input
HA
BARD1
SIRT2
C
IP
IgG BARD1
FLAG-SIRT2 + + IB:
IP
BRCA1
BARD1
FLAG
Input
BRCA1
BARD1
FLAG
D
IP
IgG SIRT2
IB:
BRCA1
SIRT2
BRCA1
SIRT2
E
IP
IgG SIRT2 IB:
IP
BARD1
SIRT2
Input
BARD1
SIRT2
F
Nicotinamide - - - +
NAD+ - - + +
HA-BRCA1 + + + +
FLAG-SIRT2 - + + +
IB:
Ac-K
HA
FLAG
G
HA-BRCA1 - + + +
FLAG-SIRT2 WT - - + -
FLAG-SIRT2 H187Y - - - +
IB:
IP: HA
Ac-K
HA
Input
SIRT2
GAPDH
H
Nicotinamide - + - -
NAD+ - + + +
GFP-BARD1 + + + +
FLAG-SIRT2 WT - + + -
FLAG-SIRT2 H187Y - - - +
IB:
Ac-K
GFP
FLAG

Figure 3.1. SIRT2 Interacts with and Deacetylates the BRCA1-BARD1 Complex

(A) Immunoprecipitation (IP) of FLAG-SIRT2 pulls down HA-BRCA1 and endogenous BARD1 in HEK293T cells. **(B)** IP of HA-BRCA1 pulls down GFP-SIRT2 and endogenous BARD1 in HEK293T cells. **(C)** IP of endogenous BARD1 pulls down FLAG-SIRT2 and endogenous BRCA1 in HEK293T cells. **(D)** Endogenous SIRT2 IP in HeLa cells pulls down endogenous BRCA1. **(E)** Endogenous SIRT2 IP in HCT116 cells pulls down endogenous BARD1. **(F)** FLAG-SIRT2 deacetylates HA-BRCA1 in HEK293T cells *in vitro*. NAD+ is a necessary SIRT2 cofactor and nicotinamide inhibits class III deacetylases, including SIRT2. **(G)** FLAG-SIRT2 WT but not H187Y decreases HA-BRCA1 acetylation in cells. **(H)** FLAG-SIRT2 deacetylates GFP-BARD1 *in vitro*.

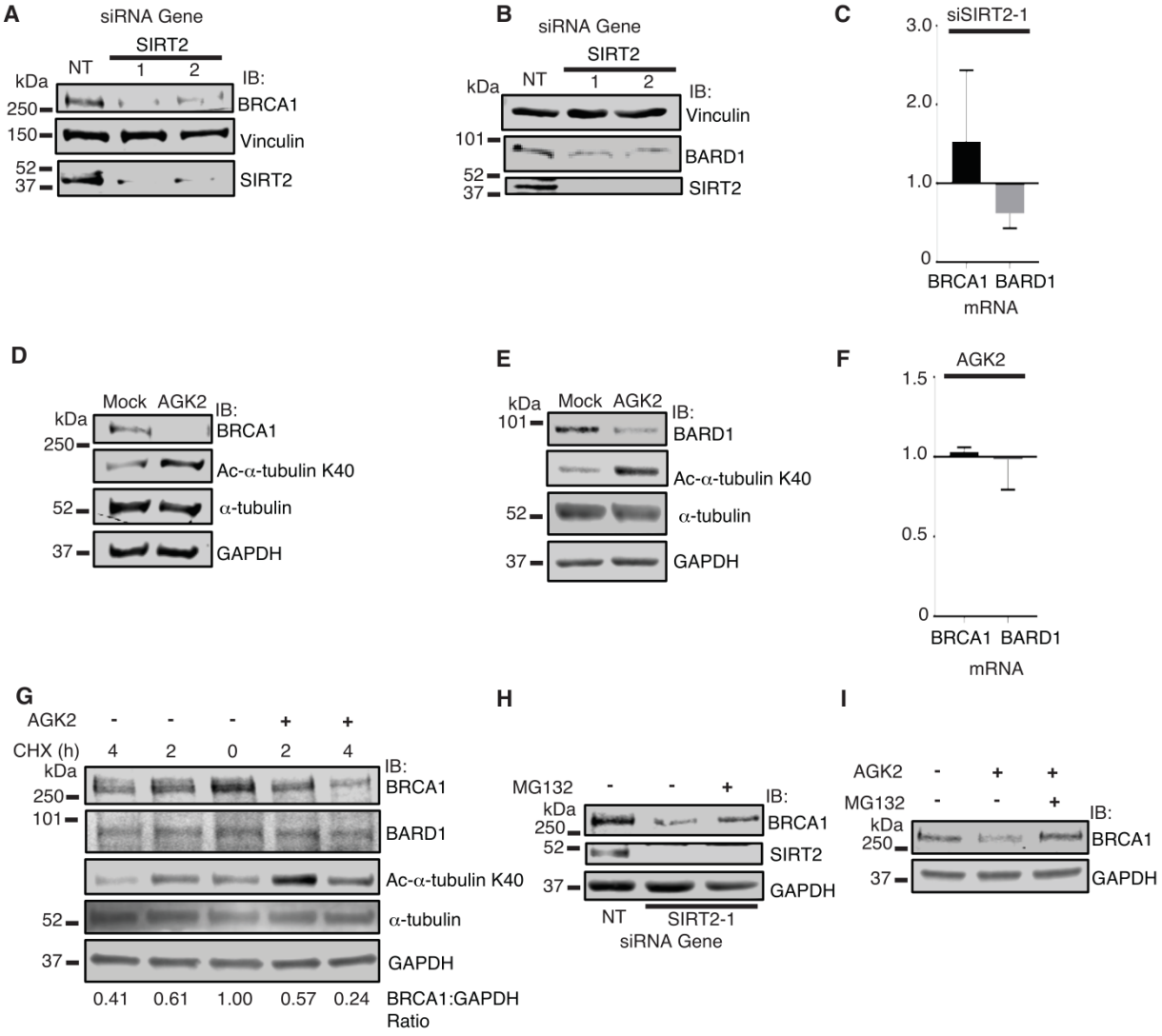
A
siRNA Gene
SIRT2
NT
1
2
kDa
IB:
250
BRCA1
150
Vinculin
52
37
SIRT2
B
siRNA Gene
SIRT2
NT
1
2
kDa
IB:
Vinculin
101
BARD1
52
37
SIRT2
C
siSIRT2-1
3.0
2.0
1.0
0
BRCA1 BARD1
mRNA
D
Mock AGK2
kDa
IB:
250
BRCA1
Ac-α-tubulin K40
52
α-tubulin
37
GAPDH
E
kDa
Mock AGK2
IB:
101
BARD1
Ac-α-tubulin K40
52
α-tubulin
37
GAPDH
F
AGK2
1.5
1.0
0.5
0
BRCA1 BARD1
mRNA
G
AGK2 - - - + +
CHX (h) 4 2 0 2 4
kDa
IB:
250
BRCA1
101
BARD1
Ac-α-tubulin K40
52
α-tubulin
37
GAPDH
0.41 0.61 1.00 0.57 0.24
BRCA1:GAPDH
Ratio
H
MG132 - - +
IB:
kDa
250
BRCA1
52
SIRT2
37
GAPDH
NT
SIRT2-1
siRNA Gene
I
AGK2 - + +
MG132 - - +
IB:
kDa
250
BRCA1
37
GAPDH

Figure 3.2. SIRT2 Deacetylase Activity Promotes BRCA1-BARD1 Stability

(A-C) SIRT knockdown decreases BRCA1 and BARD1 protein levels but does not significantly change mRNA levels in HCT116 cells. (**D-F)** AGK2 treatment for 24 hours decreases BRCA1 and BARD1 protein levels but shows no significant change in mRNA levels in HCT116 cells. **(G)** HEK293T cells were treated with cycloheximide with or without AGK2 for 0, 2, or 4 hours. AGK2 increased the rate of BRCA1 degradation in HEK293T cells as compare to a control. The normalized ratio of BRCA1 to GAPDH are listed for each lane below. **(H)** Treatment with MG132 for 6 hours alleviates BRCA1 levels after SIRT2 knockdown in HCT116 cells. **(I)** Treatment with MG132 for 6 hours alleviates BRCA1 levels treated with AGK2 in HCT116 cells. For **(C)** and **(F)**, mean and standard deviation from three replicas is shown. For **(D)** and **(E)**, acetylated α-tubulin acts as a positive control for AGK2 treatment.

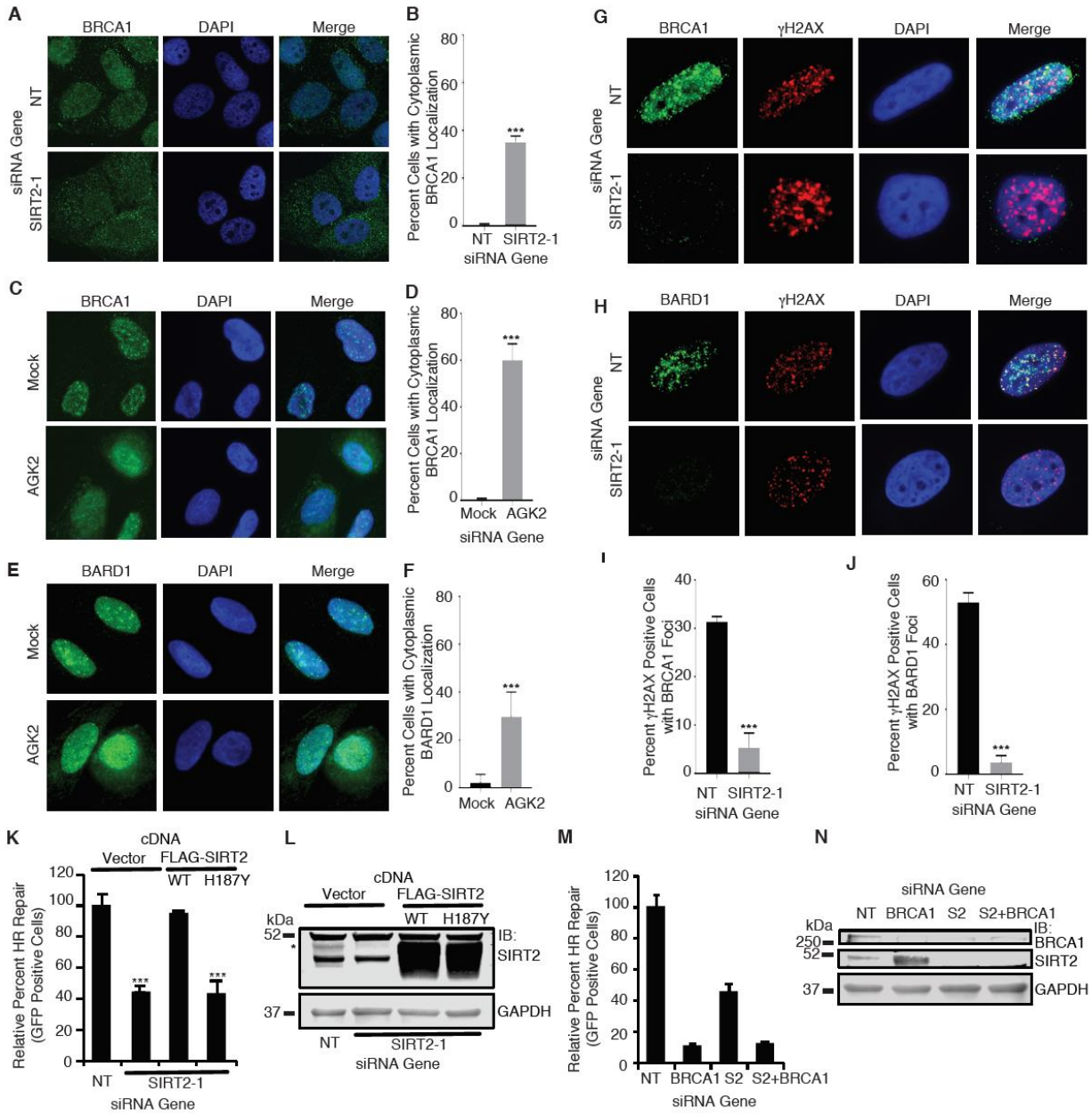
A
BRCA1
DAPI
Merge
siRNA Gene
NT
SIRT2-1
B
Percent Cells with Cytoplasmic BRCA1 Localization
NT
SIRT2-1
siRNA Gene
C
Mock
AGK2
D
Mock
AGK2
E
BARD1
F
Percent Cells with Cytoplasmic BARD1 Localization
G
γH2AX
H
I
Percent γH2AX Positive Cells with BRCA1 Foci
J
Percent γH2AX Positive Cells with BARD1 Foci
K
cDNA
Vector
FLAG-SIRT2
WT
H187Y
Relative Percent HR Repair (GFP Positive Cells)
L
kDa
52
37
IB:
SIRT2
GAPDH
M
BRCA1
S2
S2+BRCA1
N
250

Figure 3.3 SIRT2 Deacetylase Activity Promotes BRCA1-BARD1 Nuclear Retention, Recruitment to DNA Damage Sites, and Homologous Recombination

(A-D) SIRT2 knockdown or inhibition with 10 μM AGK2 for 4 hours in U2OS cells significantly increases cytoplasmic BRCA1. **(E-F)** SIRT2 inhibition with 10 μM AGK2 for 4 hours in U2OS cells significantly increases the amount of cytoplasmic BARD1. **(G-J)** BRCA1 and BARD1 recruitment to foci 4 hours after 10 Gy of IR is significantly impaired in U2OS cells after SIRT2 knockdown. **(K-L)** DR-GFP assay in U2OS cells showing that SIRT2 knockdown impairs HRR, which can be rescued by siRNA resistant FLAG-SIRT2 WT but not H187Y. The asterisk indicates where SIRT2 runs. **(M-N)** DR-GFP assay in U2OS cells indicating that combined SIRT2 and BRCA1 knockdown does not further impair HRR compared with knockdown of SIRT2 or BRCA1 alone. For the above quantitation, mean and standard deviation from three replicas is shown. ***: $p < .005$.

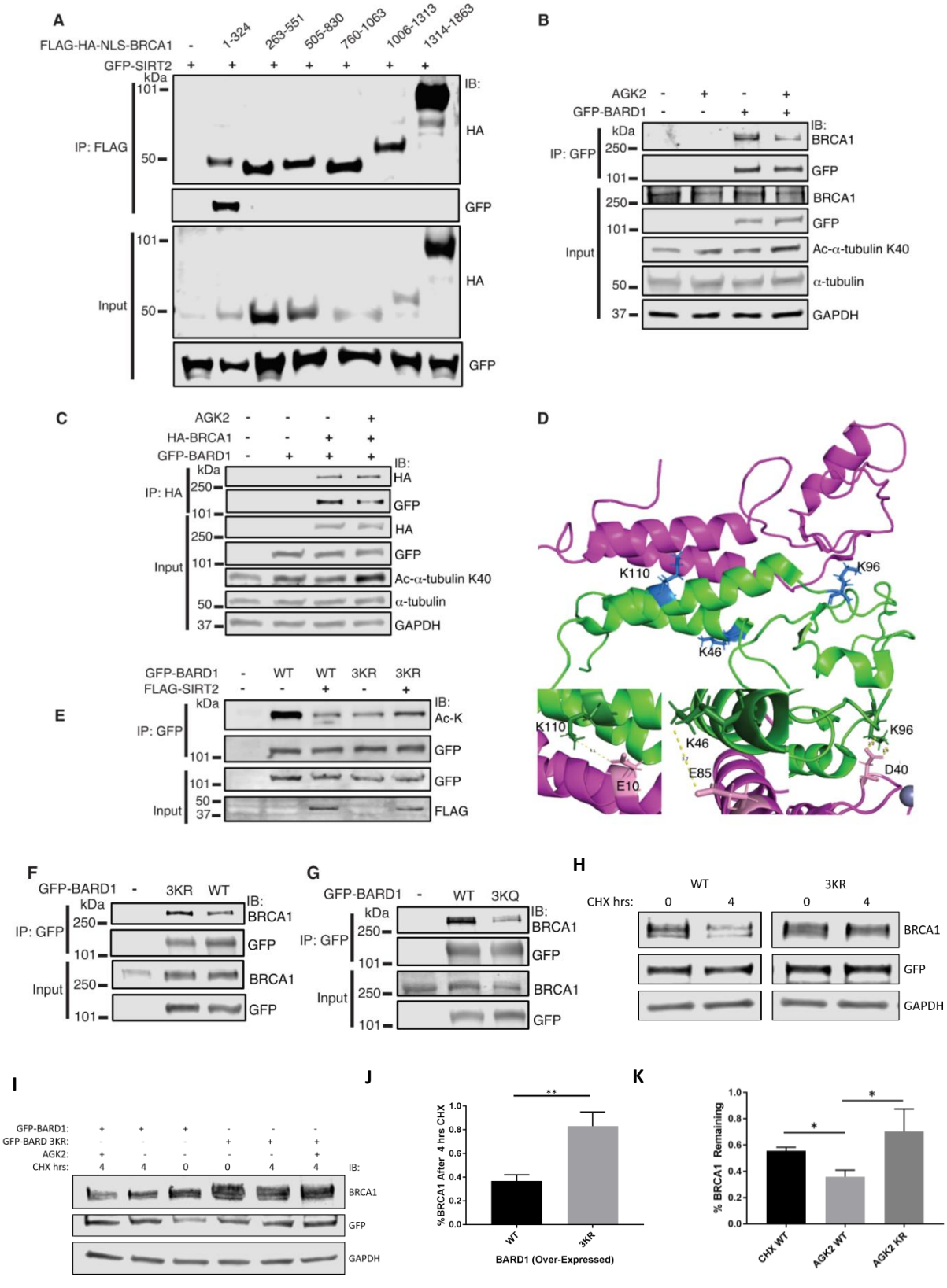
A
FLAG-HA-NLS-BRCA1 - 1-324 263-551 505-830 760-1063 1006-1313 1314-1863
GFP-SIRT2 + + + + + + +
kDa
IP: FLAG
IB:
HA
GFP
Input
HA
GFP
B
AGK2 - + - +
GFP-BARD1 - - + +
IP: GFP
IB:
BRCA1
GFP
Input
BRCA1
GFP
Ac-α-tubulin K40
α-tubulin
GAPDH
C
AGK2 - - - +
HA-BRCA1 - - + +
GFP-BARD1 - + + +
IP: HA
IB:
HA
GFP
Input
HA
GFP
Ac-α-tubulin K40
α-tubulin
GAPDH
D
K110
K96
K46
E10
E85
D40
E
GFP-BARD1 - WT WT 3KR 3KR
FLAG-SIRT2 - - + - +
IP: GFP
IB:
Ac-K
GFP
Input
GFP
FLAG
F
GFP-BARD1 - 3KR WT
IP: GFP
IB:
BRCA1
GFP
Input
BRCA1
GFP
G
GFP-BARD1 - WT 3KQ
IP: GFP
IB:
BRCA1
GFP
Input
BRCA1
GFP
H
WT 3KR
CHX hrs: 0 4 0 4
BRCA1
GFP
GAPDH
I
GFP-BARD1: + + + - - -
GFP-BARD 3KR: - - - + + +
AGK2: + - - - - +
CHX hrs: 4 4 0 0 4 4
IB:
BRCA1
GFP
GAPDH
J
%BRCA1 After 4 hrs CHX
**
WT
3KR
BARD1 (Over-Expressed)
K
% BRCA1 Remaining
*
*
CHX WT
AGK2 WT
AGK2 KR

