The BARD1 BRCT Domain in Tumor Suppression and Genome Stability

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Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy under the Executive Committee of the Graduate School of Arts and Sciences

COLUMBIA UNIVERSITY
2018
ABSTRACT

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BRCA1 preserves genome integrity through both homology-directed repair (HDR) and stalled fork protection (SFP). In vivo, BRCA1 exists as a heterodimer with the BARD1 tumor suppressor, and both proteins harbor a C-terminal BRCT domain with a phospho-recognition surface. Most pathogenic lesions of BRCA1 and BARD1 disrupt their respective BRCT domains, and BRCA1 BRCT phospho-recognition is required for its tumor suppression activity. Here we evaluate mice with mutations (Bard1^{S563F} and Bard1^{K607A}) that ablate Bard1 BRCT phospho-recognition. Although not affecting HDR, these mutations impair BRCA1/BARD1 recruitment to stalled replication forks, resulting in stalled fork degradation, chromosomal instability, and sensitivity to PARP inhibitors. However, Bard1^{S563F/S563F} and Bard1^{K607A/K607A} mice are not tumor-prone, indicating that ablation of SFP activity alone is insufficient for spontaneous tumor susceptibility. Nevertheless, since SFP, unlike HDR, is also impaired in Brca1/Bard1 heterozygous-mutant cells, SFP and HDR may contribute to distinct stages of tumor development in BRCA1/BARD1 mutation carriers.
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ACKNOWLEDGEMENTS

There are so many people who have mentored, encouraged, challenged, and inspired me in my now 2+ decades of schooling that it is impossible to thank them all in this short acknowledgements section. This thesis truly would not have been possible had it not been for countless people who took an interest in me and helped me along the way.

I would like to first thank my advisor and mentor Dr. Richard Baer. In addition to being one of the nicest people you will ever meet, Dr. Baer is a fantastic mentor. In the 4.5 years I have been a member of Dr. Baer’s lab, I have grown immensely as a direct result of his mentorship. Despite Dr. Baer’s very busy schedule, he was always willing to discuss an experiment that wasn’t quite working or review some of my writing. I am extremely grateful to Dr. Baer for the generosity he showed with his time. At the same time, Dr. Baer was able to strike the perfect balance between guiding my project and letting me figure things out on my own, which was absolutely vital to my growth as a scientist. I am very lucky to have had the experience of working for Dr. Baer, and I am forever indebted to him for the time and energy he put in on my behalf.

I would also like to thank the members of my dissertation committee: Dr. Jean Gautier, Dr. Adolfo Ferrando, Dr. Shan Zha, and Dr. Alberto Ciccia. Their advice and mentorship has been invaluable for the development of my project and my development as a scientist. I am, again, quite lucky to have had a committee of people whose work I deeply admire willing to spend their time and energy on my development.

I am also fortunate to have worked with such a great group of people in Dr. Baer’s lab. In particular, I would like to thank Foon Wu-Baer, whose technical expertise is unmatched and has been vital to the development of this project (in fact, there would not be a project without Foon, as she was responsible for making the mouse lines!). I am also thankful for how well-organized Foon kept the lab, despite being very busy with her own projects. I never once had to hunt for reagents or supplies, saving countless hours of frustration over the course of my time in the lab. I would also like to thank Dr. Michiko Horiguchi for her help with experiments, and wish her good luck as she prepares to take over the project. Dr. Yana Miteva, for always been a great bench-mate and a consistent source of both emotional and
scientific support. Finally, Lydia Tschoe, for helping with mouse maintenance, PCRs, and working to keep the lab so well-organized.

I would also like to sincerely thank our collaborators in Dr. Alberto Ciccia’s lab. In particular, Dr. Angelo Taglialatela and Dr. Silvia Alvarez-Nañez, for their help with the DNA fiber assay and Dr. Giuseppe Leuzzi for his help with the alkaline comet assay.

I have been supported by a great number of people outside of the lab at Columbia, including Zaia Sivo from the Integrated Program who always made sure I met deadlines in a timely fashion. From the MD/PhD program, I would like to thank Jeffrey Brandt, Dr. Ronald Liem, Dr. Patrice Spitalnik, Dr. Steve Reiner, and Dr. Michael Shelanski for their continued support.

I am very lucky to have been inspired by many teachers prior to my graduate study, if it weren’t for them I may have ended up choosing a career path far less rewarding and stimulating. In particular, I would like to thank my high school AP biology teacher, Jerry Lentz, whose passion for teaching biology ignited my interest in science. I would also like to thank my advisor and mentor from my undergraduate studies, Dr. Anna Greka, who showed me that bench research is much more than just something to put on a medical school application. Dr. Greka inspired me to enter the MD/PhD program, and I am lucky to have been mentored by such a first-rate physician-scientist.

Last but not least, I would like to thank my family, who have never been anything but 100% supportive of me. I am thankful for my parents, David and Elaine, whose hard work, sacrifice, and support made it possible for me attend college and graduate school. I am thankful for my two sisters, Bethany and Melissa, and I’m sorry that you had to go to so many of my sporting events growing up. Finally, I would like to thank my wife, Elise, who agreed to marry me this year even though we had to postpone our honeymoon so I could finish this thesis. I am consistently inspired by her generosity and dedication to those who are less fortunate. I am so lucky to have found a partner as wonderful as her.
For Roberta Billing and Teri Grabner

Whose absence reminds me that research is so much more than cells in a plate or mice in a cage
CHAPTER I

INTRODUCTION
A. BRCA in breast cancer

Approximately 250,000 new cases of breast cancer are diagnosed every year, making it the most common cancer among women in the United States. Furthermore, breast cancer is the second leading cause of cancer mortality in women in the U.S., resulting in an estimated 40,000 deaths per year and more than 450,000 deaths worldwide (La Vecchia et al., 2010; Siegel, Miller, & Jemal, 2017). Therefore, breast cancer is a major public health concern that continues to affect the lives of a large proportion of the global population.

Research carried out over the past few decades has revealed that breast cancers can be classified into several discrete molecular and immunological subtypes that carry important therapeutic and prognostic implications. The three most commonly used pathological markers in breast cancer diagnosis are the estrogen receptor (ER), progesterone receptor (PR), and HER2/NEU growth factor receptor (Allred, 2010; Prat et al., 2015). Of note, expression of ER and PR predict response to hormonal therapies such as tamoxifen and aromatase inhibitors, which have been shown to prolong survival and prevent recurrence in ER⁺PR⁺ breast cancers (Early Breast Cancer Trialists' Collaborative, 2005). On the other hand, HER2/Neu, while initially used as an indicator of poor outcome, is now a target of therapy using trastuzumab, a humanized monoclonal antibody which improves overall survival and prevents disease recurrence in HER2⁺ breast cancers (Engel & Kaklamani, 2007; Pegram et al., 1998; Piccart-Gebhart et al., 2005). In recent years, gene expression profiling of breast cancers has revealed the genetic signatures that underlie the pathological markers discussed above. Briefly, five distinct breast cancer molecular subtypes that correlate with ER/PR/HER2 expression status were initially identified: luminal A, luminal B, HER2-positive, normal-like, and basal-like (Perou et al., 2000), (Sorlie et al., 2001). This molecular subtyping, combined with immunohistochemistry expression data, facilitates the selection of optimal treatment regimens for individual patients (Dai et al., 2015; Sotiriou & Pusztai, 2009).

The development of modern targeted therapies like tamoxifen and trastuzumab, combined with the ability to predict who will respond to them, has benefited a large number of patients. Nonetheless, a substantial subset of breast cancers is intrinsically resistant to these drugs. Most notably, this includes the "triple-negative" breast cancers, which lack expression of ER, PR, and HER2. Most triple-negative breast cancers fall into the basal-like molecular subtype, and, as a result of their resistance to targeted
therapies, are treated with classical chemotherapy regimens (Foulkes, Smith, & Reis-Filho, 2010). As such, triple-negative breast cancers are associated with worse clinical outcomes when compared with the other breast cancer subtypes (Hennigs et al., 2016; Liedtke et al., 2008). Therefore, continued research into the molecular basis of triple-negative breast cancer is a high priority, as it may yield new drug targets that improve patient outcomes.

One of the main risk factors for developing breast cancer is family history, with up to a two-fold increase in risk for women who have a first-degree relative with breast cancer (Trentham-Dietz et al., 2014). Approximately 5-10% of all breast cancer cases are thought to be hereditary based on familial association (Foulkes, 2008). The most common breast cancer susceptibility genes are \textit{BRCA1} and \textit{BRCA2}, which collectively account for up to 25% of familial breast cancers (Easton, 1999). Inheritance of a single mutant \textit{BRCA1} allele increases a woman's risk of breast cancer 10-30 fold, resulting in a 50-80\% lifetime risk of developing breast cancer (Antoniou et al., 2003; Roy, Chun, & Powell, 2011). Furthermore, \textit{BRCA1}-mutant breast cancers are associated with triple-negative and basal-like molecular phenotypes (Foulkes et al., 2003; van der Kolk et al., 2010). As a result, these tumors are largely resistant to modern targeted therapies.

A closer look at the molecular details of \textit{BRCA1}-mutant tumors reveals that the wild type allele is very commonly inactivated, leaving both \textit{BRCA1} genes nonfunctional (Maxwell et al., 2017). Therefore, \textit{BRCA1} acts as a classic tumor suppressor. \textit{BRCA1}-mutant tumors are also characterized by a high degree of genome instability and mutation of certain other tumor suppressors such as \textit{p53}. A key feature of \textit{BRCA1}-mutant tumors is their sensitivity to chemotherapeutic agents that damage DNA directly or inhibit the repair of damaged DNA, such as poly(ADP-ribose) polymerase inhibitors (PARPi) (Byrski et al., 2010; Fong et al., 2009). However, despite initial sensitivity to these agents, \textit{BRCA1}-mutant tumors rapidly evolve resistance (Lord & Ashworth, 2013). Therefore, understanding the mechanisms of resistance and how to circumvent them is an active area of \textit{BRCA1} research.

Although \textit{BRCA1} is rarely mutated in sporadic breast cancer, there is increasing evidence that the \textit{BRCA1} pathway is functionally disrupted in many sporadic cases of basal-like and triple-negative breast cancer (Lord & Ashworth, 2016; N. C. Turner et al., 2007). Furthermore, mutations in the \textit{BRCA1} pathway continue to be found in other cancer types such as prostate, pancreatic, and, in particular, ovarian cancer.
Although common mechanisms of tumor formation in BRCA1 pathway-disrupted cancers are suspected, the exact molecular processes by which BRCA1 pathway disruption leads to tumorigenesis remain unclear. Clearly, a better understanding of the underlying molecular workings of the BRCA1 pathway should reveal novel drug targets and potential mechanisms for therapy of basal-like breast cancers. These scientific advances would have far reaching consequences for patients with either familial or sporadic breast cancer.