Figure 3.4. BARD1 RING Domain Deacetylation by SIRT2 Promotes BRCA1-BARD1 Heterodimerization

(A) IP of BRCA1 fragments spanning FL BRCA1 indicates that GFP-SIRT2 pulls down BRCA1 (1-324). **(B)** Co-IP of GFP-BARD1 before and after 4 hours of AGK2 treatment shows decreased interaction with endogenous BRCA1. **(C)** Co-IP of HA-BRCA1 after 4 hours of AGK2 treatment shows decreased interaction with GFP-BARD1. **(D)** Structure of the BRCA1-BARD1 RING domain heterodimer, indicating potential charged interactions between BARD1 K46, K96, and K110 with corresponding BRCA1 residues. BARD1 K96 is ~2.2 Å from BRCA1 D40 which may indicate a salt bridge interaction. BARD1 K110 is in close proximity, ~5.1 Å, to BRCA1 E10. BARD1 K46 is ~5.7 Å from BRCA1 E85. **(E)** GFP-BARD1 WT is acetylated in cells and mutation of lysines K46, K96, and K110 to arginines (3KR) significantly decreases acetylation. FLAG-SIRT2 decreases GFP-BARD1 WT acetylation to a comparable level as that of GFP-BARD1 3KR but does not further decrease acetylation of GFP-BARD1 3KR. **(F)** Co-IP of GFP-BARD1 3KR shows increased pulldown of endogenous BRCA1 compared to GFP-BARD1 WT. **(G)** Co-IP of GFP-BARD1 3KQ shows decreased interaction with BRCA1 compared to GFP-BARD1 WT. **(H)** In HEK-293T cells, over-expression of GFP-BARD1 3KR significantly decreases the rate of BRCA1 degradation after 4 hours of cycloheximide treatment compared to over-expression of GFP-BARD1 WT. **(I)** Similarly, GFP-BARD1 3KR rescues the increased BRCA1 degradation rate after 4 hours of cycloheximide and AGK2 treatment compared to GFP-BARD1 WT. **(J and K)** Quantification of **(H)** and **(I)**, respectively. * indicates $p < .05$, ** indicates $p < .01$. Error bars shown indicate S.D. The experiments above were done in HEK 293T cells.

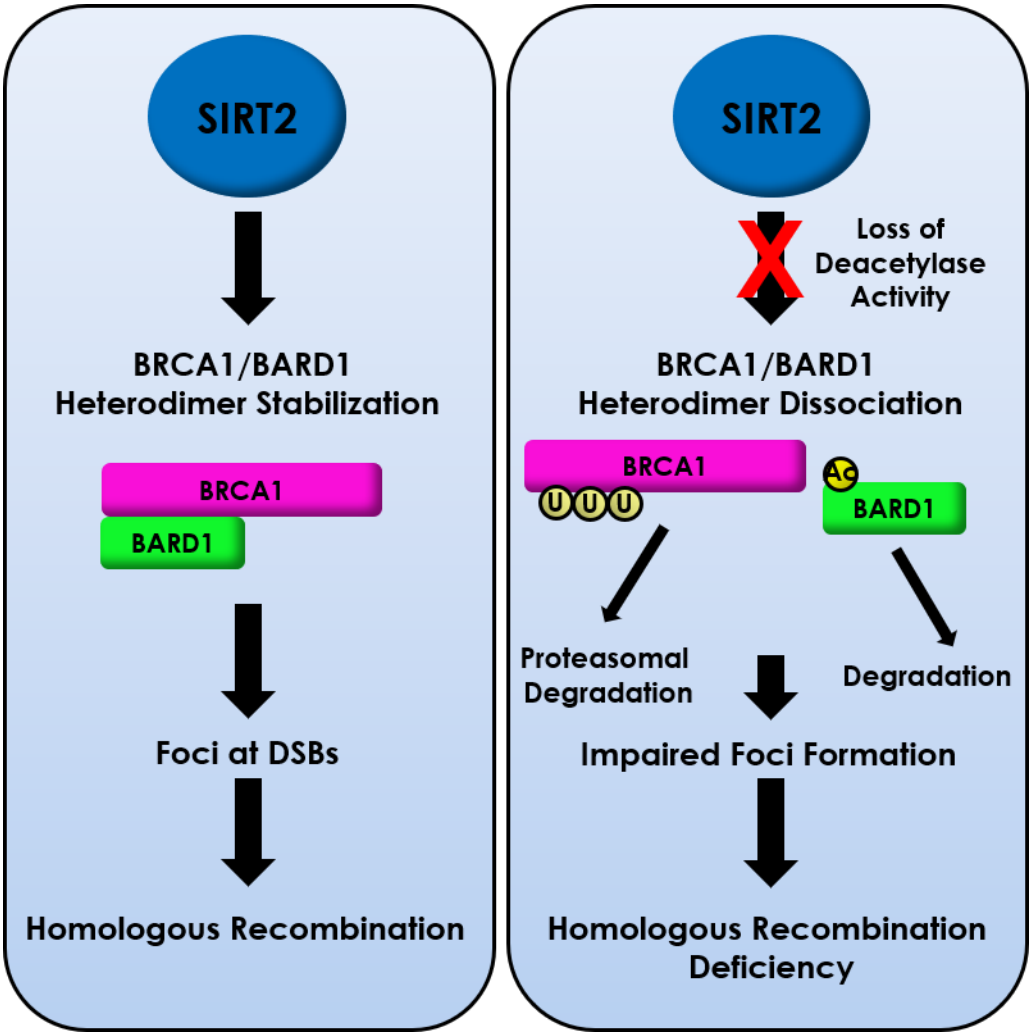
SIRT2
BRCA1/BARD1
Heterodimer Stabilization
BRCA1
BARD1
Foci at DSBs
Homologous Recombination
SIRT2
Loss of
Deacetylase
Activity
BRCA1/BARD1
Heterodimer Dissociation
BRCA1
U U U
Ac
BARD1
Proteasomal
Degradation
Degradation
Impaired Foci Formation
Homologous Recombination
Deficiency

Figure 3.5. Model

Model showing BARD1 RING domain deacetylation by SIRT2 promoting BRCA1-BARD1 heterodimerization, thereby facilitating stability, recruitment, and function in HRR.

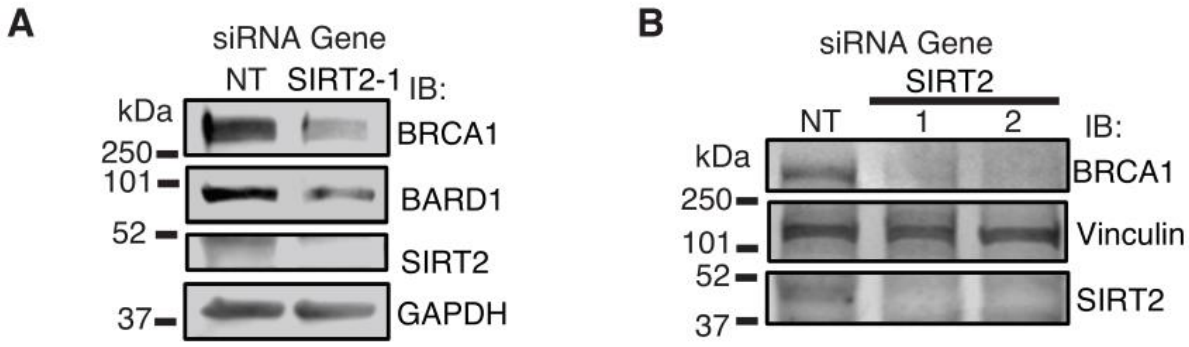
A
siRNA Gene
NT
SIRT2-1
IB:
kDa
250
101
52
37
BRCA1
BARD1
SIRT2
GAPDH
B
siRNA Gene
SIRT2
NT
1
2
IB:
kDa
250
101
52
37
BRCA1
Vinculin
SIRT2

Figure 3.S1. Figure 3.2 Supplemental

(A) Western blot analysis showing a decrease in BRCA1 and BARD1 levels after SIRT2 knockdown in U2OS cells. **(B)** BRCA1 levels decrease after SIRT2 knockdown in U2OS cells using two SIRT2 siRNAs with different target sequences.

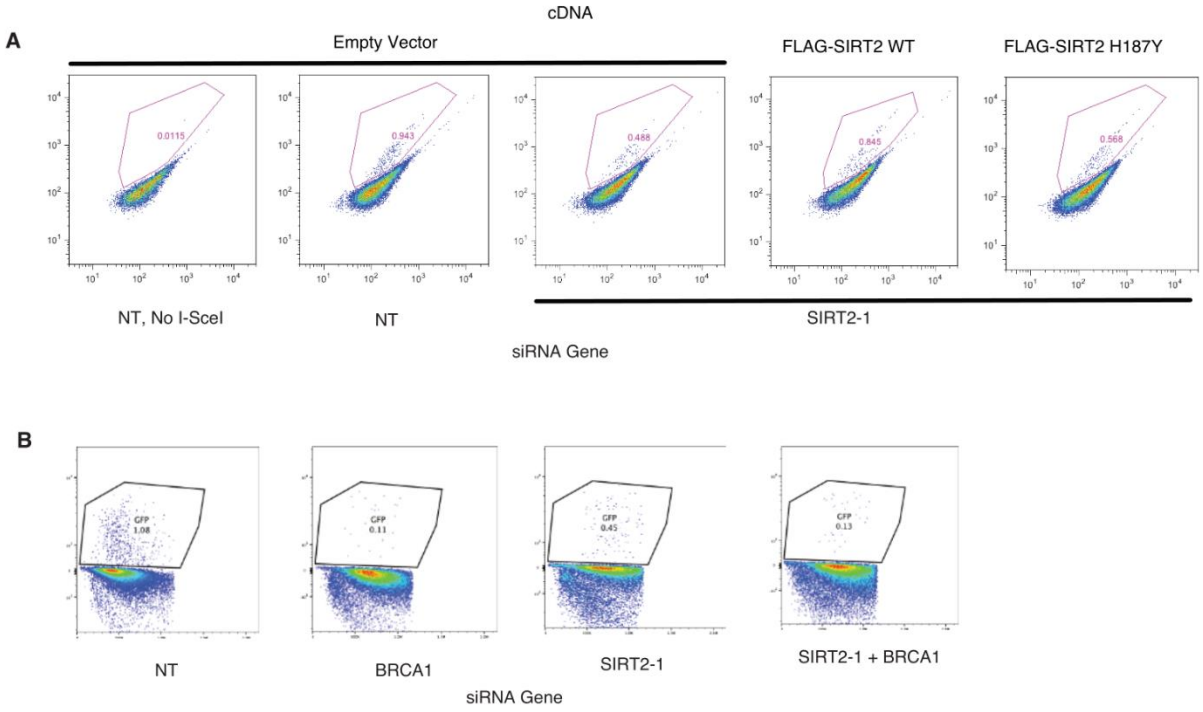
A
cDNA
Empty Vector
FLAG-SIRT2 WT
FLAG-SIRT2 H187Y
0.0115
0.943
0.488
0.845
0.568
NT, No I-SceI
NT
SIRT2-1
siRNA Gene
B
GFP
1.08
GFP
0.11
GFP
0.45
GFP
0.13
NT
BRCA1
SIRT2-1
SIRT2-1 + BRCA1
siRNA Gene

Figure 3.S2. Figure 3.3 Supplemental

(A) Flow analysis showing gating of the GFP-positive cells for the DR-GFP assay done in Figure 3K. **(B)** Flow analysis showing gating of the GFP-positive cells for the DR-GFP assay done in Figure 3M.

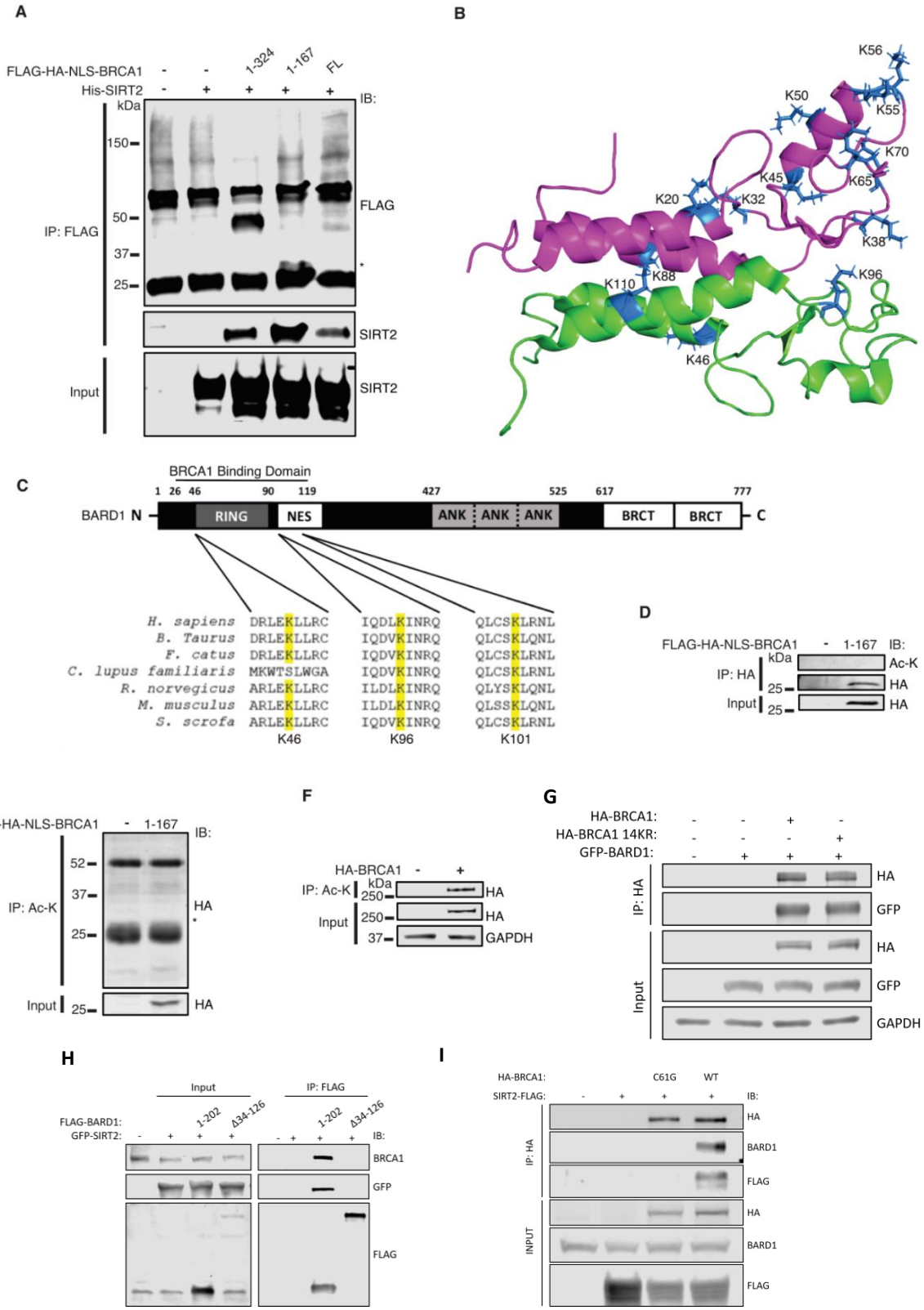
A
FLAG-HA-NLS-BRCA1 - - 1-324 1-167 FL
His-SIRT2 - + + + +
IB:
kDa
150
50
37
25
IP: FLAG
FLAG
SIRT2
Input
SIRT2
B
K56
K50
K55
K70
K45
K65
K20
K32
K38
K88
K110
K96
K46
C
BRCA1 Binding Domain
1 26 46 90 119 427 525 617 777
BARD1 N
RING
NES
ANK
ANK
ANK
BRCT
BRCT
C
H. sapiens DRLEKLLRC IQDLKINRQ QLCSKLRNL
B. Taurus DRLEKLLRC IQDVKINRQ QLCSKLQNL
F. catus DRLEKLLRC IQDVKINRQ QLCSKLRNL
C. lupus familiaris MKWTSLWGA IQDVKINRQ QLCSKLRNL
R. norvegicus ARLEKLLRC ILDLKINRQ QLYSKLQNL
M. musculus ARLEKLLRC ILDLKINRQ QLSSKLQNL
S. scrofa ARLEKLLRC IQDVKINRQ QLCSKLRNL
K46 K96 K101
D
FLAG-HA-NLS-BRCA1 - 1-167 IB:
IP: HA
kDa
25
Ac-K
HA
Input
25
HA
E
FLAG-HA-NLS-BRCA1 - 1-167 IB:
52
37
25
IP: Ac-K
HA
*
Input
25
HA
F
HA-BRCA1 - +
IP: Ac-K
kDa
250
HA
Input
250
HA
37
GAPDH
G
HA-BRCA1: - - + -
HA-BRCA1 14KR: - - - +
GFP-BARD1: - + + +
IP: HA
HA
GFP
Input
HA
GFP
GAPDH
H
Input
IP: FLAG
FLAG-BARD1:
GFP-SIRT2:
1-202
Δ34-126
1-202
Δ34-126
IB:
BRCA1
GFP
FLAG
I
HA-BRCA1: C61G WT
SIRT2-FLAG: - + + + IB:
IP: HA
HA
BARD1
FLAG
INPUT
HA
BARD1
FLAG

Figure 3.S3. Figure 3.4 Supplemental

(A) A co-IP of the FLAG-HA-NLS-BRCA1 fragments containing residues 1-324 and 1-167 from the N terminus show that His-SIRT2 interacts within the first 167 residues of BRCA1. **(B)** A crystal structure of the interaction between BRCA1 and BARD1 showing all lysines in the BRCA1/BARD1 binding domains. BRCA1 contains 10 lysines within its BARD1-binding region (K20, K32, K38, K45, K50, K55, K56, K65, K70, and K88), while BARD1 contains three lysines in its BRCA1-binding region (K46, K96, and K110). **(C)** Schematic representation of BARD1 structural domains and evolutionary conservation of BARD1 K46, K96, and K110. **(D)** An IP of the FLAG-HA-NLS-BRCA1 fragment containing residues 1-167 shows no evidence of acetylation. **(E)** An IP of acetyl-lysine does not show any evidence of pulldown of the 1-167 BRCA1 fragment. **(F)** IP of acetyl-lysine pulls down full-length HA-BRCA1. All experiments were done in HEK293T cells. **(G)** Mutation of the 14 lysines to arginine within the first 167 residues of HA-BRCA1 shows no change in interaction with GFP-BARD1 via IP. **(H)** GFP-SIRT2 co-IPs with the N-terminus of FLAG-BARD1 (1-202), but not FLAG-BARD1 without the RING domain (Δ34-126). **(I)** The HA-BRCA1 mutant C61G, which does not bind BARD1, is unable to pull down SIRT2-FLAG in a co-IP, compared to HA-BRCA1 WT.