B. BRCA1 in maintenance of genome stability

Since its discovery in 1990, BRCA1 has been implicated in a wide range of cellular functions (Hall et al., 1990). These functions include, but are not limited to: transcriptional regulation, chromatin remodeling, protein ubiquitination, cell cycle checkpoint activation, stalled replication fork protection, centrosome duplication, maintenance of genome stability, and homology-directed repair (Jiang & Greenberg, 2015; Moynahan & Jasin, 2010; Nagaraju & Scully, 2007; Roy et al., 2011; Venkitaraman, 2014). The pleiotropic nature of BRCA1 has therefore made it challenging to determine which BRCA1 functions are or are not required for its role as a tumor suppressor. Two functions that have received much attention in recent years are the repair of double-strand DNA breaks (DSB) by homology-directed repair (HDR) and protection of stalled DNA replication forks from nucleolytic degradation (Moynahan, Chiu, Koller, & Jasin, 1999; Schlacher, Wu, & Jasin, 2012). HDR and stalled fork protection (SFP) are believed to be vital to the tumor suppressor function of BRCA1 due to the role of both pathways in maintaining genome stability (Cortez, 2015; Moynahan & Jasin, 2010). Furthermore, gross chromosomal instability and sensitivity to DNA-damaging agents are hallmarks of BRCA1-mutant cells and tumors. Additionally, BRCA2 has been shown to play a role in both HDR and SFP, suggesting the possibility of shared mechanisms of tumor suppression in both BRCA1 and BRCA2 mutation carriers (Lomonosov, Anand, Sangrithi, Davies, & Venkitaraman, 2003; Moynahan, Pierce, & Jasin, 2001; Schlacher et al., 2011; Ying, Hamdy, & Helleday, 2012).

DSB repair by the HDR pathway involves the coordinated action of numerous DNA damage response proteins, including BRCA1 and BRCA2. DSBs can arise through endogenous processes, such as replication fork collapse, or may be induced by a variety of exogenous agents, such as ionizing
radiation (Greenberg, 2008). Since DSBs are a particularly toxic form of DNA damage that can lead to genome instability and cell death, cells rely on multiple repair pathways to resolve them, including HDR, non-homologous end joining (NHEJ), and microhomology-mediated end joining (MMEJ). To repair DSBs in a relatively error-free manner, HDR relies on the presence of an undamaged sister chromatid to use as a template for DNA synthesis, and thus it occurs primarily during the S and G2 phases of the cell cycle (Moynahan & Jasin, 2010). This contrasts with NHEJ and MMEJ, which may function throughout the cell cycle and are potentially error prone (Symington & Gautier, 2011).

HDR initially entails the recruitment of various factors to DSB ends, such as the MRE11/RAD50/NBS1 (MRN) complex (including the MRE11 nuclease), CtIP, and the EXO1 nuclease (Figure 1) (Mimitou & Symington, 2008; Sartori et al., 2007; Zhu, Chung, Shim, Lee, & Ira, 2008). These factors will nucleolytically resect the DNA end in a 5’-to-3’ manner to generate a 3’ single-strand DNA (ssDNA) overhang. Moreover, by inhibiting NHEJ, DNA end resection commits the cell to the HDR pathway of DSB repair (Symington & Gautier, 2011). The protein RPA is then assembled onto the ssDNA overhang to form a RPA/ssDNA nucleoprotein filament, which, in turn, can induce S and G2 cell cycle checkpoints through recruitment and activation of the ATR kinase (Cimprich & Cortez, 2008; Zhou & Elledge, 2000). Next, a complex of PALB2 and BRCA2 promotes the replacement of RPA with the RAD51 recombinase (Lisby, Barlow, Burgess, & Rothstein, 2004; Sugawara, Wang, & Haber, 2003; Xia et al., 2006). The resulting RAD51/ssDNA filament can invade a homologous duplex DNA, typically on the sister chromatid, to form a D-loop within which DNA synthesis can proceed past the break site, allowing error-free repair of the DSB (West, 2003).

Loss of HDR function is believed to lead to genome instability by channeling DSB repair to more error-prone pathways, such as NHEJ or MMEJ, resulting in the accumulation of chromosomal aberrations (Symington & Gautier, 2011). Interestingly, deletion of the NHEJ factor 53BP1 is able to rescue HDR function in Brca1-mutant cells, likely by restoring the balance between NHEJ and HDR (Bunting et al., 2010). Further evidence for the importance of HDR in maintaining genome stability and tumor suppression can be found in the identification of cancer-associated loss-of-function mutations in many components of the HDR pathway in addition to BRCA1 and BRCA2, including RAD51C, BARD1, PALB2, and ATM (Couch et al., 2015).
Figure 1. DNA double-strand break repair pathways. Repair of double-strand breaks (DSB) can proceed through homology-directed repair (HDR or HR, right), or more error-prone mechanisms such as non-homologous end joining (NHEJ, left), or microhomology-mediated end joining (MMEJ, not shown). A key initial step in determining repair pathway choice is the 5’-to-3’ resection of DNA ends by nucleases such as MRE11 and EXO1. In the absence of resection, the blunt DNA ends may be ligated together through NHEJ, a process that occasionally generates junctional deletions/insertions of nucleotides, and even gross chromosomal rearrangements. Upon resection, the resulting ssDNA overhang is coated with RPA to form a RPA/ssDNA nucleoprotein filament, which in turn serves to activate ATR signaling and the S and G2 cell cycle checkpoints. Subsequent replacement of RPA with RAD51 to form the RAD51/ssDNA filament is facilitated by BRCA2. This filament can then invade the homologous duplex of the sister chromatid to form a D-loop, which can then be used as a template for error-free repair across the break. (Adapted from Moynahan and Jasin, 2010)
While its precise role in HDR is not completely clear, there is extensive evidence implicating BRCA1 in this pathway. First, BRCA1 is highly phosphorylated in cells exposed to DNA-damaging agents that induce DSBs, such as ionizing radiation. Furthermore, immunofluorescent staining experiments show that BRCA1 forms nuclear foci at sites of DNA damage, together with other HDR components, such as RAD51, BARD1, and BRCA2 (Cortez, Wang, Qin, & Elledge, 1999; Jin et al., 1997; Scully, Chen, Ochs, et al., 1997). Indeed, BRCA1 has also been shown to interact directly with additional HDR factors like CtIP and PALB2 (Scully, Chen, Plug, et al., 1997; Sharan et al., 1997; Yu & Baer, 2000; Yu, Wu, Bowcock, Aronheim, & Baer, 1998; F. Zhang et al., 2009). More direct evidence for a role for BRCA1 in HDR emerged through the use of reporter constructs that can measure in vivo repair of an induced double-strand break (Rouet, Smih, & Jasin, 1994). These experiments demonstrated definitively that BRCA1-deficient cells show a defect in HDR (Moynahan et al., 1999). Furthermore, reconstitution of BRCA1-mutant cells with wild type BRCA1 readily restored HDR and genome stability (Moynahan, Cui, & Jasin, 2001).

From a mechanistic standpoint, BRCA1 is believed to have distinct roles in different steps of HDR. The antagonistic relationship between BRCA1 and 53BP1, an NHEJ factor that suppresses DNA resection, suggests that BRCA1 can act at the early stages of HDR by influencing whether a DSB is processed through either the NHEJ or HDR pathway. On the other hand, the association of BRCA1 with PALB2/BRCA2/Rad51 complexes implies a subsequent role for BRCA1 during assembly of the Rad51 ssDNA filament. BRCA1 has also been shown to stimulate RAD51 recombinase activity, facilitating strand invasion and synapsis (Zhao et al., 2017). Thus, HDR is a key pathway for maintaining genome stability in the face of DNA damage, and BRCA1 is a vital component of this pathway. However, whether the HDR function of BRCA1 is actually required for tumor suppression has not been formally proven.

More recently, BRCA1 has also been implicated in the stalled fork protection (SFP) pathway (Ciccia & Elledge, 2010; Ciccia & Symington, 2016; Cortez, 2015; Errico & Costanzo, 2012; Zeman & Cimprich, 2014). Indeed, many components of the HDR pathway, including RAD51, BRCA2, and RPA, are also involved in SFP (Kolinjivadi, Sannino, de Antoni, Techer, et al., 2017). Replication fork stalling occurs when the cell is exposed to replication stress, leading to pausing of the DNA polymerase and potentially the uncoupling of the polymerase from the MCM helicase. If a stalled fork is not adequately
protected from cellular nucleases, then it may be degraded and ultimately collapse before the replication block can be overcome and replication restarted. The collapse of a replication fork may in turn lead to incomplete replication, DSB formation, and chromosomal instability. Replication stress can be generated by exogenous agents, including DNA inter-strand crosslinking reagents, topoisomerase inhibitors that create protein-DNA adducts, and drugs that deplete nucleotide pools. Replication stress can also occur from endogenous sources, such as oncogene activation, collision of the replication fork with the transcriptional machinery, or DNA adducts created by byproducts of cellular metabolism (Ciccia & Elledge, 2010; Cortez, 2015; Errico & Costanzo, 2012; Zeman & Cimprich, 2014). Since replication fork stalling is a relatively common during cell division, protecting stalled forks from degradation and collapse is critical for proper maintenance of genome stability.

Upon induction of replication stress, the uncoupling of the DNA polymerase from the replicative helicase generates long stretches of ssDNA, upon which RPA monomers assemble to form RPA/ssDNA nucleoprotein filaments (Figure 2) (Byun, Pacek, Yee, Walter, & Cimprich, 2005). The RPA/ssDNA filament then serves to activate the ATR kinase, which in turn induces the intra-S-phase checkpoint and coordinates the cellular response to the stalled replication fork (Zou & Elledge, 2003). At this point, stalled forks may be remodeled into “regressed” or “reversed” forks, in which the two nascent strands have annealed and the two parental strands re-annealed to form a “chicken-foot” structure (Atkinson & McGlynn, 2009). This fork regression may be catalyzed by a number of DNA helicases and translocases such as WRN, BLM, SMARCAL1, HTLF, and ZRANB3. (Achar, Balogh, & Haracska, 2011; Ciccia et al., 2009; Ciccia et al., 2012; Machwe, Xiao, Groden, & Orren, 2006). While fork regression may benefit the cell by limiting the amount of ssDNA or allowing the replisome to bypass a lesion on the parental strand by template switching, the regressed fork is also a substrate for degradation by nucleases such as MRE11 (Cortez, 2015; Kolinjivadi, Sannino, De Antoni, Zadorozhny, et al., 2017; Schlacher et al., 2011; Taglialetela et al., 2017). Interestingly, assembly of RAD51 acts to protect reversed forks from nucleolytic degradation, thereby inhibiting replication fork collapse and genome instability (Schlacher et al., 2011). Thus, it is likely that helicase/translocase-mediated fork reversal exists in a delicate balance: too little fork reversal and stalled replication forks may fail to restart; too much fork reversal and MRE11-mediated degradation and collapse may predominate (Cortez, 2015). In support of this hypothesis, recent work
Figure 2. Stalled DNA replication fork protection pathway. A stalled replication fork (left) can undergo reversal into a “chicken foot” structure (top) catalyzed by helicases and translocases such as BLM, HTLF, SMARCAL1, WRN, and ZRANB3. The resulting reversed fork helps to limit the amount of ssDNA and catalyze fork restart via template switching of the stalled nascent strand onto the uninstalled nascent strand. Adequate fork protection, which may lead to fork restart after the replication block is bypassed or removed (right), is catalyzed by loading of RAD51 onto the reversed fork. In the absence of adequate fork protection, the reversed fork structure is a target for degradation by the MRE11 nuclease, which can lead to replication fork collapse and genome instability (bottom). (Adapted from Ciccia and Symington, 2016)
demonstrates that SFP defects and chromosomal instability can be rescued by inhibiting factors that promote fork remodeling, such as HTLF, SMARCAL1, and ZRANB3 (Kolinjivadi, Sannino, De Antoni, Zadorozhny, et al., 2017; Taglialatela et al., 2017; Vujanovic et al., 2017).

Early evidence for the role of BRCA1/2 at stalled replication forks came from immunocytochemistry experiments by Scully et al. (Scully, Chen, Ochs, et al., 1997). In their study, it was observed that BRCA1 forms nuclear foci in cycling, unperturbed cells. However, when these cells were exposed to a replication stress-inducing agent (hydroxyurea), these BRCA1 foci dissipated and BRCA1 staining re-localized to PCNA-staining nuclear structures thought to represent replication factories. More recently, this observation was confirmed using a biochemical method called isolation of proteins on nascent DNA (iPOND), showing that BRCA1 and its obligate binding partner, BARD1, are both highly enriched at stalled replication forks (Dungrawala et al., 2015). A role for BRCA2 in SFP was also proposed after it was observed that BRCA2-mutant cells accumulate DNA damage after treatment with hydroxyurea (Lomonosov et al., 2003). Almost a decade later, in a series of seminal papers, Schlacher et al. reported that both BRCA1 and BRCA2 promote SFP by preventing MRE11-dependent nucleolytic degradation of the stalled fork (Schlacher et al., 2011; Schlacher et al., 2012). Additionally, Schlacher et al. showed that BRCA2 is required for RAD51 loading onto stalled replication forks (Schlacher et al., 2011). While the role of BRCA1 in SFP has been firmly established, it is still unclear how BRCA1 contributes mechanistically to fork protection.

Although BRCA1 plays a vital role in both HDR and SFP, the molecular mechanisms by which it promotes these pathways remain unclear. Nonetheless, research into these processes has already yielded clinical benefit, particularly with the development of PARP inhibitors as therapeutic agents for BRCA-mutant tumors (Bryant et al., 2005; Farmer et al., 2005; Fong et al., 2009). Indeed, the extreme sensitivity of BRCA1-deficient cells to PARP inhibitors may stem from the combined effects of PARP inhibitors (PARPi) on HDR and SFP. Initially, PARPi treatment was thought to exploit the HDR deficiency of BRCA1-mutant cells by inhibiting single-strand break repair (SSB), resulting in a synthetic lethal interaction. Thus, since BRCA1-deficient cells are already deficient in DSB repair by HDR, inhibition of SSB repair would lead to a toxic accumulation of DNA damage. Supporting this hypothesis is the clinical observation that BRCA1 tumors often evolve resistance to PARPi treatment by restoring HDR (Lord &
Ashworth, 2013). However, PARPi treatment can also stall replication forks by generating DNA-protein adducts, and further analyses revealed that the sensitivity of BRCA1-mutant cells to PARPi correlates well with the ability of the PARP inhibitor to stabilize toxic PARP1-DNA or PARP2-DNA complexes (Helleday, 2011; Pommier, O’Connor, & de Bono, 2016). Moreover, recent reports suggest that restoration of the SFP pathway in BRCA1-deficient cells is a potential mechanism of PARPi resistance (Ray Chaudhuri et al., 2016; Taglialatela et al., 2017). As such, continued research into the HDR and SFP pathways remains a high priority, as it may provide additional insights into the mechanisms of PARPi resistance and how to combat it. Additionally, the central question regarding whether SFP, HDR, or both are required for the tumor suppressor functions of BRCA1 is still unresolved. Insight into this issue should help clarify the longstanding question of how loss of BRCA1 leads to tumorigenesis.

C. The BRCA1 protein

*BRCA1* encodes a large nuclear protein of 1863 amino acids in humans and 1812 amino acids in mice (Bennett et al., 1995; Miki et al., 1994). BRCA1 shows a distinct expression pattern that varies with the cell cycle stage. Specifically, BRCA1 expression levels are low in G0 or G1, increase at the G1/S transition, and remain high throughout S, G2, and M (Chen et al., 1996). BRCA1 can be visualized by immunofluorescent staining as nuclear foci during the S and G2 phases of the cell cycle. However, when cells are exposed to genotoxic agents, these BRCA1 foci disperse and BRCA1 staining reappears at sites of DNA damage (Scully, Chen, Ochs, et al., 1997).

The BRCA1 protein contains an N-terminal RING domain, a coiled-coil domain, and two C-terminal BRCT repeats that together comprise the BRCT domain (Figure 3). The RING domain, a cysteine-rich sequence that coordinates two atoms of zinc, is present in over 300 distinct human proteins (Freemont, Hanson, & Trowsdale, 1991; W. Li et al., 2008). The RING domain has been shown to be a critical component of most E3 ubiquitin ligases (Deshaies & Joazeiro, 2009). Indeed, the RING domain of BRCA1 readily catalyzes ubiquitination, and this activity remains the only known intrinsic enzymatic function of BRCA1 (Hashizume et al., 2001). While ubiquitin conjugation is classically associated with protein degradation, BRCA1 has been shown to catalyze non-degradative polyubiquitination (i.e., non-K48 linkages), which may play a role in other cellular processes such as protein recruitment, cell
Figure 3. The BRCA1/BARD1 heterodimer. BRCA1 and BARD1 interact through their N-terminal RING domains to form an obligate, stoichiometric heterodimer. Both proteins possess two tandem C-terminal BRCT repeats, while BARD1 also contains four ankyrin repeats. The BRCT repeats of BRCA1 form a single phosphoprotein-recognition domain that interacts with the phosphorylated isoforms of Abraxas, Bach1, CtIP, and UHRF1 in a substoichiometric, mutually-exclusive fashion. BRCA1 also contains a coiled-coil domain, which facilitates its interaction with PALB2, which in turn binds to BRCA2, thereby allowing a physical association between BRCA1 and BRCA2.
signaling, and chromatin remodeling (Densham & Morris, 2017; Dickson et al., 2016; Wu-Baer, Lagrazon, Yuan, & Baer, 2003). Despite this, the E3 ubiquitin ligase activity of BRCA1 is dispensable for HDR and tumor suppression (Reid et al., 2008; Shakya et al., 2011).

The coiled-coil domain is a relatively common protein interaction motif and, in the case of BRCA1, has been shown to facilitate its interaction with PALB2 (F. Zhang et al., 2009). Through this interaction with PALB2, BRCA1 forms a complex with BRCA2, which also binds directly to a distinct region of the PALB2 polypeptide. The association of BRCA1 with PALB2 through its coiled-coil domain is required for the recruitment of BRCA2/Rad51 to DSBs and assembly of Rad51/ssDNA filaments (Sy, Huen, & Chen, 2009; F. Zhang et al., 2009).

BRCT domains are phosphoprotein-recognition motifs found in over 20 human proteins, most of which function in the DNA damage response (Gerloff, Woods, Farago, & Monteiro, 2012). The BRCT domain of BRCA1 interacts, in a mutually exclusive manner, with several different phosphoproteins to form distinct BRCA1 complexes. Of note, four of these phospho-ligands (Abraxas/CCDC98, BACH1/BRIP1/FANCJ, CtIP, and UHRF1) have been implicated in homology-directed repair of DNA double-strand breaks (Cantor et al., 2001; Kim, Huang, & Chen, 2007; Z. Liu, Wu, & Yu, 2007; Wang et al., 2007; Yu & Chen, 2004; Yu, Chini, He, Mer, & Chen, 2003; Yu et al., 1998; H. Zhang et al., 2016). Indeed, as discussed in depth below, the phosphoprotein recognition ability of the BRCA1 BRCT domain is itself required for HDR and tumor suppression (Shakya et al., 2011).

Most, if not all, BRCA1 polypeptides exist in vivo as a stable heterodimer with the BARD1 protein (Jin et al., 1997; L. C. Wu et al., 1996; Yu & Baer, 2000). At 777 amino acids, BARD1 is a smaller protein than BRCA1, but shares structural homology with BRCA1 in that it also harbors an N-terminal RING domain and two C-terminal BRCT repeats (Figure 3). Additionally, BARD1 has four ankyrin repeats near its C-terminal end that interact with the CstF-50 polyadenylation factor and may therefore play a role in RNA processing (Edwards et al., 2008; Fox et al., 2008; Kleiman & Manley, 1999; L. C. Wu et al., 1996).

BRCA1 and BARD1 interact through sequences that encompass their respective RING domains (Brzovic, Rajagopal, Hoyt, King, & Klevit, 2001; L. C. Wu et al., 1996). Since the stability and nuclear retention of BRCA1 is dependent on its association with BARD1 and vice versa, the two proteins form an
obligate stoichiometric heterodimer in vivo (Baer & Ludwig, 2002; Fabbro, Rodriguez, Baer, & Henderson, 2002; Yu & Baer, 2000). In keeping with the obligate nature of their interaction, BRCA1 and BARD1 colocalize in nuclear foci during S and G2 phase (Jin et al., 1997). This is in contrast to other known BRCA1-binding proteins (e.g., PALB2, CtIP, Abraxas, BACH1, and UHRF1), which associate with BRCA1 in a sub-stoichiometric manner.

Not only is the interaction of BRCA1 and BARD1 important for the stability and localization of both polypeptides, formation of the BRCA1/BARD1 heterodimer is also vital for the functions of BRCA1. Specifically, the interaction with BARD1 is required for the E3 ubiquitin ligase activity of BRCA1, as well as its role in mitotic spindle assembly, homology-directed repair, and, as will be discussed further below, tumor suppression (Hashizume et al., 2001; Laufer et al., 2007; Shakya et al., 2008; Westermark et al., 2003; Wu-Baer et al., 2003). Like BRCA1-null cells, BARD1-null cells are defective for HDR and display gross chromosomal instability (Laufer et al., 2007; E. E. McCarthy, Celebi, Baer, & Ludwig, 2003; Westermark et al., 2003). Furthermore, the phenotypes of Brca1 knockout, Bard1 knockout, and Brca1/Bard1 double-knockout mice are strikingly similar, with embryonic lethality occurring between embryonic days E7.5 and E8.5 as a result of an impairment in cell proliferation (Hakem et al., 1996; Ludwig, Chapman, Papaioannou, & Efstratiadis, 1997; E. E. McCarthy et al., 2003). Taken together, the evidence strongly suggests that most, if not all, functions attributed to BRCA1 are mediated by the BRCA1/BARD1 heterodimer.