Chapter 4: Discussion and Future Directions

4.1 Connecting SIRT2 with BARD1 and BRCA1

Identifying the proteins involved in the DDR and understanding the regulatory mechanisms that control these pathways is a crucial part of understanding how cancer develops, grows, and spreads. In healthy cells, the normal DDR functions to defend and protect genomic integrity in the face of DNA damage or other types of cellular stress. Deficiencies in the related pathways, whether inherited or acquired, can have disastrous effects that lead to an inability to repair DNA damage and subsequently an accumulation of mutations, which significantly increases the likelihood of developing cancer during one's lifetime. Yet, DNA repair pathways have been found to also work against us in the context of cancer treatments where in many cases, proficient DNA repair pathways contribute to therapeutic resistance in cancer cells by protecting the cells against genomic destruction. However, given cancer cells tend to rely heavily on DNA repair pathways to avoid death, it has opened up the door where the proteins involved in these pathways have proven to be efficacious targets for treatment and continue to offer promising future results in the creation of novel treatments. Our knowledge on the DDR and the role it plays in the development and progression of cancer continues to grow, and as we gain more insight into these pathways, we also have begun to understand how to use this knowledge to our advantage in the clinic in the prevention and treatment of cancer.

The past few years have been rife with new information on DDR-related proteins, including BRCA1 and BARD1. Within the past two decades since the initial discovery and identification of these two proteins, there has been amazing progress in bringing to light their associated roles in DNA repair, apoptosis, and maintenance of genomic stability, which has contributed to our

understanding of the DDR. Similarly, further research has contributed to our understanding of the role of sirtuins, including SIRT2, and their functions in the DDR to maintaining genomic integrity. Great strides have also been made in understanding at the molecular level how these proteins carry out their functions, are regulated by complex and sometimes overlapping pathways, and may be associated with preventing and/or promoting tumorigenesis or disease progression, depending on the cellular context. Nevertheless, there is still a great amount of information we have yet to elucidate when it comes to understanding how cells maintain homeostasis.

The work presented here expands upon our current understanding of the molecular dynamic that occurs between SIRT2, BRCA1, and BARD1. A loss of SIRT2 or inhibition of SIRT2 enzymatic activity leads to a decrease in BRCA1 and BARD1 protein levels. This effect seems to happen at the protein-level, as RT-PCR analyses show that BRCA1 and BARD1 mRNA levels are not significantly altered after loss of SIRT2 levels or enzymatic activity. Furthermore, after SIRT2 deficiency, BRCA1 shows an increased rate of degradation after cycloheximide treatment, though levels can be rescued with proteasomal inhibition. BRCA1 and BARD1 protein destabilization seems to be caused by an impairment in BRCA1-BARD1 heterodimerization, as SIRT2 enzymatic inhibition shows in immunoprecipitation assays that BRCA1 and BARD1 pull down less of one another and in IF studies that BRCA1 and BARD1 become more localized to the cytoplasm compared to control conditions. Furthermore, SIRT2 knockdown causes an impairment in HRR efficiency. These findings are significant as they demonstrate for the first time that SIRT2 is involved in the HRR pathway and provide a potential point of regulation within the HRR pathway, where loss of SIRT2 leads to an impairment in the proper heterodimerization between BRCA1 and BARD1, subsequently promoting BRCA1 and BARD1 protein degradation and localization to the

cytoplasm. Indeed, these results indicate that SIRT2 helps promote BRCA1-BARD1 interaction and complex formation through deacetylation. Mutation of K46, K96, and K110 to arginines, a deacetylated-lysine mimic, in BARD1 show BRCA1 and this mutant BARD1 have increased binding in HEK293T cells compared to wild-type BARD1. In contrast, mutation of these same lysines to glutamine, an acetyl-lysine mimic, shows impaired interact between BRCA1 and this mutated BARD1. Supporting this hypothesis is also the observation that over-expression of SIRT2 decreases the acetylation of BARD1 to levels similar to that of the 3KR BARD1 mutant on western blot analyses, suggesting that these sites are indeed the major targets of SIRT2. BRCA1 and BARD1 also fail to form foci at sites of DNA damage after IR, which could be attributed to an impairment in recruitment to sites of damage or a decrease in protein levels, or a mix of both.

Our lab has previously shown that SIRT2 is a protein involved in the DDR through its role as a signaling protein early on in the RSR; other work in the lab has also shown that SIRT2 is involved in NHEJ through the regulation of DNA-PKcs via deacetylation (data unpublished). In conjunction with the work presented here, all of these observations support the notion that SIRT2 may be one of the first responders after DNA damage has occurred, acting early-on in the DDR to promote repair even before a pathway choice has been made. Moreover, as SIRT2 seems to impact BRCA1 and BARD1 stability even before damage has occurred, there is the implication that SIRT2 is not just a player in the DDR, but also as a tumor suppressor that acts as a cellular guardian to maintain cellular homeostasis under normal conditions. However, these results are not necessarily surprising when looking at the other sirtuins family members and their varied functions before and after cellular stress: SIRT1 has been linked to multiple different DNA repair pathways, such as BER, NER, NHEJ, and HRR, and also is one of the first proteins at sites of damage to promote chromatin

remodeling and DNA-repair-associated protein recruitment. SIRT6 has also been associated with multiple DNA repair pathways including BER and HRR and works to maintain telomere stability. Similarly, the mitochondrial sirtuins aid in normal metabolic homeostasis in multiple pathways while also working to prevent crisis when stress arises. Thus, it makes sense that SIRT2 would likewise be involved in the normal maintenance of the cell and potentially act as a first responder upstream of the points of diverging pathways when normal function is disrupted, such as in the case of DNA damage where the cell needs to decide how to repair the damage.

4.2 BARD1: Player Two

What is particularly curious about these findings is that BARD1, rather than BRCA1, seems to be major target of SIRT2 in terms of promoting BRCA1-BARD1 complex stability. Much of the research over the past two decades has focused on BRCA1 rather than BARD1, and even in cases where BARD1 has been studied, it is usually in the context of BRCA1 (the irony is not lost that this is true within this work too). This uneven focus is readily apparent when comparing what has been uncovered about the two proteins since their discoveries, both at a molecular level and at a clinical level. In some regard, this lopsided divide in attention seems entirely justified: As previously discussed, BRCA1 was identified first through genetic studies of families who showed an unusual propensity for cancer development, while BARD1 was first discovered as a binding partner of BRCA1 through biochemical means rather than through clinical analysis. Furthermore, there is an overall impression that BRCA1 is considered more “clinically relevant” of the two proteins and thus could be considered the more interesting study subject. The observation that cancer-associated BRCA1 mutations have been easier to identify may tie into the high probability that certain BRCA1 mutations seem to have a high penetrance, as well as the fact that BRCA1 has

identifiable mutational “hot-spots” within its domains. In contrast, BARD1 mutations seem to have a more subtle and highly disputed clinical effect, and have been much harder to identify, especially as there seem to be no mutational “hot-spots” regions in BARD1 like there are in BRCA1. Even identified “deleterious” mutations in BARD1, such as the nonsense mutation Q564*, is only considered to be a low/medium risk for developing breast cancer, and with little to no connection to ovarian cancer, though a few mutations have been linked to nephroblastoma susceptibility.[407, 408] Given the inconclusive data, BARD1 has even been left off of the recommended multigene panel proposed by the UK Caner Genetics Group for testing breast cancer patients, casting BARD1 even further into BRCA1’s shadow.[409]

Nevertheless, BARD1 should not be forgotten just because its relevance to cancer progression is less readily apparent than that of BRCA1. The studies on BARD1 are still relatively premature, meaning there is still much work to do to uncover the elusive role BARD1 plays in cancer predisposition as well as in the cell, both alone and in conjunction with BRCA1. As previously mentioned, it is very possible that certain BARD1 mutations are embryonic lethal and thus never make it to the point of being passed down through the generations, such as is the case with homozygous BRCA1 deletion. Moreover, BARD1 is still a major binding partner of BRCA1 and thus some mutations that affect its binding to BRCA1 would be expected to show clinical “BRCA-like” phenotypes that are usually associated with BRCA1 loss, as is evidenced by the knockout studies that have been done in mice. In the context of the results presented here, it’s suggested that though BARD1 may be the primary target for SIRT2, impairment of association with BRCA1 leads to decreased BRCA1 protein stability and thus the potential to replicate a BRCA1 deficiency through SIRT2 dysregulation. It is therefore not unreasonable to believe that there are other

regulatory mechanisms that target BARD1 that affects its relationship with BRCA1, or its independent role in the cell, that will prove to be invaluable when considering the contribution BARD1 has towards maintaining a healthy cell, protecting against mutation after DNA damage, and determining effective cancer treatments.

4.3 Future Considerations

BARD1 and SIRT2: What More?

Biology is rarely as clean as it is made out to be in the scientific literature and in textbooks, meaning there are other considerations to be made surrounding these data. One question that logically follows this work is: Are there other sites SIRT2 targets for deacetylation on BARD1? And what do they do? Mass spectrometry done in the lab has suggested that there may be other lysines on BARD1 outside of the BRCA1-BARD1 interacting region that SIRT2 may deacetylate, including lysines K130, K596, K650, K693, though at this point, it is unclear if these are true hits, and if they are, what their significance may be. It is possible that these sites, while not in the BRCA1-binding region, may be close enough to impact heterodimerization, either through electrostatic interactions or through an effect on the overall protein structure. However, these sites may also have nothing to do with the interaction of BARD1 with BRCA1 and instead could regulate other functions of BARD1. It is also reasonable to guess that the conditions being used in our lab or the methods we have used (e.g. western blot and mass spectrometry) may not be suitable for detecting a change in acetylation status at certain lysine sites, perhaps because of high background due to other sites of acetylation that mask more minor acetylation contributors or a low basal level of acetylation at these sites under the tested conditions. It is also noteworthy to mention that a consensus sequence has not been found for SIRT2 targets, or any other particular

substrate determinants, which implies SIRT2 has the potential to deacetylate lysines indiscriminately if in close enough proximity when preferred targets are unavailable.[325] If this is the case, it may add another layer of complexity to finding all lysines that are targeted for deacetylation on BARD1 and determining their significance. Thus, these findings warrant further investigation to unravel the full extent of the regulation of BARD1 by SIRT2.

Is BRCA1 a SIRT2 Target?

In the same vein, it is unclear the significance of BRCA1 deacetylation by SIRT2. The direct relationship between sirtuins and BRCA1 has already been established in that SIRT1 is known to deacetylate BRCA1 at lysine K830, but the regulatory consequence seems to be related to checkpoint signaling rather than protein stability as seen with SIRT2. Nevertheless, given there is some degree of conservation between the different sirtuin family members, the fact that BRCA1 is also a substrate of SIRT2 is not necessarily surprising. However, while we have demonstrated that SIRT2 is able to deacetylate full-length BRCA1 both *in vitro* and *in cells*, we have as of yet been unable to verify the specific sites of SIRT2 deacetylation or their impact on the function of BRCA1. Regarding the BRCA1 instability phenotype seen with SIRT2 deficiency, no acetylation has been found in our lab within the N-terminus of BRCA1 that would be fall in the BARD1-binding region. Like with BARD1, mass spectrometry has identified putative sites of SIRT2 deacetylation on BRCA1, namely on lysines K175, K193, and K223, but these sites once again fall outside the BARD1-interaction domain. Follow-up studies have also failed to find evidence that these lysines contribute to acetylation on BRCA1, though there are a number of potential technical explanations. First, in our hands, BRCA1 does not seem to have high levels of acetylation under basal conditions in HEK293T cells, which has necessitated the over-expression of the HATS

CBP, P300, and PCAF to get a signal that can be detected on western blot. As previously suggested as is the case of BARD1, on BRCA1 there may be lysines that make up the majority of the acetylation signal that masks a weaker signal from other acetylated lysines, such that mutation of a minor contributor would not show a significant difference in the overall acetylation signal on a western blot, especially given the potential number of acetylation sites on a protein as large as BRCA1. Second, the conditions may not be optimal to induce acetylation at these sites, which could occur in relatively low frequency or under transient conditions, making it difficult to capture a large enough pool for detection. Even more, the HATs used to induce acetylation may target alternative sites to the ones SIRT2 naturally targets. Though our data show SIRT2 is able to remove the acetylation put on BRCA1 by the three aforementioned HATs, that does not preclude the possibility that SIRT2 targets lysines acetylated by other, yet-to-be-identified HATs. Interestingly, the data suggests that the acetylation put on by these three HATs does not significantly impact BRCA1 protein stability, as over-expression of the HATs does not increase the rate of BRCA1 degradation after cycloheximide treatment compared to the non-acetylated BRCA1. But, as already suggested, these sites may not be relevant to the BRCA1 instability phenotype seen by SIRT2 knockdown or could even simply be an artifact of the experiment due to protein over-expression or an inherent promiscuity in acting on acetylated-lysine substrates by SIRT2. These observations could also be another indication that BARD1 is indeed the main mechanism by which BRCA1-BARD1 heterodimerization is impacted by loss of SIRT2 deacetylation activity. There is also the possibility that SIRT2 targets acetylated K830 similar to SIRT1 and is redundantly

involved in promoting proper checkpoint signaling via BRCA1. In any case, further research will be required to disentangle the direct impact of SIRT2 on BRCA1.

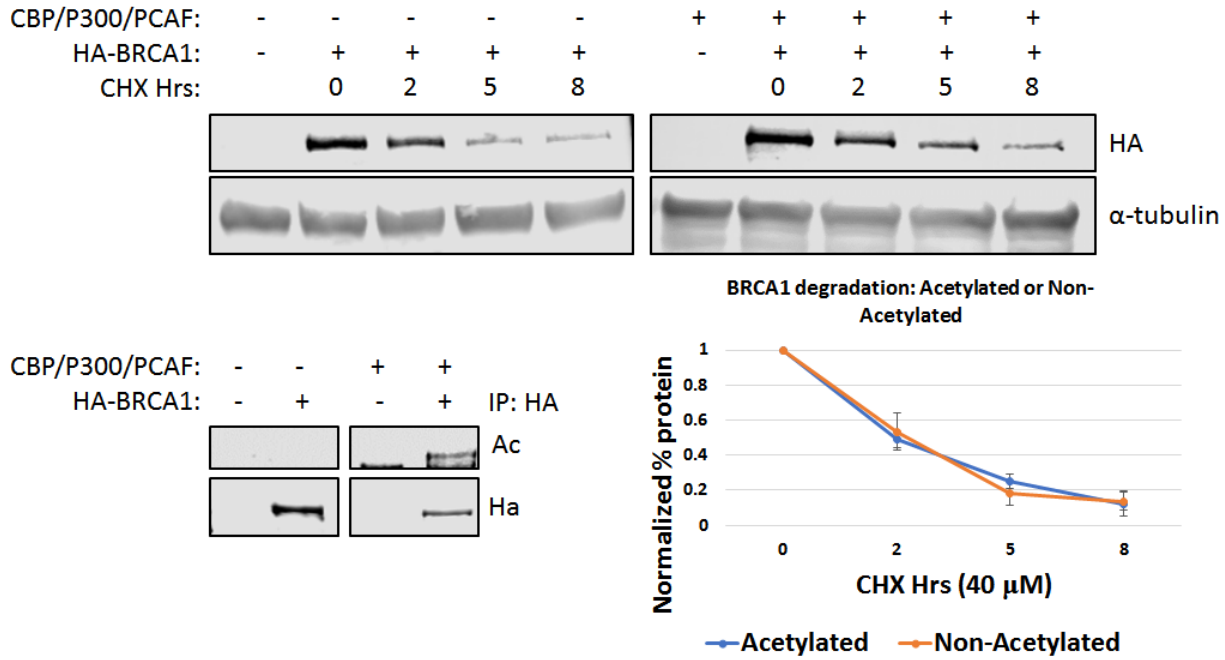

Figure 4.1. BRCA1 Acetylation and Stability. Acetylation of BRCA1 with CBP, p300, and PCAF does not seem to significantly change the protein stability of HA-BRCA1 in 293T cells as seen after treating the cells with 50 µM of cycloheximide. N = 2 for the acetylated group, N = 3 for the non-acetylated group.

Other Conditions and Pathways

Another consideration is whether or not the deacetylation of BARD1 occurs only under normal conditions to promote BRCA1-BARD1 heterodimerization or if this activity is upregulated after a trigger, such as after DNA damage. The results in this work show that under conditions of no stress, SIRT2 regulates BRCA1-BARD1 complex formation. But what about under conditions of stress? Would IR induce SIRT2 to deacetylate BARD1 and promote BRCA1-BARD1 binding to promote HRR? There is the distinct possibility that SIRT2 is activated after DNA damage to deacetylate BARD1 to promote interaction with BRCA1, and subsequently, efficient HRR. Given SIRT2 seems to be activated by replication stress, and other sirtuins have been shown to activate

in response to DNA damage, it is reasonable to think that SIRT2 may similarly activate after DSBs to upregulate other HRR-related activities. There are plenty of experiments that should be done to follow up on the work that has been done here. It should be determined if BARD1 acetylation decreases after IR, and if so, additionally after SIRT2 enzymatic inhibition. If BARD1 acetylation fails to decrease after IR with SIRT2 enzymatic inhibition, this would be strong evidence supporting the hypothesis that SIRT2 deacetylates BARD1 in response to DNA damage to promote interaction with BRCA1 in HRR. A failure to stop a decrease in the acetylation signal, however, would pose two possibilities: First, that SIRT2 is not the deacetylase responsible for this phenomenon. Second, there is another deacetylase that acts redundantly with SIRT2. In either case, it would be interesting to see if this BARD1 acetylation is important for HRR. Follow-up studies using nicotinamide, which inhibits the sirtuin family (i.e. class III HATs), and TSA, which inhibits the class I and II HATs, would be useful in narrowing down the deacetylase family responsible for BARD1 deacetylation after IR should BARD1 deacetylation decrease after DNA damage. However, we have found that even after IR, BRCA1 levels are still decreased with AGK2 treatment or SIRT2 knockdown (Figure 4A and B). Preliminary evidence suggests BARD1 levels also decrease after SIRT2 deficiency before and after IR, though may become more stable after IR (Figure 4.2C). More experiments will need to be done to validate this finding.

Experiments that focus on the cell-cycle should also be performed to further elucidate the mechanism by which SIRT2 affects BRCA1 and BARD1. BRCA1, BARD1, and SIRT2 are all well-established to be cell-cycle regulated proteins, and it is only natural to suspect that the regulatory interplay between these three proteins would also be tied to the different stages of the cell cycle. One question that should be answered is whether or not BRCA1 and BARD1 levels are

decreased after SIRT2 inhibition throughout all of the cell cycle or if this phenomenon is limited to certain phases, such as S or M where BRCA1 and BARD1 levels tend to be at their peak. This question may be somewhat difficult to answer for BRCA1 during G_1, as in many cases, G_1 BRCA1 levels are low enough to make detection via western blot difficult, though this technical difficulty could be addressed by identifying a cell type that has relatively higher levels of BRCA1 during G_1.

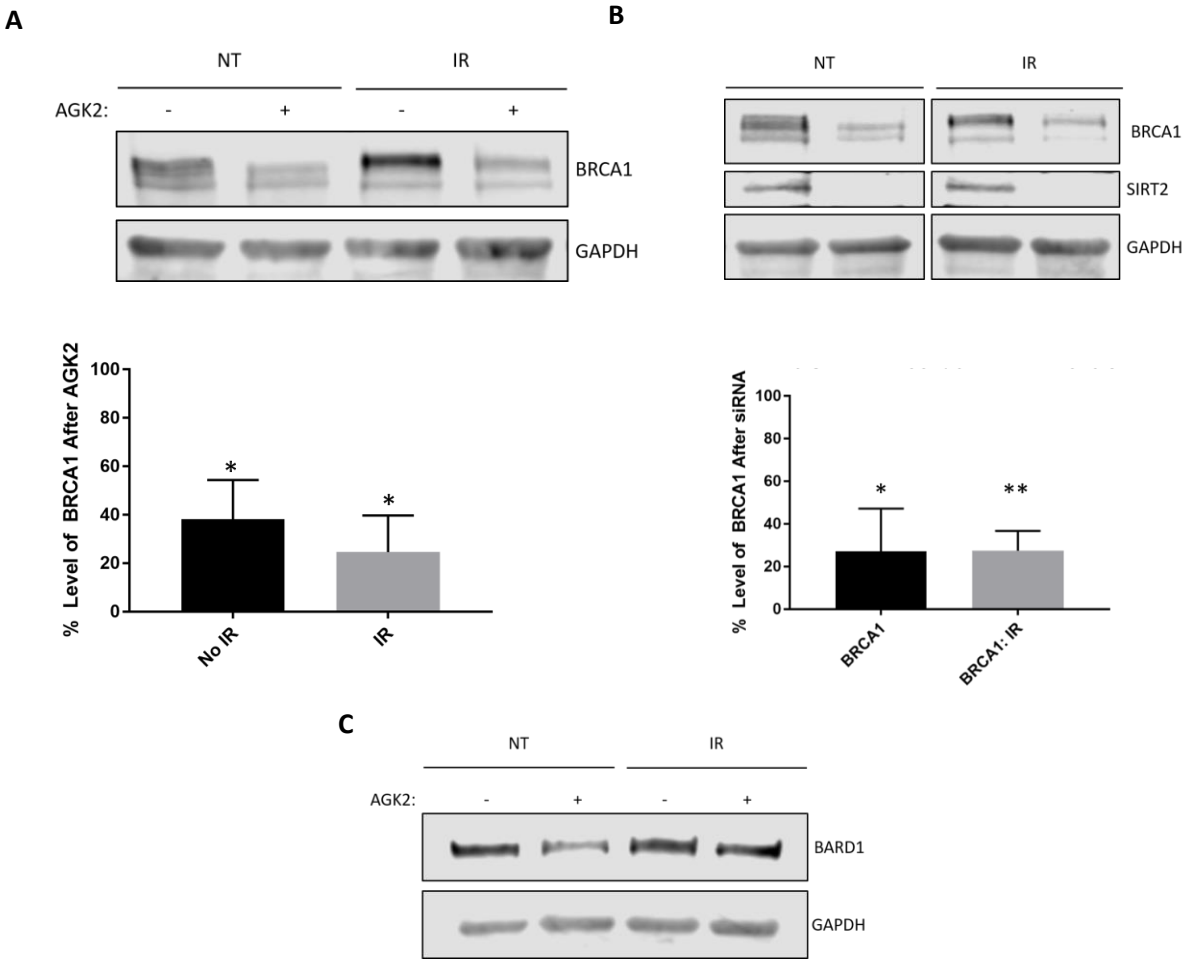

Figure 4.2. BRCA1 and BARD1 Stability after SIRT2 Inhibition and IR. After **(A)** 24 hours of 32 µM of AGK2 treatment and **(B)** SIRT2 knockdown BRCA1 levels significantly decrease both before and after 4 hours of IR recovery using 10 Gy. **(C)** indicates that there may be stabilization of BARD1 after AGK2 and IR treatment. N = 3. Error bars indicate S.D., * indicates $p < .05$, ** indicates $p < .01$.

As previously mentioned, SIRT2 may regulate BRCA1 and BARD1 through other indirect pathways, such as through the APC/C complex. SIRT2 has already been shown to affect APC/C activity, whereas the APC/C complex ubiquitinates and promotes degradation of BARD1. It would be reasonable to assume that these findings would suggest that as SIRT2 levels decrease, APC/C activity would decrease, thus leading to increased stabilization of BARD1 and subsequently BRCA1. Yet, within this work we show that BARD1 levels decrease after SIRT2 inhibition. Does this mean the aforementioned hypothesis is incorrect? Not necessarily. Pathways are often overlapping and connected, that are under a delicate balance that depends on a multitude of factors and environmental context. It is possible that SIRT2 knockdown and decreased APC/C complex activity may only affect BARD1 during certain phases of the cell cycle, or that the loss of stability through decreased ability to bind to BRCA1 is stronger than the gain of stability through decreased APC/C complex activity. As there is relatively limited information the pathways that regulate both *BARD1* mRNA and BARD1 protein stability, there may be other E3 ligases that promote BARD1 degradation in addition to the APC/C complex that are unaffected by SIRT2 levels. There are many hypotheses that could be made on the interplay between SIRT2, APC/C, BARD1, and BRCA1, but these have yet to be tested and warrant further investigation. It would be interesting to see the full extent to which SIRT2 regulates BRCA1 and BARD1 protein stability both directly and indirectly through teasing out the varied pathways.

SIRT2 Regulation and Involvement

The upstream regulation of SIRT2 is still somewhat a mystery. SIRT2 is a mostly cytoplasmic protein, yet somehow still gets into the nucleus and activates different pathways in ways that have

yet to be determined. With this in mind, is worth asking the flip question to what has been shown in this work: Given SIRT2 regulates BRCA1 and BARD1, could BRCA1 and/or BARD1 conversely regulate SIRT2? Given BRCA1 is involved in reciprocal regulatory pathways with other proteins at both the transcriptional and post-translational level, it would not be unexpected that BRCA1 would similarly be involved in a regulatory loop involving SIRT2. Indeed, knockdown of BRCA1 in DR-GFP U2OS cells, seems to significantly increase SIRT2 protein levels. If this observed phenotype is indeed true, this would indicate BRCA1 (and potentially BARD1) are involved in a negative feedback loop with SIRT2, where SIRT2 promotes expression of BRCA1 (and BARD1), while at the same time being downregulated as BRCA1 levels increase. This type of regulation would contribute to the ability of the cell to carefully modulate the level and activity of different proteins within the same pathway, so homeostasis is properly maintained under normal conditions but ready to act when the need arises.

The role of BARD1 in the regulation of SIRT2, however, is less clear. With BRCA1, BARD1 has E3 ligase activity, making it possible SIRT2 could actually be a target of BRCA1 and BARD1. While this work shows that SIRT2 is bound in a complex with BRCA1 and BARD1, where SIRT2 deacetylates BARD1, it has not been investigated whether SIRT2 binds BRCA1 and BARD1 as a substrate itself rather than enzyme. In addition to the possible transcriptional regulation, this would add yet another layer of regulation in the SIRT2-BRCA1-BARD1 axis if SIRT2 could be shown as an E3 ligase target of BRCA1-BARD1. BRCA1-BARD1 ubiquitination of its substrates, however, has been associated to protein signaling rather than promoting degradation through the proteasome. Therefore, should the BRCA1-BARD1 complex target SIRT2 for ubiquitination, this could indicate a potential mechanism for upstream SIRT2 regulation in response to DNA damage.

Future experiments should also work to further establish the roles SIRT2 could play in other DNA damage repair pathways. Right now, our lab has implicated SIRT2 in the repair of DNA DSBs, such as in the case of HRR, and NHEJ, and during replication fork stalls through the RSR, while another lab has implicated SIRT2 in NER. But what about the other types of DNA damage? Given SIRT2 seems to lie upstream of multiple pathways, it is likely that SIRT2 may work to influence DNA repair pathway choice. It would indeed be interesting to determine if SIRT2 influences DNA repair pathway choice, or, alternatively, if SIRT2 indiscriminately activates a multitude of different DNA repair pathways to prime for whatever damage has been done and provide options for the cell to adequately respond.

In any case, there is still yet a lot of work to be done in this area of research. There is still much potential to continue to elucidate the role the DDR, SIRT2, BRCA1, and BARD1 have in promoting proper DNA repair after damage, as well as the mechanisms by which they work. There is little doubt of their relevance in the formation of cancer, but there are a lot of unanswered questions as to how dysregulation, mutation, or altered function leads to carcinogenesis and influences the efficacy of current treatments. Hopefully, the more of this subject we can understand, the better we will be able to prevent and treat cancer.

Chapter 5: References

1. Yao, Y. and W. Dai, *Genomic Instability and Cancer.* J Carcinog Mutagen, 2014. **5**.
2. Zindler, J.D., et al., *Increasing the Therapeutic Ratio of Stereotactic Ablative Radiotherapy by Individualized Isotoxic Dose Prescription.* J Natl Cancer Inst, 2016. **108**(2).
3. Nijman, S.M., *Synthetic lethality: general principles, utility and detection using genetic screens in human cells.* FEBS Lett, 2011. **585**(1): p. 1-6.
4. O'Connor, M.J., *Targeting the DNA Damage Response in Cancer.* Mol Cell, 2015. **60**(4): p. 547-60.
5. Brown, J.S., et al., *Targeting DNA Repair in Cancer: Beyond PARP Inhibitors.* Cancer Discov, 2017. **7**(1): p. 20-37.
6. Shaheen, M., et al., *Synthetic lethality: exploiting the addiction of cancer to DNA repair.* Blood, 2011. **117**(23): p. 6074-82.
7. Hall, M.D., M.D. Handley, and M.M. Gottesman, *Is resistance useless? Multidrug resistance and collateral sensitivity.* Trends Pharmacol Sci, 2009. **30**(10): p. 546-56.
8. Barazas, M., et al., *Radiosensitivity Is an Acquired Vulnerability of PARPi-Resistant BRCA1-Deficient Tumors.* Cancer Res, 2019. **79**(3): p. 452-460.
9. Zhang, Y., L.H. Rohde, and H. Wu, *Involvement of nucleotide excision and mismatch repair mechanisms in double strand break repair.* Curr Genomics, 2009. **10**(4): p. 250-8.
10. Dexheimer, T.S., *DNA Repair Pathways and Mechanisms*, in *DNA Repair of Cancer Stem Cells*. 2013. p. 19-32.
11. Poulsen, M.L. and M.L. Bisgaard, *MUTYH Associated Polyposis (MAP).* Curr Genomics, 2008. **9**(6): p. 420-35.
12. Lopez-Martinez, D., C.C. Liang, and M.A. Cohn, *Cellular response to DNA interstrand crosslinks: the Fanconi anemia pathway.* Cell Mol Life Sci, 2016. **73**(16): p. 3097-114.
13. Mao, Z., et al., *DNA repair by nonhomologous end joining and homologous recombination during cell cycle in human cells.* Cell Cycle, 2008. **7**(18): p. 2902-6.
14. Chang, H.H.Y., et al., *Non-homologous DNA end joining and alternative pathways to double-strand break repair.* Nat Rev Mol Cell Biol, 2017. **18**(8): p. 495-506.
15. Zhao, X., et al., *Cell cycle-dependent control of homologous recombination.* Acta Biochim Biophys Sin (Shanghai), 2017. **49**(8): p. 655-668.
16. Lee, M.V., et al., *BRCA-associated Cancers: Role of Imaging in Screening, Diagnosis, and Management.* Radiographics, 2017. **37**(4): p. 1005-1023.
17. Altmann, T. and A.R. Gennery, *DNA ligase IV syndrome; a review.* Orphanet J Rare Dis, 2016. **11**(1): p. 137.
18. Javle, M. and N.J. Curtin, *The role of PARP in DNA repair and its therapeutic exploitation.* Br J Cancer, 2011. **105**(8): p. 1114-22.
19. Jubin, T., et al., *The PARP family: insights into functional aspects of poly (ADP-ribose) polymerase-1 in cell growth and survival.* Cell Prolif, 2016. **49**(4): p. 421-37.
20. del Rivero, J. and E.C. Kohn, *PARP Inhibitors: The Cornerstone of DNA Repair-Targeted Therapies.* Oncology (Williston Park), 2017. **31**(4): p. 265-73.
21. Farmer, H., et al., *Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy.* Nature, 2005. **434**(7035): p. 917-21.
22. Eustermann, S., et al., *The DNA-binding domain of human PARP-1 interacts with DNA single-strand breaks as a monomer through its second zinc finger.* J Mol Biol, 2011. **407**(1): p. 149-70.
23. Bryant, H.E., et al., *Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase.* Nature, 2005. **434**(7035): p. 913-7.
24. Honey, K., *FDA Approves a New PARP Inhibitor for BRCA-mutant Breast Cancer*, in *CANCER RESEARCH Catalyst*. 2018, AACR: Online.

25. Nikolaev, A. and E.S. Yang, *The Impact of DNA Repair Pathways in Cancer Biology and Therapy.* Cancers (Basel), 2017. **9**(9).
26. Lim, J.S.J. and D.S.P. Tan, *Understanding Resistance Mechanisms and Expanding the Therapeutic Utility of PARP Inhibitors.* Cancers (Basel), 2017. **9**(8).
27. Konstantinopoulos, P.A. and U.A. Matulonis, *PARP Inhibitors in Ovarian Cancer: A Trailblazing and Transformative Journey.* Clin Cancer Res, 2018. **24**(17): p. 4062-4065.
28. Konstantinopoulos, P.A., et al., *Homologous Recombination Deficiency: Exploiting the Fundamental Vulnerability of Ovarian Cancer.* Cancer Discov, 2015. **5**(11): p. 1137-54.
29. Lord, C.J. and A. Ashworth, *PARP inhibitors: Synthetic lethality in the clinic.* Science, 2017. **355**(6330): p. 1152-1158.
30. Kim, Y., et al., *Reverse the Resistance to PARP Inhibitors.* Int J Biol Sci, 2017. **13**(2): p. 198-208.
31. Cruz, C., et al., *RAD51 foci as a functional biomarker of homologous recombination repair and PARP inhibitor resistance in germline BRCA-mutated breast cancer.* Ann Oncol, 2018. **29**(5): p. 1203-1210.
32. Bhullar, K.S., et al., *Kinase-targeted cancer therapies: progress, challenges and future directions.* Mol Cancer, 2018. **17**(1): p. 48.
33. Minchom, A., C. Aversa, and J. Lopez, *Dancing with the DNA damage response: next-generation anti-cancer therapeutic strategies.* Ther Adv Med Oncol, 2018. **10**: p. 1758835918786658.
34. Durant, S.T., et al., *The brain-penetrant clinical ATM inhibitor AZD1390 radiosensitizes and improves survival of preclinical brain tumor models.* Sci Adv, 2018. **4**(6): p. eaat1719.
35. Pike, K.G., et al., *The Identification of Potent, Selective, and Orally Available Inhibitors of Ataxia Telangiectasia Mutated (ATM) Kinase: The Discovery of AZD0156 (8-{6-[3-(Dimethylamino)propoxy]pyridin-3-yl}-3-methyl-1-(tetrahydro-2 H-pyran-4-yl)-1,3-dihydro-2 H-imidazo[4,5- c]quinolin-2-one).* J Med Chem, 2018. **61**(9): p. 3823-3841.
36. Aguilar-Quesada, R., et al., *Interaction between ATM and PARP-1 in response to DNA damage and sensitization of ATM deficient cells through PARP inhibition.* BMC Mol Biol, 2007. **8**: p. 29.
37. Hickson, I., et al., *Identification and characterization of a novel and specific inhibitor of the ataxia-telangiectasia mutated kinase ATM.* Cancer Res, 2004. **64**(24): p. 9152-9.
38. Reaper, P.M., et al., *Selective killing of ATM- or p53-deficient cancer cells through inhibition of ATR.* Nat Chem Biol, 2011. **7**(7): p. 428-30.
39. Sultana, R., et al., *Ataxia telangiectasia mutated and Rad3 related (ATR) protein kinase inhibition is synthetically lethal in XRCC1 deficient ovarian cancer cells.* PLoS One, 2013. **8**(2): p. e57098.
40. O'Carrigan, B., et al., *Phase I trial of a first-in-class ATR inhibitor VX-970 as monotherapy (mono) or in combination (combo) with carboplatin (CP) incorporating pharmacodynamics (PD) studies.* Journal of Clinical Oncology, 2016. **34**(15_suppl): p. 2504-2504.
41. Qiu, Z., N.L. Oleinick, and J. Zhang, *ATR/CHK1 inhibitors and cancer therapy.* Radiother Oncol, 2018. **126**(3): p. 450-464.
42. Dominguez-Kelly, R., et al., *Wee1 controls genomic stability during replication by regulating the Mus81-Eme1 endonuclease.* J Cell Biol, 2011. **194**(4): p. 567-79.
43. Callen, E., et al., *Essential role for DNA-PKcs in DNA double-strand break repair and apoptosis in ATM-deficient lymphocytes.* Mol Cell, 2009. **34**(3): p. 285-97.
44. Pospisilova, M., M. Seifrtova, and M. Rezacova, *Small molecule inhibitors of DNA-PK for tumor sensitization to anticancer therapy.* J Physiol Pharmacol, 2017. **68**(3): p. 337-344.
45. Brian, P.W.C., P.J.; Hemming, H.G.; Norris, G.L.F,, *Wortmannin, an antibiotic produced by Penicillium wortmanni.* Transactions of the British Mycological Society, 1957. **40**(3): p. 365-368.
46. Davidson, D., et al., *Small Molecules, Inhibitors of DNA-PK, Targeting DNA Repair, and Beyond.* Frontiers in Pharmacology, 2013. **4**(5).
47. Collis, S.J., et al., *The life and death of DNA-PK.* Oncogene, 2005. **24**(6): p. 949-61.