D. The BRCA1/BARD1 heterodimer and tumor suppression

The study of BRCA1/BARD1-mediated tumor suppression in mice presents a unique challenge in that, in contrast to human patients, mice that are heterozygous for Brca1 do not develop tumors (X. Liu et al., 2007). Furthermore, as discussed previously, deletion of Brca1 or Bard1 in mice results in early embryonic lethality. Therefore, it is necessary to use either hypomorphic Brca1 (or Bard1) alleles or Cre-Lox recombination of conditional Brca1 (or Bard1) alleles to study Brca1/Bard1-mediated tumor formation in mice.

Indeed, a number of groups have used mice harboring hypomorphic Brca1 alleles to carry out tumorigenesis studies. For example, Ludwig et al. created a mutant allele (Brca1<sup>tr</sup>) that encodes a C-
terminally truncated 900-amino acid polypeptide that lacks the coiled-coil and BRCT domains (Ludwig, Fisher, Ganesan, & Efstratiadis, 2001). Mice that are homozygous for this allele are born at sub-Mendelian ratios that depend on their genetic background. Of note, after long latencies, these mice developed tumors at markedly increased rates compared with their wild type and heterozygous-mutant littermates. Although most of these tumors were lymphomas and sarcomas, a small fraction (~13%) were mammary tumors. Another strategy taken to study Brca1 hypomorphic alleles has been to introduce p53 mutations, which prolong the survival of Brca1-null embryos and rescue the viability of some Brca1 hypomorphic alleles (Cressman et al., 1999; Hakem et al., 1996; Xu et al., 2001). While these Brca1/p53 double-mutant mice develop tumors with faster kinetics (increased incidence and/or decreased latency) than Brca1 single-mutant mice, once again a major drawback is the predominance of non-mammary tumors such as lymphomas and sarcomas. Therefore, while hypomorphic Brca1 alleles represent a viable strategy to study Brca1-mediated tumor suppression in mice, their usefulness is limited by the long latency to tumor formation and the preponderance of non-mammary tumors.

Another strategy to circumvent the embryonic lethality of Brca1 and Bard1 deletion to study their tumor suppressor functions is the use of Cre-Lox recombination systems. Many systems have been developed using mammary-specific promoters to drive Cre recombinase expression and conditional inactivation of Brca1. To achieve mammary-specific Cre expression, some groups have made use of promoters from the long-terminal repeat of the mouse mammary tumor virus (MMTV), the whey acidic protein (WAP) gene, the β-lactoglobulin gene, and the cytokeratin-14 (K-14) gene (Brodie et al., 2001; X. Liu et al., 2007; A. McCarthy et al., 2007; Selbert et al., 1998; Shakya et al., 2011; Shakya et al., 2008; Wagner et al., 1997; Xu et al., 1999). In particular, the WAP and MMTV promoters, which have emerged as the popular approaches, both require the induction of pregnancy in the mice to activate expression of Cre recombinase at high levels in mammary epithelial cells. Using these systems, mice harboring conditional Brca1 alleles have been shown to develop mammary tumors at relatively long latencies, thereby circumventing the problem of the predominance of non-mammary tumors in whole body hypomorphic Brca1 tumor cohorts (Shakya et al., 2008). As is the case with the Brca1 hypomorphic mutants, addition of a p53 mutation leads to a decrease in the latency of mammary tumor formation, further supporting the hypothesis that p53 plays a role in BRCA1-mediated tumorigenesis (Brodie et al.,
2001; Xu et al., 1999). Of great importance, mammary tumors derived from these conditional Brca1 models closely resemble the breast tumors that arise in human BRCA1 mutation carriers. In particular, these mouse Brca1 tumors display the triple-negative phenotype, the basal-like histopathology, and high levels of chromosomal instability (X. Liu et al., 2007; A. McCarthy et al., 2007; Shakya et al., 2011; Shakya et al., 2008; Xu et al., 1999). Therefore, Cre-lox mediated recombination systems in mice are an excellent model for the study of human BRCA1 pathway breast cancers.

Given that Bard1-null mice and cells display a similar phenotype to Brca1-null mice and cells, it was proposed that BARD1 itself plays a role in BRCA1-mediated tumor suppression (Lauer et al., 2007; E. E. McCarthy et al., 2003; Westermark et al., 2003). To address this hypothesis and circumvent the lethality of Bard1-null mice, Shakya et al. generated Brca1 and Bard1 mouse tumor cohorts by placing Cre recombinase under the control of the WAP promoter in mice harboring either conditional Brca1 or Bard1 alleles. Upon induction of pregnancy and expression of Cre recombinase, loxP sites within the alleles were excised, resulting in mammary-specific inactivation of Brca1 or Bard1. These mice were then monitored for tumor formation. In keeping with previous studies, mammary-specific conditional Brca1-null mice developed mammary tumors at long latencies ($T_{50} = 512$ days) that closely resembled human BRCA1 tumors in that they were triple negative and had a basal like-phenotype. Strikingly, the Bard1 cohort mice developed tumors at a rate that was statistically indistinguishable from that of the Brca1 mice ($T_{50} = 465$ days). Furthermore, these tumors displayed the same characteristics as the Brca1 tumors, possessing a high degree of genome instability, lacking expression of the ER, PR, and HER2/NEU, and displaying a basal-like histopathology. Additionally, double-conditional Brca1/Bard1 mutant mice developed mammary tumors that arose with the same kinetics and displayed the same phenotype as the tumors of Brca1-only or Bard1-only conditional mice ($T_{50} = 473$ days), implying that BRCA1 and BARD1 are epistatic with respect to tumor suppression.

First, these data established that BARD1 is itself a tumor suppressor. In accord with these findings, pathogenic BARD1 mutations were subsequently identified in several human non-BRCA1/2 breast cancer families (Couch et al., 2015; De Brakeleer et al., 2016; De Brakeleer et al., 2010; Ratajska et al., 2012; Sabatier et al., 2010). Therefore, BARD1 is a clinically relevant tumor suppressor. Second, the similarity of the phenotypes of the tumors derived from conditional Brca1, Bard1, and Brca1/Bard1
double-mutant mice demonstrate that the tumor suppressor functions previously ascribed to BRCA1 alone are actually mediated by the BRCA1/BARD1 heterodimer. However, the individual contributions that BRCA1 and BARD1 make to tumor suppression are largely unclear.

E. Structural requirements for BRCA1/BARD1-mediated tumor suppression

While experiments using mouse models that result in the conditional inactivation of either Brca1 or Bard1 were instrumental in proving that BRCA1 and BARD1 are tumor suppressors in mammary epithelial cells, the experiments did not elucidate which domains and/or functions of these proteins are actually required for tumor suppression. However, these questions can be addressed by pairing targeted mutations that disrupt different functional domains of Brca1 or Bard1 with a conditional-null Brca1 or Bard1 allele. Thus, upon inactivation of the conditional allele, the mammary epithelial cells will solely express the targeted mutant Brca1 or Bard1 allele. If the tumorigenicity of these mice can then be paired with insights into the functional consequences of each particular mutation (e.g., the mutation disrupts HDR or SFP function), it should be possible to achieve one of the main goals of BRCA1 research: determining which of its functions are required for tumor suppression. Importantly, the landscape of human cancer-associated BRCA1 mutations provides clues as to what aspects of the protein may be required for tumor suppression. These observations may then be formally tested in mouse models. While most of the human BRCA1 mutations are frameshift or nonsense mutations that disrupt most or all of the protein, in some families tumor susceptibility can be attributed to missense mutations. These missense mutations cluster largely in the RING and BRCT domains of BRCA1, implying that these domains are vital to the tumor suppressor functions of the BRCA1/BARD1 heterodimer (Couch et al., 2014).

As outlined above, the RING domain of BRCA1 provides two important functions to the BRCA1/BARD1 heterodimer: first, it is required for the interaction between BRCA1 and BARD1 and second, it is required for the E3 ubiquitin ligase activity of the BRCA1/BARD1 heterodimer (Brzovic et al., 2001; Hashizume et al., 2001; L. C. Wu et al., 1996; Wu-Baer et al., 2003). Indeed, the association with BARD1 is also required for the stability of BRCA1, as the BRCA1/BARD1 interaction masks a nuclear export signal within BRCA1 that, if exposed, ultimately results in its proteolytic degradation (Fabbro et al.,
Since the E3 ubiquitin ligase activity is the only known intrinsic enzymatic function of the BRCA1/BARD1 heterodimer, it was initially hypothesized that this activity was required for tumor suppression. Indeed, the pathogenic BRCA1 mutation C61G was shown to disrupt the E3 ubiquitin ligase activity and resulted in tumor formation in a conditional mouse model (Drost et al., 2011). However, since this BRCA1 mutation disrupts both its E3 ubiquitin ligase activity as well as its association with BARD1, it was unclear which function was actually mediating tumor suppression (Hashizume et al., 2001; L. C. Wu et al., 1996).

To address this question, a separation-of-function mutation (Brca1 I26A) that disrupts the E3 ubiquitin ligase activity of Brca1 without disturbing its association with Bard1 was created (Reid et al., 2008). Cells harboring this mutation were found to be competent for HDR. Upon expression of the BRCA1 I26A allele in a conditional mouse model, it was found that these mice were not prone to mammary tumors, thus demonstrating that the E3 ubiquitin ligase is dispensable for the tumor suppression functions of the BRCA1/BARD1 heterodimer (Shakya et al., 2011). Instead, the vital function that the RING domain contributes to BRCA1-mediated tumor suppression is likely to be its ability to mediate formation of the BRCA1/BARD1 heterodimer. This result further underscores the importance of BARD1 in BRCA1-mediated tumor suppression and serves as a proof of principle that targeted mutations combined with mouse models can be used to elucidate which of the BRCA1/BARD1 heterodimer’s many functions are required for tumor suppression. However, since the stability of both BRCA1 and BARD1 is dependent upon the ability of the proteins to interact with each other, and these tumorigenic BRCA1 mutations in the RING domain disrupt this interaction, they are largely uninformative as to the larger question of which functions of BRCA1/BARD1 are required for tumor suppression.

The pathogenic missense mutations of BRCA1 cluster largely, but not exclusively, within its RING and BRCT domains (Couch et al., 2014). Indeed, if the truncating and frameshift mutations are taken into account along with the missense mutations, most (>90%) of the pathogenic BRCA1 mutations either eliminate or mutate the BRCT domain. Moreover, mice that harbor a truncated Brca1 lacking its coiled-coil and BRCT domains are tumor prone, suggesting that the BRCA1 BRCT domain may indeed be critical for BRCA1/BARD1 mediated tumor suppression (Ludwig et al., 2001). Importantly, since the
BRCA1 BRCT domain is not required for the BRCA1/BARD1 interaction, BRCA1 BRCT mutants may be used to study specific contributions of BRCA1 to the BRCA1/BARD1 heterodimer.