48. Zhao, W., Y. Qiu, and D. Kong, *Class I phosphatidylinositol 3-kinase inhibitors for cancer therapy.* Acta Pharm Sin B, 2017. **7**(1): p. 27-37.
49. Mahadevan, D., et al., *Phase I pharmacokinetic and pharmacodynamic study of the pan-PI3K/mTORC vascular targeted pro-drug SF1126 in patients with advanced solid tumours and B-cell malignancies.* Eur J Cancer, 2012. **48**(18): p. 3319-27.
50. Yanai, M., et al., *DNA-PK Inhibition by NU7441 Enhances Chemosensitivity to Topoisomerase Inhibitor in Non-Small Cell Lung Carcinoma Cells by Blocking DNA Damage Repair.* Yonago Acta Med, 2017. **60**(1): p. 9-15.
51. Gianfaldoni, S., et al., *An Overview on Radiotherapy: From Its History to Its Current Applications in Dermatology.* Open Access Maced J Med Sci, 2017. **5**(4): p. 521-525.
52. Nickoloff, J.A., et al., *Translational research in radiation-induced DNA damage signaling and repair.* Translational Cancer Research, 2017: p. S875-S891.
53. Sishc, B.J. and A.J. Davis, *The Role of the Core Non-Homologous End Joining Factors in Carcinogenesis and Cancer.* Cancers (Basel), 2017. **9**(7).
54. Franco, S., et al., *DNA-PKcs and Artemis function in the end-joining phase of immunoglobulin heavy chain class switch recombination.* J Exp Med, 2008. **205**(3): p. 557-64.
55. Woodbine, L., et al., *An Artemis polymorphic variant reduces Artemis activity and confers cellular radiosensitivity.* DNA Repair (Amst), 2010. **9**(9): p. 1003-10.
56. Zhao, Y., et al., *Preclinical evaluation of a potent novel DNA-dependent protein kinase inhibitor NU7441.* Cancer Res, 2006. **66**(10): p. 5354-62.
57. Gavande, N.S., et al., *DNA repair targeted therapy: The past or future of cancer treatment?* Pharmacol Ther, 2016. **160**: p. 65-83.
58. Barker, H.E., et al., *CHK1 Inhibition Radiosensitizes Head and Neck Cancers to Paclitaxel-Based Chemoradiotherapy.* Mol Cancer Ther, 2016. **15**(9): p. 2042-54.
59. Tao, Y., et al., *Radiosensitization by Chir-124, a selective CHK1 inhibitor: effects of p53 and cell cycle checkpoints.* Cell Cycle, 2009. **8**(8): p. 1196-205.
60. Borst, G.R., et al., *Targeted radiosensitization by the Chk1 inhibitor SAR-020106.* Int J Radiat Oncol Biol Phys, 2013. **85**(4): p. 1110-8.
61. Jackson, J.R., et al., *An indolocarbazole inhibitor of human checkpoint kinase (Chk1) abrogates cell cycle arrest caused by DNA damage.* Cancer Res, 2000. **60**(3): p. 566-72.
62. Zhang, H., et al., *SIRT2 directs the replication stress response through CDK9 deacetylation.* Proc Natl Acad Sci U S A, 2013. **110**(33): p. 13546-51.
63. Head, P.E., et al., *Sirtuin 2 mutations in human cancers impair its function in genome maintenance.* J Biol Chem, 2017. **292**(24): p. 9919-9931.
64. Zhang, H., et al., *ATRIP Deacetylation by SIRT2 Drives ATR Checkpoint Activation by Promoting Binding to RPA-ssDNA.* Cell Rep, 2016. **14**(6): p. 1435-1447.
65. Daddacha, W., et al., *SAMHD1 Promotes DNA End Resection to Facilitate DNA Repair by Homologous Recombination.* Cell Rep, 2017. **20**(8): p. 1921-1935.
66. Hall, J.M., et al., *Linkage of early-onset familial breast cancer to chromosome 17q21.* Science, 1990. **250**(4988): p. 1684-9.
67. Miki, Y., et al., *A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1.* Science, 1994. **266**(5182): p. 66-71.
68. Smith, T.M., et al., *Complete genomic sequence and analysis of 117 kb of human DNA containing the gene BRCA1.* Genome Res, 1996. **6**(11): p. 1029-49.
69. Xu, C.F., et al., *Distinct transcription start sites generate two forms of BRCA1 mRNA.* Hum Mol Genet, 1995. **4**(12): p. 2259-64.
70. Xu, C.F., J.A. Chambers, and E. Solomon, *Complex regulation of the BRCA1 gene.* J Biol Chem, 1997. **272**(34): p. 20994-7.

71. Friedman, L.S., et al., *Novel inherited mutations and variable expressivity of BRCA1 alleles, including the founder mutation 185delAG in Ashkenazi Jewish families.* Am J Hum Genet, 1995. **57**(6): p. 1284-97.
72. Orban, T.I. and E. Olah, *Emerging roles of BRCA1 alternative splicing.* Mol Pathol, 2003. **56**(4): p. 191-7.
73. Orban, T.I. and E. Olah, *Expression profiles of BRCA1 splice variants in asynchronous and in G1/S synchronized tumor cell lines.* Biochem Biophys Res Commun, 2001. **280**(1): p. 32-8.
74. Maniccia, A.W., et al., *Mitochondrial localization, ELK-1 transcriptional regulation and growth inhibitory functions of BRCA1, BRCA1a, and BRCA1b proteins.* J Cell Physiol, 2009. **219**(3): p. 634-41.
75. Lu, Y., et al., *Ubiquitination and proteasome-mediated degradation of BRCA1 and BARD1 during steroidogenesis in human ovarian granulosa cells.* Mol Endocrinol, 2007. **21**(3): p. 651-63.
76. Meza, J.E., et al., *Mapping the functional domains of BRCA1. Interaction of the ring finger domains of BRCA1 and BARD1.* J Biol Chem, 1999. **274**(9): p. 5659-65.
77. Bienstock, R.J., et al., *Molecular modeling of the amino-terminal zinc ring domain of BRCA1.* Cancer Res, 1996. **56**(11): p. 2539-45.
78. Rodriguez, J.A. and B.R. Henderson, *Identification of a functional nuclear export sequence in BRCA1.* J Biol Chem, 2000. **275**(49): p. 38589-96.
79. Thompson, M.E., C.L. Robinson-Benion, and J.T. Holt, *An amino-terminal motif functions as a second nuclear export sequence in BRCA1.* J Biol Chem, 2005. **280**(23): p. 21854-7.
80. Paull, T.T., et al., *Direct DNA binding by Brca1.* Proc Natl Acad Sci U S A, 2001. **98**(11): p. 6086-91.
81. Chen, C.F., et al., *The nuclear localization sequences of the BRCA1 protein interact with the importin-alpha subunit of the nuclear transport signal receptor.* J Biol Chem, 1996. **271**(51): p. 32863-8.
82. Koonin, E.V., S.F. Altschul, and P. Bork, *BRCA1 protein products ... Functional motifs.* Nat Genet, 1996. **13**(3): p. 266-8.
83. Williams, R.S., R. Green, and J.N. Glover, *Crystal structure of the BRCT repeat region from the breast cancer-associated protein BRCA1.* Nat Struct Biol, 2001. **8**(10): p. 838-42.
84. Williams, R.S., et al., *Structural basis for phosphorylation-dependent signaling in the DNA-damage response.* Biochem Cell Biol, 2005. **83**(6): p. 721-7.
85. Leung, C.C. and J.N. Glover, *BRCT domains: easy as one, two, three.* Cell Cycle, 2011. **10**(15): p. 2461-70.
86. Manke, I.A., et al., *BRCT repeats as phosphopeptide-binding modules involved in protein targeting.* Science, 2003. **302**(5645): p. 636-9.
87. Kim, H., J. Huang, and J. Chen, *CCDC98 is a BRCA1-BRCT domain-binding protein involved in the DNA damage response.* Nat Struct Mol Biol, 2007. **14**(8): p. 710-5.
88. Barker, D.F., X. Liu, and E.R. Almeida, *The BRCA1 and 1A1.3B promoters are parallel elements of a genomic duplication at 17q21.* Genomics, 1996. **38**(2): p. 215-22.
89. Brown, M.A., et al., *The 5' end of the BRCA1 gene lies within a duplicated region of human chromosome 17q21.* Oncogene, 1996. **12**(12): p. 2507-13.
90. Xu, C.F., et al., *Isolation and characterisation of the NBR2 gene which lies head to head with the human BRCA1 gene.* Hum Mol Genet, 1997. **6**(7): p. 1057-62.
91. Thakur, S. and C.M. Croce, *Positive regulation of the BRCA1 promoter.* J Biol Chem, 1999. **274**(13): p. 8837-43.
92. Atlas, E., et al., *GA-binding protein alpha/beta is a critical regulator of the BRCA1 promoter.* Oncogene, 2000. **19**(15): p. 1933-40.
93. Mancini, D.N., et al., *CpG methylation within the 5' regulatory region of the BRCA1 gene is tumor specific and includes a putative CREB binding site.* Oncogene, 1998. **16**(9): p. 1161-9.

94. Atlas, E., M. Stramwasser, and C.R. Mueller, *A CREB site in the BRCA1 proximal promoter acts as a constitutive transcriptional element.* Oncogene, 2001. **20**(48): p. 7110-4.
95. Boccard, F. and P. Prentki, *Specific interaction of IHF with RIBs, a class of bacterial repetitive DNA elements located at the 3' end of transcription units.* EMBO J, 1993. **12**(13): p. 5019-27.
96. Wang, A., et al., *Regulation of BRCA1 expression by the Rb-E2F pathway.* J Biol Chem, 2000. **275**(6): p. 4532-6.
97. Bindra, R.S., et al., *Hypoxia-induced down-regulation of BRCA1 expression by E2Fs.* Cancer Res, 2005. **65**(24): p. 11597-604.
98. Bindra, R.S. and P.M. Glazer, *Basal repression of BRCA1 by multiple E2Fs and pocket proteins at adjacent E2F sites.* Cancer Biol Ther, 2006. **5**(10): p. 1400-7.
99. Oberley, M.J., D.R. Inman, and P.J. Farnham, *E2F6 negatively regulates BRCA1 in human cancer cells without methylation of histone H3 on lysine 9.* J Biol Chem, 2003. **278**(43): p. 42466-76.
100. Li, Y., et al., *Expression patterns of E2F transcription factors and their potential prognostic roles in breast cancer.* Oncol Lett, 2018. **15**(6): p. 9216-9230.
101. Kanakkanthara, A., et al., *ZC3H18 specifically binds and activates the BRCA1 promoter to facilitate homologous recombination in ovarian cancer.* Nat Commun, 2019. **10**(1): p. 4632.
102. Moureau, S., et al., *A role for the p53 tumour suppressor in regulating the balance between homologous recombination and non-homologous end joining.* Open Biol, 2016. **6**(9).
103. MacLachlan, T.K., et al., *Repression of BRCA1 through a feedback loop involving p53.* J Biol Chem, 2000. **275**(41): p. 31869-75.
104. Arizti, P., et al., *Tumor suppressor p53 is required to modulate BRCA1 expression.* Mol Cell Biol, 2000. **20**(20): p. 7450-9.
105. Rauch, T., et al., *53BP1 is a positive regulator of the BRCA1 promoter.* Cell Cycle, 2005. **4**(8): p. 1078-83.
106. Baldassarre, G., et al., *Negative regulation of BRCA1 gene expression by HMGA1 proteins accounts for the reduced BRCA1 protein levels in sporadic breast carcinoma.* Mol Cell Biol, 2003. **23**(7): p. 2225-38.
107. Baker, L.A., H. Holliday, and A. Swarbrick, *ID4 controls luminal lineage commitment in normal mammary epithelium and inhibits BRCA1 function in basal-like breast cancer.* Endocr Relat Cancer, 2016. **23**(9): p. R381-92.
108. Beger, C., et al., *Identification of Id4 as a regulator of BRCA1 expression by using a ribozyme-library-based inverse genomics approach.* Proc Natl Acad Sci U S A, 2001. **98**(1): p. 130-5.
109. Chen, Y., et al., *c-Myc activates BRCA1 gene expression through distal promoter elements in breast cancer cells.* BMC Cancer, 2011. **11**: p. 246.
110. De Siervi, A., et al., *Transcriptional autoregulation by BRCA1.* Cancer Res, 2010. **70**(2): p. 532-42.
111. Jeffy, B.D., et al., *An estrogen receptor-alpha/p300 complex activates the BRCA-1 promoter at an AP-1 site that binds Jun/Fos transcription factors: repressive effects of p53 on BRCA-1 transcription.* Neoplasia, 2005. **7**(9): p. 873-82.
112. Andres, J.L., et al., *Regulation of BRCA1 and BRCA2 expression in human breast cancer cells by DNA-damaging agents.* Oncogene, 1998. **16**(17): p. 2229-41.
113. Hockings, J.K., et al., *The ligand status of the aromatic hydrocarbon receptor modulates transcriptional activation of BRCA-1 promoter by estrogen.* Cancer Res, 2006. **66**(4): p. 2224-32.
114. DiNardo, D.N., et al., *Functional analysis of CpG methylation in the BRCA1 promoter region.* Oncogene, 2001. **20**(38): p. 5331-40.
115. Butcher, D.T., et al., *DNA binding sites for putative methylation boundaries in the unmethylated region of the BRCA1 promoter.* Int J Cancer, 2004. **111**(5): p. 669-78.
116. Xu, J., et al., *CpG island methylation affects accessibility of the proximal BRCA1 promoter to transcription factors.* Breast Cancer Res Treat, 2010. **120**(3): p. 593-601.

117. Wardrop, S.L., M.A. Brown, and I. kConFab, *Identification of two evolutionarily conserved and functional regulatory elements in intron 2 of the human BRCA1 gene.* Genomics, 2005. **86**(3): p. 316-28.
118. Choudhury, A.D., H. Xu, and R. Baer, *Ubiquitination and proteasomal degradation of the BRCA1 tumor suppressor is regulated during cell cycle progression.* J Biol Chem, 2004. **279**(32): p. 33909-18.
119. Blagosklonny, M.V., et al., *Regulation of BRCA1 by protein degradation.* Oncogene, 1999. **18**(47): p. 6460-8.
120. Chen, Y., et al., *BRCA1 is a 220-kDa nuclear phosphoprotein that is expressed and phosphorylated in a cell cycle-dependent manner.* Cancer Res, 1996. **56**(14): p. 3168-72.
121. Ruffner, H. and I.M. Verma, *BRCA1 is a cell cycle-regulated nuclear phosphoprotein.* Proc Natl Acad Sci U S A, 1997. **94**(14): p. 7138-43.
122. Gudas, J.M., et al., *Cell cycle regulation of BRCA1 messenger RNA in human breast epithelial cells.* Cell Growth Differ, 1996. **7**(6): p. 717-23.
123. Vaughn, J.P., et al., *BRCA1 expression is induced before DNA synthesis in both normal and tumor-derived breast cells.* Cell Growth Differ, 1996. **7**(6): p. 711-5.
124. Wu, W., et al., *HERC2 is an E3 ligase that targets BRCA1 for degradation.* Cancer Res, 2010. **70**(15): p. 6384-92.
125. Lu, Y., et al., *The F-box protein FBXO44 mediates BRCA1 ubiquitination and degradation.* J Biol Chem, 2012. **287**(49): p. 41014-22.
126. Wang, X., et al., *HUWE1 interacts with BRCA1 and promotes its degradation in the ubiquitin-proteasome pathway.* Biochem Biophys Res Commun, 2014. **444**(4): p. 549-54.
127. Kim, S., et al., *Regulating BRCA1 protein stability by cathepsin S-mediated ubiquitin degradation.* Cell Death Differ, 2019. **26**(5): p. 812-825.
128. Ueki, T., et al., *Ubiquitination and downregulation of BRCA1 by ubiquitin-conjugating enzyme E2T overexpression in human breast cancer cells.* Cancer Res, 2009. **69**(22): p. 8752-60.
129. Lu, Q., et al., *USP9X stabilizes BRCA1 and confers resistance to DNA-damaging agents in human cancer cells.* Cancer Med, 2019. **8**(15): p. 6730-6740.
130. Hsu, L.C. and R.L. White, *BRCA1 is associated with the centrosome during mitosis.* Proc Natl Acad Sci U S A, 1998. **95**(22): p. 12983-8.
131. Hsu, L.C., T.P. Doan, and R.L. White, *Identification of a gamma-tubulin-binding domain in BRCA1.* Cancer Res, 2001. **61**(21): p. 7713-8.
132. Coene, E.D., et al., *Phosphorylated BRCA1 is predominantly located in the nucleus and mitochondria.* Mol Biol Cell, 2005. **16**(2): p. 997-1010.
133. Laulier, C., et al., *Bcl-2 inhibits nuclear homologous recombination by localizing BRCA1 to the endomembranes.* Cancer Res, 2011. **71**(10): p. 3590-602.
134. Rodriguez, J.A., W.W. Au, and B.R. Henderson, *Cytoplasmic mislocalization of BRCA1 caused by cancer-associated mutations in the BRCT domain.* Exp Cell Res, 2004. **293**(1): p. 14-21.
135. Elstrodt, F., et al., *BRCA1 mutation analysis of 41 human breast cancer cell lines reveals three new deleterious mutants.* Cancer Res, 2006. **66**(1): p. 41-5.
136. Gough, C.A., T. Gojobori, and T. Imanishi, *Cancer-related mutations in BRCA1-BRCT cause long-range structural changes in protein-protein binding sites: a molecular dynamics study.* Proteins, 2007. **66**(1): p. 69-86.
137. Li, S., et al., *Identification of a novel cytoplasmic protein that specifically binds to nuclear localization signal motifs.* J Biol Chem, 1998. **273**(11): p. 6183-9.
138. Fulcher, A.J., et al., *The BRCA-1 binding protein BRAP2 is a novel, negative regulator of nuclear import of viral proteins, dependent on phosphorylation flanking the nuclear localization signal.* FASEB J, 2010. **24**(5): p. 1454-66.

139. Plo, I., et al., *AKT1 inhibits homologous recombination by inducing cytoplasmic retention of BRCA1 and RAD51.* Cancer Res, 2008. **68**(22): p. 9404-12.
140. Qin, Y., et al., *Ubc9 mediates nuclear localization and growth suppression of BRCA1 and BRCA1a proteins.* J Cell Physiol, 2011. **226**(12): p. 3355-67.
141. Paull, T.T. and M. Gellert, *The 3' to 5' exonuclease activity of Mre 11 facilitates repair of DNA double-strand breaks.* Mol Cell, 1998. **1**(7): p. 969-79.
142. Sartori, A.A., et al., *Human CtIP promotes DNA end resection.* Nature, 2007. **450**(7169): p. 509-14.
143. Zhu, Z., et al., *Sgs1 helicase and two nucleases Dna2 and Exo1 resect DNA double-strand break ends.* Cell, 2008. **134**(6): p. 981-94.
144. Bianco, P.R., R.B. Tracy, and S.C. Kowalczykowski, *DNA strand exchange proteins: a biochemical and physical comparison.* Front Biosci, 1998. **3**: p. D570-603.
145. Renkawitz, J., C.A. Lademann, and S. Jentsch, *Mechanisms and principles of homology search during recombination.* Nat Rev Mol Cell Biol, 2014. **15**(6): p. 369-83.
146. Ranjha, L., S.M. Howard, and P. Cejka, *Main steps in DNA double-strand break repair: an introduction to homologous recombination and related processes.* Chromosoma, 2018. **127**(2): p. 187-214.
147. Maloisel, L., F. Fabre, and S. Gangloff, *DNA polymerase delta is preferentially recruited during homologous recombination to promote heteroduplex DNA extension.* Mol Cell Biol, 2008. **28**(4): p. 1373-82.
148. McVey, M., et al., *Eukaryotic DNA Polymerases in Homologous Recombination.* Annu Rev Genet, 2016. **50**: p. 393-421.
149. Bunting, S.F., et al., *53BP1 inhibits homologous recombination in Brca1-deficient cells by blocking resection of DNA breaks.* Cell, 2010. **141**(2): p. 243-54.
150. Chapman, J.R., et al., *BRCA1-associated exclusion of 53BP1 from DNA damage sites underlies temporal control of DNA repair.* J Cell Sci, 2012. **125**(Pt 15): p. 3529-34.
151. Wong, A.K., et al., *Characterization of a carboxy-terminal BRCA1 interacting protein.* Oncogene, 1998. **17**(18): p. 2279-85.
152. Yu, X., et al., *The C-terminal (BRCT) domains of BRCA1 interact in vivo with CtIP, a protein implicated in the CtBP pathway of transcriptional repression.* J Biol Chem, 1998. **273**(39): p. 25388-92.
153. Yu, X. and J. Chen, *DNA damage-induced cell cycle checkpoint control requires CtIP, a phosphorylation-dependent binding partner of BRCA1 C-terminal domains.* Mol Cell Biol, 2004. **24**(21): p. 9478-86.
154. Reczek, C.R., et al., *The interaction between CtIP and BRCA1 is not essential for resection-mediated DNA repair or tumor suppression.* J Cell Biol, 2013. **201**(5): p. 693-707.
155. Cruz-Garcia, A., A. Lopez-Saavedra, and P. Huertas, *BRCA1 accelerates CtIP-mediated DNA-end resection.* Cell Rep, 2014. **9**(2): p. 451-9.
156. Coleman, K.A. and R.A. Greenberg, *The BRCA1-RAP80 complex regulates DNA repair mechanism utilization by restricting end resection.* J Biol Chem, 2011. **286**(15): p. 13669-80.
157. Savage, K.I. and D.P. Harkin, *BRCA1, a 'complex' protein involved in the maintenance of genomic stability.* FEBS J, 2015. **282**(4): p. 630-46.
158. Hu, Y., et al., *RAP80-directed tuning of BRCA1 homologous recombination function at ionizing radiation-induced nuclear foci.* Genes Dev, 2011. **25**(7): p. 685-700.
159. Xia, B., et al., *Control of BRCA2 cellular and clinical functions by a nuclear partner, PALB2.* Mol Cell, 2006. **22**(6): p. 719-29.
160. Sy, S.M., M.S. Huen, and J. Chen, *PALB2 is an integral component of the BRCA complex required for homologous recombination repair.* Proc Natl Acad Sci U S A, 2009. **106**(17): p. 7155-60.

161. Zhang, F., et al., *PALB2 functionally connects the breast cancer susceptibility proteins BRCA1 and BRCA2.* Mol Cancer Res, 2009. **7**(7): p. 1110-8.
162. Zhang, F., et al., *MDC1 and RNF8 function in a pathway that directs BRCA1-dependent localization of PALB2 required for homologous recombination.* J Cell Sci, 2012. **125**(Pt 24): p. 6049-57.
163. Yan, J. and A.M. Jetten, *RAP80 and RNF8, key players in the recruitment of repair proteins to DNA damage sites.* Cancer Lett, 2008. **271**(2): p. 179-90.
164. Sobhian, B., et al., *RAP80 targets BRCA1 to specific ubiquitin structures at DNA damage sites.* Science, 2007. **316**(5828): p. 1198-202.
165. Kolas, N.K., et al., *Orchestration of the DNA-damage response by the RNF8 ubiquitin ligase.* Science, 2007. **318**(5856): p. 1637-40.
166. Mattiroli, F., et al., *RNF168 ubiquitinates K13-15 on H2A/H2AX to drive DNA damage signaling.* Cell, 2012. **150**(6): p. 1182-95.
167. Campbell, S.J., et al., *Molecular insights into the function of RING finger (RNF)-containing proteins hRNF8 and hRNF168 in Ubc13/Mms2-dependent ubiquitylation.* J Biol Chem, 2012. **287**(28): p. 23900-10.
168. Strauss, C., et al., *MDC1 is ubiquitylated on its tandem BRCT domain and directly binds RAP80 in a UBC13-dependent manner.* DNA Repair (Amst), 2011. **10**(8): p. 806-14.
169. Xu, B., S. Kim, and M.B. Kastan, *Involvement of Brca1 in S-phase and G(2)-phase checkpoints after ionizing irradiation.* Mol Cell Biol, 2001. **21**(10): p. 3445-50.
170. Xu, B., et al., *Phosphorylation of serine 1387 in Brca1 is specifically required for the Atm-mediated S-phase checkpoint after ionizing irradiation.* Cancer Res, 2002. **62**(16): p. 4588-91.
171. Xie, J., et al., *FANCJ/BACH1 acetylation at lysine 1249 regulates the DNA damage response.* PLoS Genet, 2012. **8**(7): p. e1002786.
172. Yarden, R.I., et al., *BRCA1 regulates the G2/M checkpoint by activating Chk1 kinase upon DNA damage.* Nat Genet, 2002. **30**(3): p. 285-9.
173. Hutchins, J.R. and P.R. Clarke, *Many fingers on the mitotic trigger: post-translational regulation of the Cdc25C phosphatase.* Cell Cycle, 2004. **3**(1): p. 41-5.
174. Muslin, A.J. and H. Xing, *14-3-3 proteins: regulation of subcellular localization by molecular interference.* Cell Signal, 2000. **12**(11-12): p. 703-9.
175. Wang, B., et al., *Abraxas and RAP80 form a BRCA1 protein complex required for the DNA damage response.* Science, 2007. **316**(5828): p. 1194-8.
176. Schlacher, K., et al., *Double-strand break repair-independent role for BRCA2 in blocking stalled replication fork degradation by MRE11.* Cell, 2011. **145**(4): p. 529-42.
177. Schlacher, K., H. Wu, and M. Jasin, *A distinct replication fork protection pathway connects Fanconi anemia tumor suppressors to RAD51-BRCA1/2.* Cancer Cell, 2012. **22**(1): p. 106-16.
178. Ying, S., F.C. Hamdy, and T. Helleday, *Mre11-dependent degradation of stalled DNA replication forks is prevented by BRCA2 and PARP1.* Cancer Res, 2012. **72**(11): p. 2814-21.
179. Xu, Y., et al., *53BP1 and BRCA1 control pathway choice for stalled replication restart.* Elife, 2017. **6**.
180. Gong, Z., et al., *BACH1/FANCJ acts with TopBP1 and participates early in DNA replication checkpoint control.* Mol Cell, 2010. **37**(3): p. 438-46.
181. Saha, J. and A.J. Davis, *Unsolved mystery: the role of BRCA1 in DNA end-joining.* J Radiat Res, 2016. **57 Suppl 1**: p. i18-i24.
182. Escribano-Diaz, C., et al., *A cell cycle-dependent regulatory circuit composed of 53BP1-RIF1 and BRCA1-CtIP controls DNA repair pathway choice.* Mol Cell, 2013. **49**(5): p. 872-83.
183. Chapman, J.R., et al., *RIF1 is essential for 53BP1-dependent nonhomologous end joining and suppression of DNA double-strand break resection.* Mol Cell, 2013. **49**(5): p. 858-71.

184. Zhong, Q., et al., *BRCA1 facilitates microhomology-mediated end joining of DNA double strand breaks.* J Biol Chem, 2002. **277**(32): p. 28641-7.
185. Zhang, J., et al., *Chk2 phosphorylation of BRCA1 regulates DNA double-strand break repair.* Mol Cell Biol, 2004. **24**(2): p. 708-18.
186. Zhuang, J., et al., *Checkpoint kinase 2-mediated phosphorylation of BRCA1 regulates the fidelity of nonhomologous end-joining.* Cancer Res, 2006. **66**(3): p. 1401-8.
187. Wang, H.C., et al., *Ataxia telangiectasia mutated and checkpoint kinase 2 regulate BRCA1 to promote the fidelity of DNA end-joining.* Cancer Res, 2006. **66**(3): p. 1391-400.
188. Wei, L., et al., *Rapid recruitment of BRCA1 to DNA double-strand breaks is dependent on its association with Ku80.* Mol Cell Biol, 2008. **28**(24): p. 7380-93.
189. Thompson, E.G., H. Fares, and K. Dixon, *BRCA1 requirement for the fidelity of plasmid DNA double-strand break repair in cultured breast epithelial cells.* Environ Mol Mutagen, 2012. **53**(1): p. 32-43.
190. Dohrn, L., et al., *BRCA1-mediated repression of mutagenic end-joining of DNA double-strand breaks requires complex formation with BACH1.* Biochem J, 2012. **441**(3): p. 919-26.
191. Wang, Y., et al., *BASC, a super complex of BRCA1-associated proteins involved in the recognition and repair of aberrant DNA structures.* Genes Dev, 2000. **14**(8): p. 927-39.
192. Kleiman, F.E., et al., *BRCA1/BARD1 inhibition of mRNA 3' processing involves targeted degradation of RNA polymerase II.* Genes Dev, 2005. **19**(10): p. 1227-37.
193. Starita, L.M., et al., *BRCA1/BARD1 ubiquitinate phosphorylated RNA polymerase II.* J Biol Chem, 2005. **280**(26): p. 24498-505.
194. Hartman, A.R. and J.M. Ford, *BRCA1 induces DNA damage recognition factors and enhances nucleotide excision repair.* Nat Genet, 2002. **32**(1): p. 180-4.
195. Takimoto, R., et al., *BRCA1 transcriptionally regulates damaged DNA binding protein (DDB2) in the DNA repair response following UV-irradiation.* Cancer Biol Ther, 2002. **1**(2): p. 177-86.
196. Saha, T., et al., *Transcriptional regulation of the base excision repair pathway by BRCA1.* J Biol Chem, 2010. **285**(25): p. 19092-105.
197. Fabbro, M., et al., *BRCA1-BARD1 complexes are required for p53Ser-15 phosphorylation and a G1/S arrest following ionizing radiation-induced DNA damage.* J Biol Chem, 2004. **279**(30): p. 31251-8.
198. MacLachlan, T.K., R. Takimoto, and W.S. El-Deiry, *BRCA1 directs a selective p53-dependent transcriptional response towards growth arrest and DNA repair targets.* Mol Cell Biol, 2002. **22**(12): p. 4280-92.
199. Andrews, H.N., et al., *BRCA1 regulates the interferon gamma-mediated apoptotic response.* J Biol Chem, 2002. **277**(29): p. 26225-32.
200. Jiang, J., et al., *p53-dependent BRCA1 nuclear export controls cellular susceptibility to DNA damage.* Cancer Res, 2011. **71**(16): p. 5546-57.
201. Heldring, N., et al., *Estrogen receptors: how do they signal and what are their targets.* Physiol Rev, 2007. **87**(3): p. 905-31.
202. Zheng, L., et al., *BRCA1 mediates ligand-independent transcriptional repression of the estrogen receptor.* Proc Natl Acad Sci U S A, 2001. **98**(17): p. 9587-92.
203. Fan, S., et al., *Role of direct interaction in BRCA1 inhibition of estrogen receptor activity.* Oncogene, 2001. **20**(1): p. 77-87.
204. Kawai, H., et al., *Direct interaction between BRCA1 and the estrogen receptor regulates vascular endothelial growth factor (VEGF) transcription and secretion in breast cancer cells.* Oncogene, 2002. **21**(50): p. 7730-9.
205. Aiyar, S.E., et al., *Attenuation of estrogen receptor alpha-mediated transcription through estrogen-stimulated recruitment of a negative elongation factor.* Genes Dev, 2004. **18**(17): p. 2134-46.

206. Chen, G.C., et al., *Rb-associated protein 46 (RbAp46) inhibits transcriptional transactivation mediated by BRCA1.* Biochem Biophys Res Commun, 2001. **284**(2): p. 507-14.
207. Yarden, R.I. and L.C. Brody, *BRCA1 interacts with components of the histone deacetylase complex.* Proc Natl Acad Sci U S A, 1999. **96**(9): p. 4983-8.
208. Harte, M.T., et al., *BRD7, a subunit of SWI/SNF complexes, binds directly to BRCA1 and regulates BRCA1-dependent transcription.* Cancer Res, 2010. **70**(6): p. 2538-47.
209. Hosey, A.M., et al., *Molecular basis for estrogen receptor alpha deficiency in BRCA1-linked breast cancer.* J Natl Cancer Inst, 2007. **99**(22): p. 1683-94.
210. Ma, Y., et al., *BRCA1 regulates acetylation and ubiquitination of estrogen receptor-alpha.* Mol Endocrinol, 2010. **24**(1): p. 76-90.
211. Harkin, D.P., et al., *Induction of GADD45 and JNK/SAPK-dependent apoptosis following inducible expression of BRCA1.* Cell, 1999. **97**(5): p. 575-86.
212. Li, S., et al., *Binding of CtIP to the BRCT repeats of BRCA1 involved in the transcription regulation of p21 is disrupted upon DNA damage.* J Biol Chem, 1999. **274**(16): p. 11334-8.
213. Furuta, S., et al., *Removal of BRCA1/CtIP/ZBRK1 repressor complex on ANG1 promoter leads to accelerated mammary tumor growth contributed by prominent vasculature.* Cancer Cell, 2006. **10**(1): p. 13-24.
214. Zheng, L., et al., *Sequence-specific transcriptional corepressor function for BRCA1 through a novel zinc finger protein, ZBRK1.* Mol Cell, 2000. **6**(4): p. 757-68.
215. Kennedy, R.D., et al., *BRCA1 and c-Myc associate to transcriptionally repress psoriasin, a DNA damage-inducible gene.* Cancer Res, 2005. **65**(22): p. 10265-72.
216. Li, H., T.H. Lee, and H. Avraham, *A novel tricomplex of BRCA1, Nmi, and c-Myc inhibits c-Myc-induced human telomerase reverse transcriptase gene (hTERT) promoter activity in breast cancer.* J Biol Chem, 2002. **277**(23): p. 20965-73.
217. Savage, K.I., et al., *Identification of a BRCA1-mRNA splicing complex required for efficient DNA repair and maintenance of genomic stability.* Mol Cell, 2014. **54**(3): p. 445-59.
218. Aprelikova, O.N., et al., *BRCA1-associated growth arrest is RB-dependent.* Proc Natl Acad Sci U S A, 1999. **96**(21): p. 11866-71.
219. Shao, N., et al., *Induction of apoptosis by the tumor suppressor protein BRCA1.* Oncogene, 1996. **13**(1): p. 1-7.
220. Martin, S.A. and T. Ouchi, *BRCA1 phosphorylation regulates caspase-3 activation in UV-induced apoptosis.* Cancer Res, 2005. **65**(23): p. 10657-62.
221. Husain, A., et al., *BRCA1 up-regulation is associated with repair-mediated resistance to cis-diamminedichloroplatinum(II).* Cancer Res, 1998. **58**(6): p. 1120-3.
222. Harte, M.T., et al., *NF-kappaB is a critical mediator of BRCA1-induced chemoresistance.* Oncogene, 2014. **33**(6): p. 713-723.
223. Xiong, J., et al., *BRCA1 inhibition of telomerase activity in cultured cells.* Mol Cell Biol, 2003. **23**(23): p. 8668-90.
224. Ballal, R.D., et al., *BRCA1 localization to the telomere and its loss from the telomere in response to DNA damage.* J Biol Chem, 2009. **284**(52): p. 36083-98.
225. Wu, L.C., et al., *Identification of a RING protein that can interact in vivo with the BRCA1 gene product.* Nat Genet, 1996. **14**(4): p. 430-40.
226. Fox, D., 3rd, et al., *Crystal structure of the BARD1 ankyrin repeat domain and its functional consequences.* J Biol Chem, 2008. **283**(30): p. 21179-86.
227. Edwards, R.A., et al., *The BARD1 C-terminal domain structure and interactions with polyadenylation factor CstF-50.* Biochemistry, 2008. **47**(44): p. 11446-56.
228. Birrane, G., et al., *Crystal structure of the BARD1 BRCT domains.* Biochemistry, 2007. **46**(26): p. 7706-12.