F. The BRCT domains of BRCA1 and BARD1

As was briefly discussed above, the evolutionarily-conserved BRCT (BRCA1 C-Terminus) domain was initially identified in BRCA1 but has since been found in over 20 human proteins (Bork et al., 1997; Gerloff et al., 2012; Q. Wu, Jubb, & Blundell, 2015). Many of the proteins which possess a BRCT domain, such as 53BP1, PARP1, MDC1, and TOPBP1, function in the DNA damage response. BRCT domains may exist as a single unit, or, as in the case of both BRCA1 and BARD1, two tandem units that form a single structural domain. BRCT domains, like that of BRCA1, are thought to function primarily as phospho-recognition surfaces, but have also been reported to bind DNA and poly(ADP-ribose) (PAR) (M. Li & Yu, 2013; Manke, Lowery, Nguyen, & Yaffe, 2003; Pleschke, Kleczkowska, Strohm, & Althaus, 2000; Yamane & Tsuruo, 1999; Yu et al., 2003). Given the importance of kinases like ATM, ATR, CHK1, and CHK2 in regulating the DNA damage response, the recognition of specific phosphorylation events by the BRCT domains may promote formation of protein complexes which ultimately coordinate DNA repair.

Structural studies have revealed the form and function of the BRCA1 BRCT domain. The BRCA1 BRCT domain exists as two repeats arranged in a head to tail fashion and joined by a 23-amino acid linker region, and the resulting structure is stabilized by interactions between the hydrophobic regions of both BRCT repeats (Figure 4) (Williams, Green, & Glover, 2001). The BRCA1 BRCT domain preferentially recognizes phospho-serine peptides with the pSXXF sequence motif (in which "p" indicates phosphorylation and "X" indicates an undetermined amino acid) (Manke et al., 2003; Rodriguez, Yu, Chen, & Songyang, 2003; Yu et al., 2003). Two binding pockets, a hydrophilic pocket in the N-terminal BRCT repeat and a hydrophobic pocket in the C-terminal repeat, form the structural basis for recognition of pSXXF-containing peptides. Specifically, the phospho-serine residue of pSXXF fits into the hydrophilic pocket of the N-terminal BRCT repeat while the phenylalanine is coordinated by the hydrophobic pocket. Three highly conserved residues, S1655, T1700, and K1702, as well as the amino group in the backbone of G1656, make up the hydrophilic pocket in the N-terminal BRCT repeat and coordinate binding to the phospho-serine peptide through hydrogen bonds. Thus, the phospho-recognition ability of the BRCA1
Figure 4. The BRCA1 BRCT domain. The BRCA1 BRCT domain is comprised of two BRCT repeats arranged in a head to tail fashion. The interaction of hydrophobic regions at their interface (two yellow alpha helixes, center) stabilizes the association between the two repeats. The BRCA1 BRCT domain preferentially binds phospho-serine peptides with the motif pSXXF (blue). The phospho-serine residue of pSXXF is coordinated by a hydrophilic pocket in the N-terminal BRCT repeat comprised of four highly conserved residues: S1655, G1656, T1700, and K1702 (in humans). The phenylalanine of the pSXXF motif is coordinated by a hydrophobic binding pocket which includes the M1775 residue in the C-terminal BRCT repeat. Human cancer-associated missense mutations have been identified in the S1655 and M1775 residues. (Adapted from Glover et al., 2004)
BRCT domain can be disrupted by mutation of either S1655, T1700, or K1702 (Clapperton et al., 2004; Shiozaki, Gu, Yan, & Shi, 2004; Williams, Lee, Hau, & Glover, 2004). Interestingly, some pathogenic BRCA1 missense mutations alter key structural features of the BRCT domain involved in coordinating the phosphopeptide. In particular, the M1775R mutation disrupts the hydrophobic pocket that coordinates the phenylalanine in the pSXXF motif while the S1655F mutation disrupts one of the phospho-serine interacting residues (Figure 4).

To address the hypothesis that the phospho-recognition ability of the BRCA1 BRCT domain is required for tumor suppression, the equivalent mutation to $BRCA1^{S1655F}$ was introduced into mouse Brca1 ($Brca1^{S1598F}$) (Shakya et al., 2011). In contrast to Brca1-null mice, homozygous $Brca1^{S1598F/S1598F}$ mice are viable, but born at sub-Mendelian ratios. Furthermore, these mice display developmental defects characteristic of Brca1 hypomorphic mutations, such as growth retardation, kinked tails, and white spots on the abdomen and hind limbs. Strikingly, mice harboring the $Brca1^{S1598F}$ mutation along with a mammary-specific Brca1 conditional allele ($Brca1^{S1598F/co}$) developed mammary tumors at a rate that was statistically indistinguishable from that of mammary-specific conditional Brca1-null mice ($Brca1^{co/co}$). Moreover, the mammary tumors derived from Brca1$^{S1598F/co}$ mice displayed the same triple-negative basal-like phenotype as the mammary tumors of conditional Brca1- or Bard1-null mice. Therefore, the phospho-recognition ability of the BRCA1 BRCT domain is required for the tumor suppressor functions of the BRCA1/BARD1 heterodimer.

In light of these results, insights into which functions of the BRCA1/BARD1 heterodimer are required for tumor suppression may be acquired by considering the phospho-dependent protein interactions mediated by the BRCA1 BRCT domain. As discussed above, the BRCA1 BRCT domain can interact with the phosphorylated forms of at least four different proteins implicated in HDR: Abraxas/CCDC98, BACH1/BRIP1/FANCJ, CtIP, and UHRF1 (Cantor et al., 2001; Kim et al., 2007; Z. Liu et al., 2007; Wang et al., 2007; Yu & Chen, 2004; Yu et al., 2003; Yu et al., 1998; H. Zhang et al., 2016). In contrast to the interaction between BRCA1 with BARD1, these interactions are all sub-stoichiometric.

Moreover, since the BRCA1 BRCT domain may only bind one phospho-protein at a time, these interactions are mutually exclusive and thus result in the formation of distinct BRCA1 complexes within the cell. Importantly, $Brca1^{S1598F/S1598F}$ cells are deficient for HDR (Shakya et al., 2011). However, it is
currently unknown whether the phospho-recognition ability of the BRCA1 BRCT domain is required for stalled fork protection. Therefore, it cannot yet be definitively concluded whether the HDR function alone accounts for the tumor suppressor function of the BRCA1/BARD1 heterodimer.

Like BRCA1, BARD1 possesses two C-terminal BRCT repeats that together comprise a BRCT structural domain. Structural studies have revealed that the two BARD1 BRCT repeats are oriented in a head to tail fashion and their association is stabilized by the interaction of hydrophobic surfaces at their interfaces (Figure 5A). Like BRCA1, the BARD1 BRCT domain contains two pockets, a hydrophilic pocket in the N-terminal repeat and a more hydrophobic pocket in the C-terminal repeat. The hydrophilic pocket in the N-terminal repeat structurally resembles the BRCA1 BRCT phospho-binding pocket in that it retains the four conserved residues predicted to make hydrogen bonds with a phosphoserine peptide (S1655, G1656, T1700, and K1702 in human BRCA1; S575, G576, T617, and K619 in human BARD1) (Figure 5B). Therefore, disruption of S575, G576, T617, or K619 is predicted to ablate the phospho-recognition ability of the BARD1 BRCT domain. However, the hydrophobic pocket in the C-terminal BARD1 BRCT repeat is very different from the BRCA1 C-terminal hydrophobic pocket, as the BARD1 BRCT domain contains multiple histidine and serine residues not present in BRCA1. This structural difference in the hydrophobic pocket likely explains the differing protein binding affinities of the two BRCT domains (Birrane, Varma, Soni, & Ladias, 2007; Edwards et al., 2008). As such, the BARD1 BRCT domain has not been reported to bind any of the known BRCA1 BRCT phospho-ligands (e.g., Abraxas, BACH1, CtIP, UHRF1).

Initially, the BARD1 BRCT domain was believed to bind phosphoserine peptides with the motif pS[D/E][D/E]E, similar to the interaction of BRCA1 with pSXXF motifs (Rodriguez et al., 2003). However subsequent studies revealed very low binding affinity for phosphopeptides containing pS[D/E][D/E]E, indicating that the interaction of the BARD1 BRCT domain with this motif is either highly transient or non-existent in vivo (Birrane et al., 2007; Edwards et al., 2008). Indeed, no confirmed phosphoprotein ligands of the BARD1 BRCT domain have yet been identified.

The lack of known phosphoprotein ligands for the BARD1 BRCT domain has been an obstacle in uncovering its functions. Recently, however, Li and Yu demonstrated that the BARD1 BRCT domain can bind poly(ADP-ribose) (PAR) (M. Li & Yu, 2013), a nucleic acid-like polymer formed upon polymerization
Figure 5. The BARD1 BRCT domain. A) A ribbon diagram shows that the BARD1 BRCT domain is comprised of two BRCT repeats, arranged in a head-to-tail fashion, that interact via the hydrophobic surfaces at their interface (blue helices, center). Like the BRCA1 BRCT domain, the BARD1 BRCT domain forms a hydrophilic pocket in its N-terminal repeat (P₁) and a hydrophobic pocket in its C-terminal repeat (P₂). B) Superposition of the BARD1 (blue) and BRCA1 (orange) hydrophilic and hydrophobic binding pockets. A pSXXF peptide is shown in green. The BRCA1 and BARD1 domains show significant structural homology in their hydrophilic pockets with potential phosphate-binding residues in human BARD1 (S575, G576, T617, K619) that directly correspond to the pSer-binding residues in human BRCA1 (S1655, G1656, T1700, K1702). In contrast, the hydrophobic binding pockets of BRCA1 and BARD1 show significant structural differences, as the BARD1 BRCT domain contains a proline residue (P687) in place of the phenylalanine-coordinating M1775 residue of BRCA1. (Adapted from Edwards et al., 2008)
of NAD$^+$ (nicotinamide adenine dinucleotide) by certain enzymes of the poly(ADP-ribose) polymerase (PARP) family. While PAR has been implicated in many different cellular pathways, PAR polymers are rapidly assembled at both DSBs and stalled replication forks (Ali et al., 2012; Bryant et al., 2009; Leung, 2014; Schreiber, Dantzer, Ame, & de Murcia, 2006; Yang, Cortes, Patnaik, Jasin, & Wang, 2004). The ability of the BARD1 BRCT domain to bind PAR in a phospho-dependent manner, a function not attributed to BRCA1, suggests a unique role for BARD1 in the HDR and/or SFP functions of the BRCA1/BARD1 heterodimer. Furthermore, mutation of the conserved K619 residue in the BARD1 BRCT domain ablated its interaction with PAR, suggesting that the same residues originally thought to coordinate phosphopeptide binding (S575, G576, T617, or K619) are likely to be required to bind the diphosphate linkages of PAR (M. Li & Yu, 2013).