229. Rodriguez, J.A., et al., *Nuclear-cytoplasmic shuttling of BARD1 contributes to its proapoptotic activity and is regulated by dimerization with BRCA1.* Oncogene, 2004. **23**(10): p. 1809-20.
230. Schuchner, S., et al., *Nuclear targeting and cell cycle regulatory function of human BARD1.* J Biol Chem, 2005. **280**(10): p. 8855-61.
231. Irminger-Finger, I., M. Ratajska, and M. Pilyugin, *New concepts on BARD1: Regulator of BRCA pathways and beyond.* Int J Biochem Cell Biol, 2016. **72**: p. 1-17.
232. Ren, B., et al., *E2F integrates cell cycle progression with DNA repair, replication, and G(2)/M checkpoints.* Genes Dev, 2002. **16**(2): p. 245-56.
233. Creekmore, A.L., et al., *Estrogen receptor alpha regulates expression of the breast cancer 1 associated ring domain 1 (BARD1) gene through intronic DNA sequence.* Mol Cell Endocrinol, 2007. **267**(1-2): p. 106-15.
234. Pilyugin, M. and I. Irminger-Finger, *Long non-coding RNA and microRNAs might act in regulating the expression of BARD1 mRNAs.* Int J Biochem Cell Biol, 2014. **54**: p. 356-67.
235. Choudhury, A.D., et al., *Hyperphosphorylation of the BARD1 tumor suppressor in mitotic cells.* J Biol Chem, 2005. **280**(26): p. 24669-79.
236. Hayami, R., et al., *Down-regulation of BRCA1-BARD1 ubiquitin ligase by CDK2.* Cancer Res, 2005. **65**(1): p. 6-10.
237. Song, L. and M. Rape, *Regulated degradation of spindle assembly factors by the anaphase-promoting complex.* Mol Cell, 2010. **38**(3): p. 369-82.
238. Gautier, F., et al., *Identification of an apoptotic cleavage product of BARD1 as an autoantigen: a potential factor in the antitumoral response mediated by apoptotic bodies.* Cancer Res, 2000. **60**(24): p. 6895-900.
239. Irminger-Finger, I., et al., *In vitro repression of Brca1-associated RING domain gene, Bard1, induces phenotypic changes in mammary epithelial cells.* J Cell Biol, 1998. **143**(5): p. 1329-39.
240. Feki, A., et al., *BARD1 expression during spermatogenesis is associated with apoptosis and hormonally regulated.* Biol Reprod, 2004. **71**(5): p. 1614-24.
241. Irminger-Finger, I., et al., *Identification of BARD1 as mediator between proapoptotic stress and p53-dependent apoptosis.* Mol Cell, 2001. **8**(6): p. 1255-66.
242. Jefford, C.E., et al., *Nuclear-cytoplasmic translocation of BARD1 is linked to its apoptotic activity.* Oncogene, 2004. **23**(20): p. 3509-20.
243. Feki, A., et al., *BARD1 induces apoptosis by catalysing phosphorylation of p53 by DNA-damage response kinase.* Oncogene, 2005. **24**(23): p. 3726-36.
244. Ryser, S., et al., *Distinct roles of BARD1 isoforms in mitosis: full-length BARD1 mediates Aurora B degradation, cancer-associated BARD1beta scaffolds Aurora B and BRCA2.* Cancer Res, 2009. **69**(3): p. 1125-34.
245. Kleiman, F.E. and J.L. Manley, *Functional interaction of BRCA1-associated BARD1 with polyadenylation factor CstF-50.* Science, 1999. **285**(5433): p. 1576-9.
246. Kleiman, F.E. and J.L. Manley, *The BARD1-CstF-50 interaction links mRNA 3' end formation to DNA damage and tumor suppression.* Cell, 2001. **104**(5): p. 743-53.
247. Nazeer, F.I., et al., *p53 inhibits mRNA 3' processing through its interaction with the CstF/BARD1 complex.* Oncogene, 2011. **30**(27): p. 3073-83.
248. Yim, E.K., et al., *Novel interaction between HPV E6 and BARD1 (BRCA1-associated ring domain 1) and its biologic roles.* DNA Cell Biol, 2007. **26**(10): p. 753-61.
249. Spahn, L., et al., *Interaction of the EWS NH2 terminus with BARD1 links the Ewing's sarcoma gene to a common tumor suppressor pathway.* Cancer Res, 2002. **62**(16): p. 4583-7.
250. Dechend, R., et al., *The Bcl-3 oncoprotein acts as a bridging factor between NF-kappaB/Rel and nuclear co-regulators.* Oncogene, 1999. **18**(22): p. 3316-23.

251. Li, M. and X. Yu, *Function of BRCA1 in the DNA damage response is mediated by ADP-ribosylation.* Cancer Cell, 2013. **23**(5): p. 693-704.
252. Joukov, V., et al., *Functional communication between endogenous BRCA1 and its partner, BARD1, during Xenopus laevis development.* Proc Natl Acad Sci U S A, 2001. **98**(21): p. 12078-83.
253. Hashizume, R., et al., *The RING heterodimer BRCA1-BARD1 is a ubiquitin ligase inactivated by a breast cancer-derived mutation.* J Biol Chem, 2001. **276**(18): p. 14537-40.
254. Brzovic, P.S., et al., *Binding and recognition in the assembly of an active BRCA1/BARD1 ubiquitin-ligase complex.* Proc Natl Acad Sci U S A, 2003. **100**(10): p. 5646-51.
255. Fabbro, M., et al., *BARD1 induces BRCA1 intranuclear foci formation by increasing RING-dependent BRCA1 nuclear import and inhibiting BRCA1 nuclear export.* J Biol Chem, 2002. **277**(24): p. 21315-24.
256. Brzovic, P.S., et al., *BRCA1 RING domain cancer-predisposing mutations. Structural consequences and effects on protein-protein interactions.* J Biol Chem, 2001. **276**(44): p. 41399-406.
257. Henderson, B.R., *Regulation of BRCA1, BRCA2 and BARD1 intracellular trafficking.* Bioessays, 2005. **27**(9): p. 884-93.
258. Brzovic, P.S., et al., *Structure of a BRCA1-BARD1 heterodimeric RING-RING complex.* Nat Struct Biol, 2001. **8**(10): p. 833-7.
259. Drost, R., et al., *BRCA1 RING function is essential for tumor suppression but dispensable for therapy resistance.* Cancer Cell, 2011. **20**(6): p. 797-809.
260. Mallery, D.L., C.J. Vandenberg, and K. Hiom, *Activation of the E3 ligase function of the BRCA1/BARD1 complex by polyubiquitin chains.* EMBO J, 2002. **21**(24): p. 6755-62.
261. Nishikawa, H., et al., *BRCA1-associated protein 1 interferes with BRCA1/BARD1 RING heterodimer activity.* Cancer Res, 2009. **69**(1): p. 111-9.
262. Stark, J.M., et al., *Genetic steps of mammalian homologous repair with distinct mutagenic consequences.* Mol Cell Biol, 2004. **24**(21): p. 9305-16.
263. Reid, L.J., et al., *E3 ligase activity of BRCA1 is not essential for mammalian cell viability or homology-directed repair of double-strand DNA breaks.* Proc Natl Acad Sci U S A, 2008. **105**(52): p. 20876-81.
264. Chen, A., et al., *Autoubiquitination of the BRCA1*BARD1 RING ubiquitin ligase.* J Biol Chem, 2002. **277**(24): p. 22085-92.
265. Xia, Y., et al., *Enhancement of BRCA1 E3 ubiquitin ligase activity through direct interaction with the BARD1 protein.* J Biol Chem, 2003. **278**(7): p. 5255-63.
266. Wu-Baer, F., et al., *The BRCA1/BARD1 heterodimer assembles polyubiquitin chains through an unconventional linkage involving lysine residue K6 of ubiquitin.* J Biol Chem, 2003. **278**(37): p. 34743-6.
267. Sankaran, S., et al., *Identification of domains of BRCA1 critical for the ubiquitin-dependent inhibition of centrosome function.* Cancer Res, 2006. **66**(8): p. 4100-7.
268. Shinagawa, H., Y. Miki, and K. Yoshida, *BRCA1-mediated ubiquitination inhibits topoisomerase II alpha activity in response to oxidative stress.* Antioxid Redox Signal, 2008. **10**(5): p. 939-49.
269. Sordet, O., et al., *Hyperphosphorylation of RNA polymerase II in response to topoisomerase I cleavage complexes and its association with transcription- and BRCA1-dependent degradation of topoisomerase I.* J Mol Biol, 2008. **381**(3): p. 540-9.
270. Joukov, V., et al., *The BRCA1/BARD1 heterodimer modulates ran-dependent mitotic spindle assembly.* Cell, 2006. **127**(3): p. 539-52.
271. Starita, L.M., et al., *BRCA1-dependent ubiquitination of gamma-tubulin regulates centrosome number.* Mol Cell Biol, 2004. **24**(19): p. 8457-66.

272. Dizin, E. and I. Irminger-Finger, *Negative feedback loop of BRCA1-BARD1 ubiquitin ligase on estrogen receptor alpha stability and activity antagonized by cancer-associated isoform of BARD1.* Int J Biochem Cell Biol, 2010. **42**(5): p. 693-700.
273. Calvo, V. and M. Beato, *BRCA1 counteracts progesterone action by ubiquitination leading to progesterone receptor degradation and epigenetic silencing of target promoters.* Cancer Res, 2011. **71**(9): p. 3422-31.
274. Sato, K., et al., *A DNA-damage selective role for BRCA1 E3 ligase in claspin ubiquitylation, CHK1 activation, and DNA repair.* Curr Biol, 2012. **22**(18): p. 1659-66.
275. Densham, R.M. and J.R. Morris, *The BRCA1 Ubiquitin ligase function sets a new trend for remodelling in DNA repair.* Nucleus, 2017. **8**(2): p. 116-125.
276. Thakar, A., J. Parvin, and J. Zlatanova, *BRCA1/BARD1 E3 ubiquitin ligase can modify histones H2A and H2B in the nucleosome particle.* J Biomol Struct Dyn, 2010. **27**(4): p. 399-406.
277. Densham, R.M., et al., *Human BRCA1-BARD1 ubiquitin ligase activity counteracts chromatin barriers to DNA resection.* Nat Struct Mol Biol, 2016. **23**(7): p. 647-55.
278. Kalb, R., et al., *BRCA1 is a histone-H2A-specific ubiquitin ligase.* Cell Rep, 2014. **8**(4): p. 999-1005.
279. Zhu, Q., et al., *BRCA1 tumour suppression occurs via heterochromatin-mediated silencing.* Nature, 2011. **477**(7363): p. 179-84.
280. Gowen, L.C., et al., *Brca1 deficiency results in early embryonic lethality characterized by neuroepithelial abnormalities.* Nat Genet, 1996. **12**(2): p. 191-4.
281. Shakya, R., et al., *The basal-like mammary carcinomas induced by Brca1 or Bard1 inactivation implicate the BRCA1/BARD1 heterodimer in tumor suppression.* Proc Natl Acad Sci U S A, 2008. **105**(19): p. 7040-5.
282. McCarthy, E.E., et al., *Loss of Bard1, the heterodimeric partner of the Brca1 tumor suppressor, results in early embryonic lethality and chromosomal instability.* Mol Cell Biol, 2003. **23**(14): p. 5056-63.
283. Broca, P., *Traité des tumeurs*. 1866.
284. Godet, I. and D.M. Gilkes, *BRCA1 and BRCA2 mutations and treatment strategies for breast cancer.* Integr Cancer Sci Ther, 2017. **4**(1).
285. Kuchenbaecker, K.B., et al., *Risks of Breast, Ovarian, and Contralateral Breast Cancer for BRCA1 and BRCA2 Mutation Carriers.* JAMA, 2017. **317**(23): p. 2402-2416.
286. Clark, S.L., et al., *Structure-Function Of The Tumor Suppressor BRCA1.* Comput Struct Biotechnol J, 2012. **1**(1).
287. Ruffner, H., et al., *Cancer-predisposing mutations within the RING domain of BRCA1: loss of ubiquitin protein ligase activity and protection from radiation hypersensitivity.* Proc Natl Acad Sci U S A, 2001. **98**(9): p. 5134-9.
288. Santivasi, W.L., et al., *Association between cytosolic expression of BRCA1 and metastatic risk in breast cancer.* Br J Cancer, 2015. **113**(3): p. 453-9.
289. Lakhani, S.R., et al., *The pathology of familial breast cancer: predictive value of immunohistochemical markers estrogen receptor, progesterone receptor, HER-2, and p53 in patients with mutations in BRCA1 and BRCA2.* J Clin Oncol, 2002. **20**(9): p. 2310-8.
290. Palacios, J., et al., *Re: Germline BRCA1 mutations and a basal epithelial phenotype in breast cancer.* J Natl Cancer Inst, 2004. **96**(9): p. 712-4; author reply 714.
291. Foulkes, W.D., et al., *Germline BRCA1 mutations and a basal epithelial phenotype in breast cancer.* J Natl Cancer Inst, 2003. **95**(19): p. 1482-5.
292. Nielsen, T.O., et al., *Immunohistochemical and clinical characterization of the basal-like subtype of invasive breast carcinoma.* Clin Cancer Res, 2004. **10**(16): p. 5367-74.
293. Melchor, L. and J. Benitez, *The complex genetic landscape of familial breast cancer.* Hum Genet, 2013. **132**(8): p. 845-63.

294. Klonowska, K., et al., *Analysis of large mutations in BARD1 in patients with breast and/or ovarian cancer: the Polish population as an example.* Sci Rep, 2015. **5**: p. 10424.
295. Thai, T.H., et al., *Mutations in the BRCA1-associated RING domain (BARD1) gene in primary breast, ovarian and uterine cancers.* Hum Mol Genet, 1998. **7**(2): p. 195-202.
296. Sauer, M.K. and I.L. Andrulis, *Identification and characterization of missense alterations in the BRCA1 associated RING domain (BARD1) gene in breast and ovarian cancer.* J Med Genet, 2005. **42**(8): p. 633-8.
297. Ishitobi, M., et al., *Mutational analysis of BARD1 in familial breast cancer patients in Japan.* Cancer Lett, 2003. **200**(1): p. 1-7.
298. Wu, J.Y., et al., *Aberrant expression of BARD1 in breast and ovarian cancers with poor prognosis.* Int J Cancer, 2006. **118**(5): p. 1215-26.
299. Huo, X., et al., *Common non-synonymous polymorphisms in the BRCA1 Associated RING Domain (BARD1) gene are associated with breast cancer susceptibility: a case-control analysis.* Breast Cancer Res Treat, 2007. **102**(3): p. 329-37.
300. Vahteristo, P., et al., *BARD1 variants Cys557Ser and Val507Met in breast cancer predisposition.* Eur J Hum Genet, 2006. **14**(2): p. 167-72.
301. De Brakeleer, S., et al., *Cancer predisposing missense and protein truncating BARD1 mutations in non-BRCA1 or BRCA2 breast cancer families.* Hum Mutat, 2010. **31**(3): p. E1175-85.
302. Ghimenti, C., et al., *Germline mutations of the BRCA1-associated ring domain (BARD1) gene in breast and breast/ovarian families negative for BRCA1 and BRCA2 alterations.* Genes Chromosomes Cancer, 2002. **33**(3): p. 235-42.
303. Karppinen, S.M., et al., *Mutation screening of the BARD1 gene: evidence for involvement of the Cys557Ser allele in hereditary susceptibility to breast cancer.* J Med Genet, 2004. **41**(9): p. e114.
304. Karppinen, S.M., et al., *Nordic collaborative study of the BARD1 Cys557Ser allele in 3956 patients with cancer: enrichment in familial BRCA1/BRCA2 mutation-negative breast cancer but not in other malignancies.* J Med Genet, 2006. **43**(11): p. 856-62.
305. Stacey, S.N., et al., *The BARD1 Cys557Ser variant and breast cancer risk in Iceland.* PLoS Med, 2006. **3**(7): p. e217.
306. Gorringe, K.L., et al., *BARD1 variants are not associated with breast cancer risk in Australian familial breast cancer.* Breast Cancer Res Treat, 2008. **111**(3): p. 505-9.
307. Jakubowska, A., et al., *BARD1 and breast cancer in Poland.* Breast Cancer Res Treat, 2008. **107**(1): p. 119-22.
308. Johnatty, S.E., et al., *The BARD1 Cys557Ser polymorphism and breast cancer risk: an Australian case-control and family analysis.* Breast Cancer Res Treat, 2009. **115**(1): p. 145-50.
309. Spurdle, A.B., et al., *Common genetic variation at BARD1 is not associated with breast cancer risk in BRCA1 or BRCA2 mutation carriers.* Cancer Epidemiol Biomarkers Prev, 2011. **20**(5): p. 1032-8.
310. Ding, D.P., et al., *Lack of association between BARD1 Cys557Ser variant and breast cancer risk: a meta-analysis of 11,870 cases and 7,687 controls.* J Cancer Res Clin Oncol, 2011. **137**(10): p. 1463-8.
311. Ratajska, M., et al., *Cancer predisposing BARD1 mutations in breast-ovarian cancer families.* Breast Cancer Res Treat, 2012. **131**(1): p. 89-97.
312. Ratajska, M., et al., *Cancer predisposing BARD1 mutations affect exon skipping and are associated with overexpression of specific BARD1 isoforms.* Oncol Rep, 2015. **34**(5): p. 2609-17.
313. ElShamy, W.M. and D.M. Livingston, *Identification of BRCA1-IRIS, a BRCA1 locus product.* Nat Cell Biol, 2004. **6**(10): p. 954-67.
314. Li, L., et al., *Identification of BARD1 splice-isoforms involved in human trophoblast invasion.* Int J Biochem Cell Biol, 2007. **39**(9): p. 1659-72.

315. Li, L., et al., *Oncogenic BARD1 isoforms expressed in gynecological cancers.* Cancer Res, 2007. **67**(24): p. 11876-85.
316. Pettigrew, C.A., et al., *Identification and functional analysis of novel BRCA1 transcripts, including mouse Brca1-Iris and human pseudo-BRCA1.* Breast Cancer Res Treat, 2010. **119**(1): p. 239-47.
317. Sporn, J.C., T. Hothorn, and B. Jung, *BARD1 expression predicts outcome in colon cancer.* Clin Cancer Res, 2011. **17**(16): p. 5451-62.
318. Zhang, Y.Q., et al., *BARD1: an independent predictor of survival in non-small cell lung cancer.* Int J Cancer, 2012. **131**(1): p. 83-94.
319. Zhang, Y.Q., et al., *Expression of oncogenic BARD1 isoforms affects colon cancer progression and correlates with clinical outcome.* Br J Cancer, 2012. **107**(4): p. 675-83.
320. Bosse, K.R., et al., *Common variation at BARD1 results in the expression of an oncogenic isoform that influences neuroblastoma susceptibility and oncogenicity.* Cancer Res, 2012. **72**(8): p. 2068-78.
321. Thomassen, M., et al., *Characterization of BRCA1 and BRCA2 splicing variants: a collaborative report by ENIGMA consortium members.* Breast Cancer Res Treat, 2012. **132**(3): p. 1009-23.
322. Lepore, I., et al., *HDAC inhibitors repress BARD1 isoform expression in acute myeloid leukemia cells via activation of miR-19a and/or b.* PLoS One, 2013. **8**(12): p. e83018.
323. Chen, J. and W.A. Weiss, *Alternative splicing in cancer: implications for biology and therapy.* Oncogene, 2015. **34**(1): p. 1-14.
324. Klar, A.J., S. Fogel, and K. Macleod, *MAR1-a Regulator of the HMa and HMalpha Loci in SACCHAROMYCES CEREVISIAE.* Genetics, 1979. **93**(1): p. 37-50.
325. Yuan, H. and R. Marmorstein, *Structural basis for sirtuin activity and inhibition.* J Biol Chem, 2012. **287**(51): p. 42428-35.
326. Imai, S. and L. Guarente, *Ten years of NAD-dependent SIR2 family deacetylases: implications for metabolic diseases.* Trends Pharmacol Sci, 2010. **31**(5): p. 212-20.
327. Avalos, J.L., K.M. Bever, and C. Wolberger, *Mechanism of sirtuin inhibition by nicotinamide: altering the NAD(+) cosubstrate specificity of a Sir2 enzyme.* Mol Cell, 2005. **17**(6): p. 855-68.
328. Sinclair, D.A. and L. Guarente, *Extrachromosomal rDNA circles--a cause of aging in yeast.* Cell, 1997. **91**(7): p. 1033-42.
329. Kaeberlein, M., M. McVey, and L. Guarente, *The SIR2/3/4 complex and SIR2 alone promote longevity in Saccharomyces cerevisiae by two different mechanisms.* Genes Dev, 1999. **13**(19): p. 2570-80.
330. Haigis, M.C. and D.A. Sinclair, *Mammalian sirtuins: biological insights and disease relevance.* Annu Rev Pathol, 2010. **5**: p. 253-95.
331. Frye, R.A., *Phylogenetic classification of prokaryotic and eukaryotic Sir2-like proteins.* Biochem Biophys Res Commun, 2000. **273**(2): p. 793-8.
332. Ford, E., et al., *Mammalian Sir2 homolog SIRT7 is an activator of RNA polymerase I transcription.* Genes Dev, 2006. **20**(9): p. 1075-80.
333. Tanno, M., et al., *Nucleocytoplasmic shuttling of the NAD+-dependent histone deacetylase SIRT1.* J Biol Chem, 2007. **282**(9): p. 6823-32.
334. Dryden, S.C., et al., *Role for human SIRT2 NAD-dependent deacetylase activity in control of mitotic exit in the cell cycle.* Mol Cell Biol, 2003. **23**(9): p. 3173-85.
335. Vaquero, A., et al., *SirT2 is a histone deacetylase with preference for histone H4 Lys 16 during mitosis.* Genes Dev, 2006. **20**(10): p. 1256-61.
336. Serrano, L., et al., *The tumor suppressor SirT2 regulates cell cycle progression and genome stability by modulating the mitotic deposition of H4K20 methylation.* Genes Dev, 2013. **27**(6): p. 639-53.
337. North, B.J., et al., *The human Sir2 ortholog, SIRT2, is an NAD+-dependent tubulin deacetylase.* Mol Cell, 2003. **11**(2): p. 437-44.

338. Michishita, E., et al., *Evolutionarily conserved and nonconserved cellular localizations and functions of human SIRT proteins.* Mol Biol Cell, 2005. **16**(10): p. 4623-35.
339. Saunders, L.R. and E. Verdin, *Sirtuins: critical regulators at the crossroads between cancer and aging.* Oncogene, 2007. **26**(37): p. 5489-504.
340. Peng, C., et al., *The first identification of lysine malonylation substrates and its regulatory enzyme.* Mol Cell Proteomics, 2011. **10**(12): p. M111 012658.
341. Du, J., et al., *Sirt5 is a NAD-dependent protein lysine demalonylase and desuccinylase.* Science, 2011. **334**(6057): p. 806-9.
342. Rardin, M.J., et al., *SIRT5 regulates the mitochondrial lysine succinylome and metabolic networks.* Cell Metab, 2013. **18**(6): p. 920-33.
343. Tan, M., et al., *Lysine glutarylation is a protein posttranslational modification regulated by SIRT5.* Cell Metab, 2014. **19**(4): p. 605-17.
344. Jiang, H., et al., *SIRT6 regulates TNF-alpha secretion through hydrolysis of long-chain fatty acyl lysine.* Nature, 2013. **496**(7443): p. 110-3.
345. Zhang, X., et al., *SIRT6 regulates Ras-related protein R-Ras2 by lysine defatty-acylation.* Elife, 2017. **6**.
346. Li, L., et al., *SIRT7 is a histone desuccinylase that functionally links to chromatin compaction and genome stability.* Nat Commun, 2016. **7**: p. 12235.
347. Cha, Y., et al., *Metabolic control of primed human pluripotent stem cell fate and function by the miR-200c-SIRT2 axis.* Nat Cell Biol, 2017. **19**(5): p. 445-456.
348. Gomes, P., T. Fleming Outeiro, and C. Cavadas, *Emerging Role of Sirtuin 2 in the Regulation of Mammalian Metabolism.* Trends Pharmacol Sci, 2015. **36**(11): p. 756-768.
349. Janke, C. and J.C. Bulinski, *Post-translational regulation of the microtubule cytoskeleton: mechanisms and functions.* Nat Rev Mol Cell Biol, 2011. **12**(12): p. 773-86.
350. Hubbert, C., et al., *HDAC6 is a microtubule-associated deacetylase.* Nature, 2002. **417**(6887): p. 455-8.
351. Akella, J.S., et al., *MEC-17 is an alpha-tubulin acetyltransferase.* Nature, 2010. **467**(7312): p. 218-22.
352. Shida, T., et al., *The major alpha-tubulin K40 acetyltransferase alphaTAT1 promotes rapid ciliogenesis and efficient mechanosensation.* Proc Natl Acad Sci U S A, 2010. **107**(50): p. 21517-22.
353. North, B.J. and E. Verdin, *Interphase nucleo-cytoplasmic shuttling and localization of SIRT2 during mitosis.* PLoS One, 2007. **2**(8): p. e784.
354. Eldridge, M.J.G., et al., *Active nuclear import of the deacetylase Sirtuin-2 is controlled by its C-terminus and importins.* Sci Rep, 2020. **10**(1): p. 2034.
355. Inoue, T., et al., *SIRT2, a tubulin deacetylase, acts to block the entry to chromosome condensation in response to mitotic stress.* Oncogene, 2007. **26**(7): p. 945-57.
356. Bae, N.S., et al., *Human histone deacetylase SIRT2 interacts with the homeobox transcription factor HOXA10.* J Biochem, 2004. **135**(6): p. 695-700.
357. Eskandarian, H.A., et al., *A role for SIRT2-dependent histone H3K18 deacetylation in bacterial infection.* Science, 2013. **341**(6145): p. 1238858.
358. Black, J.C., et al., *The SIRT2 deacetylase regulates autoacetylation of p300.* Mol Cell, 2008. **32**(3): p. 449-55.
359. Liu, G., et al., *Loss of NAD-Dependent Protein Deacetylase Sirtuin-2 Alters Mitochondrial Protein Acetylation and Dysregulates Mitophagy.* Antioxid Redox Signal, 2017. **26**(15): p. 849-863.
360. Lemos, V., et al., *The NAD+-dependent deacetylase SIRT2 attenuates oxidative stress and mitochondrial dysfunction and improves insulin sensitivity in hepatocytes.* Hum Mol Genet, 2017. **26**(21): p. 4105-4117.

361. Silva, D.F., et al., *Mitochondrial Metabolism Power SIRT2-Dependent Deficient Traffic Causing Alzheimer's-Disease Related Pathology.* Mol Neurobiol, 2017. **54**(6): p. 4021-4040.
362. Cimprich, K.A. and D. Cortez, *ATR: an essential regulator of genome integrity.* Nat Rev Mol Cell Biol, 2008. **9**(8): p. 616-27.
363. Awasthi, P., M. Foiani, and A. Kumar, *ATM and ATR signaling at a glance.* J Cell Sci, 2016. **129**(6): p. 1285.
364. Cortez, D., et al., *ATR and ATRIP: partners in checkpoint signaling.* Science, 2001. **294**(5547): p. 1713-6.
365. Zhang, M., et al., *SIRT2 protects peripheral neurons from cisplatin-induced injury by enhancing nucleotide excision repair.* J Clin Invest, 2020.
366. Kim, H.S., et al., *SIRT2 maintains genome integrity and suppresses tumorigenesis through regulating APC/C activity.* Cancer Cell, 2011. **20**(4): p. 487-99.
367. Chen, J., et al., *SIRT2 overexpression in hepatocellular carcinoma mediates epithelial to mesenchymal transition by protein kinase B/glycogen synthase kinase-3beta/beta-catenin signaling.* Hepatology, 2013. **57**(6): p. 2287-98.
368. Grbesa, I., et al., *Expression of sirtuin 1 and 2 is associated with poor prognosis in non-small cell lung cancer patients.* PLoS One, 2015. **10**(4): p. e0124670.
369. McGlynn, L.M., et al., *SIRT2: tumour suppressor or tumour promoter in operable breast cancer?* Eur J Cancer, 2014. **50**(2): p. 290-301.
370. Lai, C.C., et al., *Altered expression of SIRT gene family in head and neck squamous cell carcinoma.* Tumour Biol, 2013. **34**(3): p. 1847-54.
371. Nguyen, P., et al., *SIRT2 interacts with beta-catenin to inhibit Wnt signaling output in response to radiation-induced stress.* Mol Cancer Res, 2014. **12**(9): p. 1244-53.
372. Yao, H., E. Ashihara, and T. Maekawa, *Targeting the Wnt/beta-catenin signaling pathway in human cancers.* Expert Opin Ther Targets, 2011. **15**(7): p. 873-87.
373. Yang, M.H., et al., *HDAC6 and SIRT2 regulate the acetylation state and oncogenic activity of mutant K-RAS.* Mol Cancer Res, 2013. **11**(9): p. 1072-7.
374. Liu, P.Y., et al., *The histone deacetylase SIRT2 stabilizes Myc oncoproteins.* Cell Death Differ, 2013. **20**(3): p. 503-14.
375. Lahusen, T.J., et al., *BRCA1 function in the intra-S checkpoint is activated by acetylation via a pCAF/SIRT1 axis.* Oncogene, 2018. **37**(17): p. 2343-2350.
376. Tibbetts, R.S., et al., *Functional interactions between BRCA1 and the checkpoint kinase ATR during genotoxic stress.* Genes Dev, 2000. **14**(23): p. 2989-3002.
377. Foulkes, W.D., *Inherited susceptibility to common cancers.* N Engl J Med, 2008. **359**(20): p. 2143-53.
378. Roy, R., J. Chun, and S.N. Powell, *BRCA1 and BRCA2: different roles in a common pathway of genome protection.* Nat Rev Cancer, 2011. **12**(1): p. 68-78.
379. Silver, D.P. and D.M. Livingston, *Mechanisms of BRCA1 tumor suppression.* Cancer Discov, 2012. **2**(8): p. 679-84.
380. Tarsounas, M. and P. Sung, *The antitumorigenic roles of BRCA1-BARD1 in DNA repair and replication.* Nat Rev Mol Cell Biol, 2020.
381. Venkitaraman, A.R., *Cancer suppression by the chromosome custodians, BRCA1 and BRCA2.* Science, 2014. **343**(6178): p. 1470-5.
382. Moynahan, M.E., et al., *Brca1 controls homology-directed DNA repair.* Mol Cell, 1999. **4**(4): p. 511-8.
383. Moynahan, M.E., T.Y. Cui, and M. Jasin, *Homology-directed dna repair, mitomycin-c resistance, and chromosome stability is restored with correction of a Brca1 mutation.* Cancer Res, 2001. **61**(12): p. 4842-50.

384. Snouwaert, J.N., et al., *BRCA1 deficient embryonic stem cells display a decreased homologous recombination frequency and an increased frequency of non-homologous recombination that is corrected by expression of a brca1 transgene.* Oncogene, 1999. **18**(55): p. 7900-7.
385. Scully, R., et al., *Dynamic changes of BRCA1 subnuclear location and phosphorylation state are initiated by DNA damage.* Cell, 1997. **90**(3): p. 425-35.
386. Xu, X., et al., *Centrosome amplification and a defective G2-M cell cycle checkpoint induce genetic instability in BRCA1 exon 11 isoform-deficient cells.* Mol Cell, 1999. **3**(3): p. 389-95.
387. Kawai, S. and A. Amano, *BRCA1 regulates microRNA biogenesis via the DROSHA microprocessor complex.* J Cell Biol, 2012. **197**(2): p. 201-8.
388. Chang, S. and S.K. Sharan, *BRCA1 and microRNAs: emerging networks and potential therapeutic targets.* Mol Cells, 2012. **34**(5): p. 425-32.
389. Hatchi, E., et al., *BRCA1 recruitment to transcriptional pause sites is required for R-loop-driven DNA damage repair.* Mol Cell, 2015. **57**(4): p. 636-647.
390. Irminger-Finger, I. and C.E. Jefford, *Is there more to BARD1 than BRCA1?* Nat Rev Cancer, 2006. **6**(5): p. 382-91.
391. Jiang, Q. and R.A. Greenberg, *Deciphering the BRCA1 Tumor Suppressor Network.* J Biol Chem, 2015. **290**(29): p. 17724-32.
392. Kolinjivadi, A.M., et al., *Moonlighting at replication forks - a new life for homologous recombination proteins BRCA1, BRCA2 and RAD51.* FEBS Lett, 2017. **591**(8): p. 1083-1100.
393. Moynahan, M.E. and M. Jasin, *Mitotic homologous recombination maintains genomic stability and suppresses tumorigenesis.* Nat Rev Mol Cell Biol, 2010. **11**(3): p. 196-207.
394. Castilla, L.H., et al., *Mutations in the BRCA1 gene in families with early-onset breast and ovarian cancer.* Nat Genet, 1994. **8**(4): p. 387-91.
395. Friedman, L.S., et al., *Confirmation of BRCA1 by analysis of germline mutations linked to breast and ovarian cancer in ten families.* Nat Genet, 1994. **8**(4): p. 399-404.
396. Morris, J.R., N.H. Keep, and E. Solomon, *Identification of residues required for the interaction of BARD1 with BRCA1.* J Biol Chem, 2002. **277**(11): p. 9382-6.
397. Lee, C., et al., *Functional Analysis of BARD1 Missense Variants in Homology-Directed Repair of DNA Double Strand Breaks.* Hum Mutat, 2015. **36**(12): p. 1205-14.
398. Hu, W.L., et al., *GUARDIN is a p53-responsive long non-coding RNA that is essential for genomic stability.* Nat Cell Biol, 2018. **20**(4): p. 492-502.
399. Kim, J.L., et al., *Negative regulation of BRCA1 by transforming acidic coiled-coil protein 3 (TACC3).* Biochem Biophys Res Commun, 2018. **496**(2): p. 633-640.
400. Peng, Y., et al., *TUSC4 functions as a tumor suppressor by regulating BRCA1 stability.* Cancer Res, 2015. **75**(2): p. 378-86.
401. Finkel, T., C.X. Deng, and R. Mostoslavsky, *Recent progress in the biology and physiology of sirtuins.* Nature, 2009. **460**(7255): p. 587-91.
402. Guarente, L., *Franklin H. Epstein Lecture: Sirtuins, aging, and medicine.* N Engl J Med, 2011. **364**(23): p. 2235-44.
403. Zhang, H., P.E. Head, and D.S. Yu, *SIRT2 orchestrates the DNA damage response.* Cell Cycle, 2016. **15**(16): p. 2089-2090.
404. Zhang, H., et al., *ATRIP Deacetylation by SIRT2 Drives ATR Checkpoint Activation by Promoting Binding to RPA-ssDNA.* Cell Rep, 2016. **14**(6): p. 1435-47.
405. Outeiro, T.F., et al., *Sirtuin 2 inhibitors rescue alpha-synuclein-mediated toxicity in models of Parkinson's disease.* Science, 2007. **317**(5837): p. 516-9.
406. Pierce, A.J., et al., *XRCC3 promotes homology-directed repair of DNA damage in mammalian cells.* Genes Dev, 1999. **13**(20): p. 2633-8.

407. Suszynska, M., et al., *BARD1 is A Low/Moderate Breast Cancer Risk Gene: Evidence Based on An Association Study of the Central European p.Q564X Recurrent Mutation.* Cancers (Basel), 2019. **11**(6).
408. Fu, W., et al., *BARD1 Gene Polymorphisms Confer Nephroblastoma Susceptibility.* EBioMedicine, 2017. **16**: p. 101-105.
409. Taylor, A., et al., *Consensus for genes to be included on cancer panel tests offered by UK genetics services: guidelines of the UK Cancer Genetics Group.* J Med Genet, 2018. **55**(6): p. 372-377.