Importantly, Li and Yu showed that the phospho-dependent binding of the BARD1 BRCT domain to PAR is required for early recruitment of the BRCA1/BARD1 heterodimer to DSBs (M. Li & Yu, 2013). In short, in the context of laser- or endonuclease-induced DSBs, they observed two temporally-distinct recruitment events of the BRCA1/BARD1 heterodimer. The first event was a transient, early recruitment of the heterodimer that occurs within 20 seconds of DSB formation in a $\gamma$H2AX-independent manner and dissipates within 5-10 minutes after break formation. The second is a late recruitment that occurs within 5-10 minutes after break formation in a $\gamma$H2AX-dependent manner and remains stable for hours after DSB induction. Notably, the early recruitment event was ablated by mutation of the BARD1 BRCT domain, as well as by treatment with PARP inhibitors (PARPi). In contrast, the late recruitment event was dependent on the phospho-recognition ability of the BRCA1 BRCT domain (M. Li & Yu, 2013). Together, these data suggest that the BRCA1 and BARD1 BRCT domains work together to facilitate distinct aspects of DSB repair. However, whether the BARD1 BRCT domain is crucial for the BRCA1/BARD1 heterodimer’s roles in the HDR and SFP pathways is unknown.

Interestingly, as was the case with the human BRCA1 mutation landscape, many of the BARD1 mutations found in human breast cancer patients either ablate or disrupt the BARD1 BRCT domain (Couch et al., 2015; De Brakeleer et al., 2016; De Brakeleer et al., 2010; Ratajska et al., 2012; Sabatier et al., 2010). This suggests that the BARD1 BRCT domain may be required for BRCA1/BARD1-mediated tumor suppression. Additionally, given that the BARD1 BRCT domain is not required for the interaction with
BRCA1, these cancer-associated mutations suggest that the BARD1 BRCT domain may be contributing functions, distinct from those of BRCA1, that are required for BRCA1/BARD1-mediated tumor suppression. Further exploration of the roles of the BARD1 BRCT domain may help to clarify these fundamental questions.

G. Goals and rationale

Since BRCA1 was discovered over twenty years ago, significant progress has been made identifying the diverse functions of the BRCA1/BARD1 heterodimer. However, it is still unclear which of its plethora of cellular functions are relevant for tumor suppression. While the functions of BRCA1 in HDR and SFP are commonly thought to be important for tumor suppression, this hypothesis has never been formally proven. Thus, it is not known whether loss of SFP, HDR, or both lead to tumor formation. The use of targeted mutations that disrupt key functional domains combined with mouse models of breast cancer represents a significant opportunity to answer this longstanding question. Indeed, further insights into the mechanisms of the SFP and HDR pathways, as well as their role in tumor suppression, may identify novel drug targets for the treatment of triple-negative breast cancer.

Another fundamental unanswered question regards the individual roles of the BRCA1 and BARD1 in cellular function and tumor suppression. Numerous lines of evidence discussed above support the conclusion that BRCA1 and BARD1 act in the same cellular pathways and are reliant upon each other for their stability and function (Baer & Ludwig, 2002; Fabbro et al., 2002; Hashizume et al., 2001; Laufer et al., 2007; Shakya et al., 2008; Westermark et al., 2003; Wu-Baer et al., 2003; Yu & Baer, 2000). Therefore, the use of BRCA1-null or BARD1-null experimental systems inevitably affects the other binding partner, thus obscuring their individual functions. It is likely that BRCA1 and BARD1 each contribute unique functions to the BRCA1/BARD1 heterodimer. Indeed, the tumorigenicity of targeted mutations in the BRCT domain of BRCA1 that do not affect its association with BARD1 argues that BRCA1 contributes distinct functions to the BRCA1/BARD1 heterodimer (Ludwig et al., 2001; Shakya et al., 2011). However, it is unknown whether BARD1 contributes any distinct, vital functions to the heterodimer. While it is possible that BARD1 may play a largely housekeeping role in stabilizing BRCA1, the presence of pathogenic mutations in the BARD1 BRCT domain that are not predicted to disrupt the
association with BRCA1 argue that BARD1 makes unique contributions to the tumor suppressor functions of the BRCA1/BARD1 heterodimer (Couch et al., 2015; De Brakeleer et al., 2016; De Brakeleer et al., 2010; Ratajska et al., 2012; Sabatier et al., 2010). Once again, the use of functional analysis of targeted mutations combined with mouse models should help to tease out the distinct roles of BARD1.

In summary, the decision to study the Bard1 BRCT domains is based on three main findings: 1) the phospho-recognition ability of the BRCA1 BRCT domains is required for tumor suppression, 2) the BARD1 BRCT domains are capable of phospho-recognition and/or poly-(ADP-ribose) (PAR) binding, and 3) BARD1 mutations that disrupt the BRCT domains have been implicated as the tumor-predisposing lesion in several non-BRCA1/2 breast cancer families (Couch et al., 2015; De Brakeleer et al., 2016; De Brakeleer et al., 2010; Ratajska et al., 2012; Sabatier et al., 2010; Shakya et al., 2011). Together, these findings suggest that the BARD1 BRCT domains are critical for the tumor suppression and genome stability functions of the BRCA1/BARD1 heterodimer.

To test this hypothesis, we chose to model two distinct BARD1 BRCT point mutations in mice: Bard1-S563F and Bard1-K607A, equivalent to the S575 and K619 phospho-coordinating residues in human BARD1. Indeed, mutation of either the S563 or K607 residue is predicted by structural studies to disrupt Bard1 binding to either phosphoproteins or PAR. Thus, by modeling these mutations in mice, we sought to ascertain whether the phosphoprotein-binding or PAR-binding functions of the BARD1 BRCT domain are required for the HDR or SFP functions of the BRCA1/BARD1 heterodimer.
CHAPTER II
MATERIALS AND METHODS
A. Mouse strain generation

The mice used in this study were housed in an AAALAC-accredited facility at Columbia University Medical Center. All experiments involving mice were performed according to the Columbia University Institutional Animal Care and Use Committee-approved protocols.

To generate the transgenic mouse strains, a S563F knock-in targeting vector containing mouse *Bard1* genomic DNA was constructed by inserting a neomycin-resistance gene cassette flanked by *loxP* sites (loxP-neo-LoxP) into intron 7 and the S563F missense mutation into exon 8 (Figure 6B). Likewise, a K607A targeting construct was generated by inserting the loxP-neo-LoxP cassette into intron 8 and the K607A missense mutation into exon 9 (Figure 7B). These vectors were then electroporated into ES cells on a 129Sv background and selected with media supplemented with neomycin. The presence of the desired mutations in neomycin-resistant clones was confirmed by sequence analysis and the proper integration confirmed by Southern blot analysis (Figures 7C, E and 8C, E). Two independent clones of either *Bard1<sup>S563F-neo/+</sup>* or *Bard1<sup>K607A-neo/+</sup>* ES cells were injected into C57BL/6J blastocysts for the production of chimeric germline-transformed mice. Once again, the presence of the S563F or K607A mutation was confirmed by sequence analysis, PCR, and Southern blot using genomic DNA harvested from mouse tails (see sections D, E, and F below). The neomycin cassette was then removed by crossing heterozygous *Bard1<sup>S563F-neo/+</sup>* or *Bard1<sup>K607A-neo/+</sup>* with *Rosa<sup>Cre</sup>* mice, a mouse strain that ubiquitously expresses Cre recombinase (Figures 6D and 7D). The excision of the neomycin cassette was confirmed by Southern blot analysis. The heterozygous *Bard1<sup>S563F/+</sup>* or *Bard1<sup>K607A/+</sup>* mice were then backcrossed with pure C57BL/6J mice (Jackson Laboratory) three times to yield animals that were approximately 94% C57BL/6J (N3 backcrossed). All mouse tumor cohorts, mouse embryonic fibroblast (MEF) lines, and embryonic stem (ES) cell lines were generated using mice on this background.

B. Tumor monitoring and histopathology

Mice were monitored for tumor development on a weekly basis. Upon detection of a palpable mass or moribund appearance, the mice were sacrificed and their tissues harvested for histological analysis. For testicular histology, mice were sacrificed at 6 weeks of age. The mice were euthanized with CO2 followed by cervical dislocation in accord with the American Veterinary Medical Association...
Guidelines for the Euthanasia of Animals: 2013 Edition. After euthanasia, a piece of the tail was harvested for confirmation of the genotype by PCR or Southern blot (see Sections D, E, and F below). All major organs were collected and fixed overnight in 10% buffered formalin, followed by dehydration with 70% ethanol the next day. Tissues were then embedded in paraffin, sectioned at a thickness of 4 µm, and stained with hematoxylin and eosin for histopathological evaluation.

C. Mouse whole-body IR treatment

Five-week-old mice were treated with 7.5 Gy of radiation on a rotating platform using a Mark I Cesium-137 mouse irradiator (J.L. Shepard and associates). Survival was monitored daily for 6 weeks post treatment.

D. Genomic DNA isolation

Genomic DNA was isolated for polymerase chain reaction (PCR) or Southern blot analysis from mouse tails, embryo heads, MEFs, and ES cells. To isolate the genomic DNA, cells or the mouse tail were dissolved by incubation with 500 µL of tail buffer (50 mM Tris-HCl, pH 8.0, 0.1 M NaCl, 2 mM EDTA, 1% SDS) supplemented with 200 µg/mL of proteinase K at 56°C overnight. The next day, the dissolved cells or tails were vortexed for 5 minutes, supplemented with 200 µL 5M NaCl, and vortexed for another 5 minutes. The dissolved materials were then centrifuged at 13000 RPM for 10 minutes and the resulting supernatant transferred to a new 1.5 mL Eppendorf tube. The DNA was then precipitated using 500 µL of isopropanol and centrifuged at 13000 RPM for 5 minutes to pellet the DNA. The supernatant was subsequently removed, the pellet washed with 70% ethanol, and centrifuged at 13000 RPM for 2 minutes. The supernatant was again removed and the DNA pellet dried for 15 minutes in a speed vacuum concentrator before resuspension of the DNA in 150 µL of 1X TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).
E. Polymerase chain reaction (PCR)

All PCRs were performed in a final volume of 25 µL with Taq DNA polymerase (Invitrogen), 10X PCR reaction buffer (200 mM Tris, pH 8.4, 500 mM KCl), 50 mM MgCl₂, 10 mM dNTP, 100% dimethylsulfoxide (DMSO), 10 µM oligonucleotide DNA primers (see Table 1), and 1 µL of genomic DNA in 1X TE isolated as described above. The PCRs were then run on an Eppendorf Mastercycler Nexus GX2 gradient PCR machine. After completion of the PCR, 10 µL of the reaction was run on a 2% agarose gel in 1X TAE (40 mM Tris, 20 mM Acetate, 1 mM EDTA, pH 8.0) supplemented with 0.5 µg/mL ethidium bromide (EtBr). Finally, the gel was imaged on a Biodoc-IT UV transilluminator (UVP).

To genotype the Bard\(^{K607A}\) and Bard\(^1^+\) alleles, the Qx1-21 and Qx1-H primers (see Table 1) were used at a concentration of 10 µM with the following PCR conditions: 94˚C 3 min (1 cycle); 94˚C 30 sec, 62˚C 30 sec, 72˚C 45 sec (35 cycles); 72˚C 5 min (1 cycle); 4˚C hold. The reaction yields a 596 bp band for the Bard\(^{K607A}\) allele and a 500 bp band for the Bard\(^1^+\) allele.

To genotype the Bard\(^{S563F}\) and Bard\(^1^+\) alleles, the Qx1-13 and Qx1-D primers were used at a concentration of 10 µM with the same PCR conditions as the Bard\(^{K607A}\) allele (see above). The reaction yields a 521 bp band for the Bard\(^{S563F}\) allele and a 426 bp band for the Bard\(^1^+\) alleles.

To genotype the Brca1\(^{S1598F-LNL}\) and Brca1\(^1^+\) alleles, two separate PCR reactions were necessary. The first reaction, used to PCR the Brca1\(^{S1598F-LNL}\) allele, utilized the S1598F-2 and LNL-A primers at a concentration of 10 µM. This reaction yielded a band at 459 bp for the Brca1\(^{S1598F-LNL}\) allele, and no band for the Brca1\(^1^+\) allele. The second reaction, used to PCR the Brca1\(^1^+\) allele, utilized the S1598F-2 and S1598F-C primers at a concentration of 10 µM. This reaction yielded a band at 396 bp for the Bard\(^1^+\) allele, and no band for the Brca1\(^{S1598F-LNL}\) allele. Both reactions were carried out under the following conditions: 94˚C 3 min (1 cycle); 94˚C 30 sec, 65˚C 30 sec, 72˚C 45 sec (35 cycles); 72˚C 5 min (1 cycle); 4˚C hold.

For PCRs involving ES cell DNA, the same reaction mixtures and primers were used to amplify the respective Bard\(^1\) and Brca1 alleles. However, two different PCR conditions were run in the thermocycler in an effort to prevent DNA contamination from the primary MEF feeder cells with which the ES cells were co-cultured. For each reaction, a “long” and a “short” condition were run using the same primers. In the case of the Bard\(^1\) alleles, the “long” reaction was as follows: 94˚C 3 min (1 cycle); 94˚C
30 sec, 62°C 30 sec, 72°C 45 sec (38 cycles); 72°C 5 min (1 cycle); 4°C hold, while the “short” reaction conditions were: 94°C 3 min (1 cycle); 94°C 30 sec, 62°C 30 sec, 72°C 45 sec (25 cycles); 72°C 5 min (1 cycle); 4°C hold. In the case of the Brca1 alleles, the “long” reaction was as follows: 94°C 3 min (1 cycle); 94°C 30 sec, 65°C 30 sec, 72°C 45 sec (38 cycles); 72°C 5 min (1 cycle); 4°C hold; while the “short” reaction conditions were: 94°C 3 min (1 cycle); 94°C 30 sec, 65°C 30 sec, 72°C 45 sec (25 cycles); 72°C 5 min (1 cycle); 4°C hold.

Table 1: PCR Primers

<table>
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<th>Allele</th>
<th>Primer name</th>
<th>Primer sequence</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bard1&lt;sup&gt;K607A&lt;/sup&gt;</td>
<td>Qx1-21</td>
<td>5'-CACGTGGTTGCTGGAAATTG-3'</td>
<td>K607A: 596bp</td>
</tr>
<tr>
<td></td>
<td>Qx1-H</td>
<td>5'-ATGTAAGGAGCCAGCAGC-3'</td>
<td>Wild type: 500 bp</td>
</tr>
<tr>
<td>Bard1&lt;sup&gt;S563F&lt;/sup&gt;</td>
<td>Qx1-13</td>
<td>5’-GCAGGTGCTCTACCCTCAAC-3’</td>
<td>S563F: 521 bp</td>
</tr>
<tr>
<td></td>
<td>Qx1-D</td>
<td>5’-AACCTGGCCATCAACATG-3’</td>
<td>Wild type: 426 bp</td>
</tr>
<tr>
<td>Brca1&lt;sup&gt;S1598F-LNL&lt;/sup&gt;</td>
<td>S1598F-2</td>
<td>5’-AACATTAAGCCCACCTGACCC-3’</td>
<td>S1598F-LNL: 459 bp</td>
</tr>
<tr>
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<td>LNL-A</td>
<td>5’-TCAGCGGTGCTCTCCATCTGC-3’</td>
<td>Wild type: no band</td>
</tr>
<tr>
<td></td>
<td>S1598F-2</td>
<td>5’-AACATTAAGCCCACCTGACCC-3’</td>
<td>S1598F-LNL: no band</td>
</tr>
<tr>
<td></td>
<td>S1598F-C</td>
<td>5’-GATTTTCCAGAGGAACTGCTGC-3’</td>
<td>Wild type: 396 bp</td>
</tr>
</tbody>
</table>

F. Southern blot analysis

Southern analysis was performed by digesting genomic DNA isolated from mouse tail or ES cells (prepared as described in Section D) with the appropriate restriction enzyme. The digest was performed by incubating 10 µL of genomic DNA in 1X TE with 10 units of the appropriate restriction enzyme in a total volume of 30 µL at 37°C overnight. The following day, the complete digestion reaction was electrophoresed on a 0.8% high melt molecular biology grade agarose gel (Denville) in 1X TAE (40 mM Tris, 20 mM acetate, 1 mM EDTA, pH 8.0) supplemented with 0.5 µg/mL ethidium bromide (EtBr). After electrophoresis, the gel was incubated in depurination solution (0.25 M HCl) on a shaking incubator for 20 min at room temperature and then rinsed with ddH2O three times. The gel was then incubated in denaturing solution (0.5 M NaOH and 1.5 M NaCl) on a shaking incubator for 20 minutes at room
temperature. After denaturation, the gel was blotted onto a Biodyne B 0.45 µm nylon membrane (Pall) by upward capillary transfer overnight.

The following day, the membrane was allowed to dry on a piece of Whatman paper for 20 minutes before immobilizing by baking the membrane at 100˚C in a vacuum oven for 30 minutes. The membrane was then wet in 2X SSC (300 mM NaCl, 35 mM sodium citrate pH 7.0) and pre-hybridized in 10 mL of rapid hybridization buffer (5X SSC, 10% polyethylene glycol MW 8000, 5% poly(sodium 4-styrenesulfonate), 0.2% cetylpyridinium chloride monohydrate) supplemented with 1 mg sheared salmon sperm DNA (Thermo Fischer) at 65˚C for 30 minutes in a rotating hybridization chamber. During prehybridization, a α32P-dCTP-labeled (Perkin Elmer) probe was prepared using Prime-It II Random Primer Labeling Kit (Agilent Technologies) by first denaturing the appropriate probe fragment along with random primer mix by heating at 95˚C for 5 minutes. After denaturation, the α32P-dCTP-labeled probe was synthesized by combining the unlabeled denatured probe with α32P-dCTP and incubating with Klenow fragment enzyme (Agilent Technologies) at 37˚C for 10 minutes. Stop mix buffer (0.5 M EDTA, pH 8.0; Agilent Technologies) and 500 µg sheared salmon sperm DNA were then added to the probe mixture and incubated at 95˚C for 5 minutes before cooling on ice for 1 minute in order to halt the reaction. Next, the radiolabeled probe mixture was added to the membrane in pre-hybridization buffer and incubated at 65˚C in a rotating hybridization chamber for 2 hours. Following incubation with the radiolabeled probe, the membrane was removed from hybridization buffer and washed for 10 minutes at 65˚C in pre-warmed 2X SSC, 0.5% SDS at 65˚C on a shaking platform. Following the first wash, the membrane was washed two more times, first in pre-warmed 1X SSC, 0.1% SDS followed by pre-warmed 0.1X SSC, 0.1% SDS for 10 min each at 65˚C on a shaking platform. After washing, the membrane was dried on Whatman paper for 15 minutes, attached to a previously exposed western blot film by wrapping with plastic wrap, and placed between two amplifying screens along with Carestream Kodak BioMax MS film (Sigma). The film was then exposed overnight at -80˚C and developed the following morning.

Two separate strategies were designed to detect the properly targeted Bard1S563F allele. In the first strategy, genomic DNA from mice or ES cells was digested with EcoRV and hybridized with a probe upstream of exon 7 (“5’ probe”) (Figure 6). Bard1S563F-neo or Bard1S563F was detected as a 7.4 kb band whereas Bard1+ was detected as a 9.3 kb band. In the second strategy, which can ascertain the
presence or absence of the neomycin cassette included for selection during gene targeting and then removed subsequently via cre recombination (Bard1^{S563F-neo} vs Bard1^{S563F}), genomic DNA was digested with KpnI. The digested DNA was then hybridized with a probe downstream of exon 9 (“3’ probe”) (Figure 6), yielding bands of 9.4 kb for Bard1^+, 5.9 kb for Bard1^{S563F-neo}, and 4.0 kb for Bard1^{S563F}.

The two strategies outlined above were also applied to detect properly targeted Bard1^{K607A}. In the first strategy, genomic DNA from mice or ES cells was digested with EcoRV and hybridized with a probe upstream of exon 7 (“5’ probe”) (Figure 7). Bard1^{K607A-neo} or Bard1^{K607A} was detected as an 8.5 kb band whereas Bard1^+ was detected as a 9.3 kb band. In the second strategy, which again could distinguish between the Bard1^{K607A-neo} and Bard1^{K607A} alleles, genomic DNA was digested with KpnI. The digested DNA was then hybridized with a probe downstream of exon 9 (“3’ probe”) (Figure 7), yielding bands of 9.4 kb for Bard1^+, 5.0 kb for Bard1^{K607A-neo}, and 3.0 kb for Bard1^{K607A}.

G. Generation and immortalization of mouse embryonic fibroblasts (MEFs)

Embryos were harvested under sterile conditions from a pregnant mouse on day E13.5 post-fertilization by removing the uterine horns and placing them into a 10-cm dish with sterile 1X PBS. The individual embryos were then separated from the uterine horn and yolk sac using scissors and forceps and placed into a 6-well plate containing sterile 1X PBS. The scissors and forceps were rinsed with 70% ethanol and sterile 1X PBS between handling of each embryo to prevent cross contamination. Once the embryos were placed into separate wells of the 6-well plate, the head of the embryo was removed and placed into a 1.5 mL Eppendorf tube for isolation of genomic DNA and genotyping (see Sections D and E). Next, the liver of the embryo was removed and the remaining embryonic tissue was placed into a 15 mL tube containing 500 µL of ice-cold 1X trypsin-EDTA (0.25% trypsin/2.21 mM EDTA in Hank’s Balanced Salt Solution without sodium bicarbonate, calcium, and magnesium; Cellgro) inside a sterile tissue culture hood and incubated on ice overnight in a 4°C room.

The following morning, excess trypsin was drained from each 15 mL tube before adding 2 µL DNase I (2000 U/mL, New England Biolabs) to the embryo. The 15 mL tube was then placed into a 37°C water bath for five minutes; gently vortexing before, midway through, and after the five minute incubation period. Following the incubation, 2.5 mL of primary MEF media (Dulbecco’s Modified Eagle Medium
(DMEM; Cellgro) supplemented with 10% fetal bovine serum (FBS; Cellgro), 100 µg/mL penicillin/streptomycin, 2 mM L-glutamine, 1X nonessential amino acids (Cellgro) 1.25 µg/mL Plasmocin (InvivoGen), and 0.1 mM 2-mercaptoethanol, was used to inactivate the trypsin. Using a sterile 10 mL serological pipette, the media/embryo mixture was pipetted up and down at least 10 times to break the embryo into a single cell suspension. The 15 mL tube was then left undisturbed for 5 minutes to allow any undigested tissue to settle to the bottom. The cells in single suspension at the top of the 15 mL tube were transferred to a new sterile 15 mL tube, while another 2.5 mL of primary MEF media was added to the original tube. Once again, a 10 mL serological pipette was used to break up the undigested tissue into single cell suspension by pipetting up and down at least 10 times and then left undisturbed for five minutes, after which point the single cell suspension at the top of the tube was transferred to the second 15 mL tube. Finally, 5 mL of fresh primary MEF media was added to the 5 mL single cell suspension, mixed and plated on a 10 cm plate coated with 0.2% gelatin. The cells were washed with 1X PBS and given fresh media the following day.

To immortalize the primary MEF lines, passage two (P2) MEFs at approximately 50% confluence were transfected with SV40 large-T antigen. For transfection, 10 µg of the pMSSVLT plasmid was mixed with 25 µL of Lipofectamine 2000 (Invitrogen) in 1 mL of opti-MEM reduced serum media (Life Technologies) and incubated for 20 minutes. After incubation, the lipofectamine/DNA mix was added dropwise to primary MEFs in a 10-cm dish and returned the incubator. The next morning, the cells were washed with 1X PBS and given fresh primary MEF media. The MEFs were subsequently cultured for 10-12 passages until only immortalized cells remained (approximately 4 weeks in total). The genotype of the immortalized MEFs was confirmed by PCR prior to freezing.

H. Primary and immortalized MEF cultures

All MEFs were grown in a sterile 37˚C incubator with a humidified 5% CO2 atmosphere. Primary MEFs were cultured in Dulbecco’s Modified Eagle Medium (DMEM; Cellgro) supplemented with 10% fetal bovine serum (FBS; Cellgro), 100 µg/mL penicillin/streptomycin, 2 mM L-glutamine, 1X nonessential amino acids (Cellgro) 1.25 µg/mL Plasmocin (InvivoGen), and 0.1 mM 2-mercaptoethanol. Immortalized MEFs were cultured in DMEM supplemented with 10% FBS, 100 µg/mL penicillin/streptomycin, and 2 mM...
L-glutamine.

All MEFs were passaged by washing with 1X PBS and incubating with 1X trypsin-EDTA (0.25% trypsin/2.21 mM EDTA in Hank’s Balanced Salt Solution without sodium bicarbonate, calcium, and magnesium; Cellgro) at 37˚C for 3 minutes. Trypsin was then neutralized with the appropriate MEF medium, transferred to a 15-mL tube, and centrifuged at 1200 RPM for 3 minutes. Primary MEFs were plated on dishes coated with 0.2% gelatin.

To freeze cells, MEFs were resuspended in 2X freezing medium (80% FBS and 20% dimethylsulfoxide) and an equal volume of MEF media. Cells were then kept at -80˚C overnight in a cell freezing container (Thermo Scientific) and transferred to liquid nitrogen the next morning.

I. ES cell generation

Blastocysts were recovered from pregnant mice at embryonic day E3.5 and transferred into 96-well plates containing a layer of primary MEF feeder cells (see Section J) in ES medium (Dulbecco’s Modified Eagle Medium (DMEM; Cellgro) supplemented with 15% Hyclone ES cell screened fetal bovine serum (FBS, Fisher Scientific), 100 µg/mL penicillin/streptomycin, 2 mM L-glutamine, 1X nonessential amino acids (Cellgro), 1.25 µg/mL Plasmocin (InvivoGen), 0.1 mM 2-mercaptoethanol, and 1000 units/mL leukemia inhibitory factor (LIF, Millipore)). The Blastocysts were then left untouched for 6 days in a sterile 37˚C incubator with a humidified 5% CO2 atmosphere. After the 6-day incubation, the cells were washed with 1X PBS and incubated with 30 µL of half-strength trypsin (0.125% trypsin/2.21 mM EDTA in Hank’s Balanced Salt Solution without sodium bicarbonate, calcium, and magnesium; Cellgro) for 6 minutes at 37˚C. The trypsin was then neutralized with 180 µL of ES medium, and the cells transferred to another 96-well plate with primary MEF feeder cells. After overnight incubation at 37˚C, the cells were washed with 1X PBS and given fresh ES medium. ES cell colonies were visible approximately two days after transfer to the second 96 well plate. When confluent, the ES cells were then transferred to a 24-well plate and frozen down.
J. ES cell culture

Embryonic stem cells (ES cells) were cultured in Dulbecco's Modified Eagle Medium (DMEM; Cellgro) supplemented with 15% HyClone ES cell screened fetal bovine serum (FBS, Fisher Scientific), 100 µg/mL penicillin/streptomycin, 2 mM L-glutamine, 1X nonessential amino acids (Cellgro), 1.25 µg/mL Plasmocin (InvivoGen), 0.1 mM 2-mercaptoethanol, and 1000 units/mL leukemia inhibitory factor (LIF, Millipore), and housed in a sterile 37°C incubator with a humidified 5% CO2 atmosphere. To prevent differentiation, ES cells were cultured on a layer of mitotically inactive primary mouse embryonic fibroblasts (MEFs). Primary MEFs were seeded onto 0.2% gelatin coated plates and inactivated when approximately 90% confluent by 2-hour incubation with 5 µg/mL mitomycin C (MMC, Sigma) in primary MEF media (Dulbecco's Modified Eagle Medium (DMEM; Cellgro) supplemented with 10% fetal bovine serum (FBS; Cellgro), 100 µg/mL penicillin/streptomycin, 2 mM L-glutamine, 1X nonessential amino acids (Cellgro), 1.25 µg/mL Plasmocin (InvivoGen), and 0.1 mM 2-mercaptoethanol). Following MMC inactivation, the primary MEFs were washed twice with 1X PBS and cultured in ES cell media.

ES cells were passaged by washing with 1X PBS and incubated with 1X trypsin-EDTA (0.25% trypsin/2.21 mM EDTA in Hank's Balanced Salt Solution without sodium bicarbonate, calcium, and magnesium; Cellgro) at 37°C for 5 minutes. Trypsin was then neutralized with ES cell media, transferred to a 15-mL tube, and centrifuged at 1200 RPM for 3 minutes. Finally, the ES cells were seeded onto dishes containing a single layer of mitotically inactivated primary MEFs.

To freeze cells, ES cells were resuspended in 2X ES freezing medium (80% HyClone ES cell screened FBS and 20% dimethylsulfoxide) and an equal volume of ES media. Cells were then kept at -80°C overnight in a cell freezing container (Thermo Scientific) and transferred to liquid nitrogen the next morning.

K. Gene targeting analysis

Gene targeting of \(Bard1^{+/+}\), \(Bard1^{K/A}\), and \(Bard1^{S/F}\) ES cells at the \(Pim1\) locus was accomplished by first linearizing the p59xDR-GFP6 targeting vector, a plasmid that contains the DR-GFP construct along with a promoterless hygromycin resistance gene flanked by the \(Pim1\) sequence, with \(XhoI\) endonuclease (Pierce & Jasin, 2001). An 80% confluent 10-cm dish of ES cells growing on
mitotically inactivated primary MEFs was trypsinized for 8 minutes, neutralized with ES cell media, and centrifuged at 1200 RPM for 2 minutes. The media was aspirated and the cells resuspended in 500 µL of 1X PBS. The cells were then transferred to a 0.4 cm electroporation cuvette (USA Scientific) along with 25 µg of the linearized p59xDR-GFP targeting vector, electroporated at 0.8V/10 µF, and incubated at room temperature for 5 minutes. Following electroporation, the cells were resuspended in ES cell media, and then seeded onto three 10-cm dishes containing mitotically inactivated primary DR4 feeder MEFs (See Section J). The DR4 MEFs are cells that have been genetically engineered to contain resistance genes to neomycin, hygromycin, puromycin, and 6-thioguanin. The next day, the ES cell media was replaced with selection media (ES media supplemented with 0.2 mg/mL hygromycin B (Sigma). The ES cells were then given fresh selection media daily for the next 7-10 days, until the hygromycin-resistant ES clones were large enough to be seen with the naked eye. Individual hygromycin-resistant ES clones were then transferred from the 10-cm dish to a 96-well plate containing DR4 feeder MEFs, and each clone expanded into 48-, 12-, and finally 6-well plates. At the 96-well plate stage, genomic DNA was harvested from each hygromycin resistant clone for verification of the genotype by PCR and verification of correct targeting by Southern blot analysis (See Section F). For Southern analysis, the genomic DNA from the ES cell clones was digested with HincII, and hybridized with the “P22” probe, resulting in a 3.6 kb fragment for the wild type Pim1 allele and a 2.4 kb fragment for the targeted Pim1 allele (Moynahan, Pierce, et al., 2001).

L. DR-GFP assay

To perform the DR-GFP assay, which measures the repair of I-SceI-induced chromosomal breaks, ES cells harboring the DR-GFP reporter at the Pim1 locus (See Section K) growing exponentially on primary MEF feeder cells were harvested by trypsinization, centrifuged at 1000 RPM for 5 minutes, and resuspended in ES transfection medium (ES medium without penicillin/streptomycin, plasmocin, or LIF) at a concentration of 0.8 x 10⁶ cells per mL. While harvesting the cells, the transfection mix was prepared by combining either 0.5 µg of empty vector (pCAGGs), I-SceI expression vector, or GFP expression vector with 1.2 µL of Lipofectamine 2000 (Invitrogen) in 66 µL of opti-MEM reduced serum media (Life Technologies) and incubating for 20 minutes. After incubation, the ES cells from each clone
were seeded onto 7 wells of a gelatin-coated, feederless 24-well plate (0.2 mL total volume, \(0.16 \times 10^6\) cells per well, 3 wells for empty vector transfection, 3 wells for I-SceI transfection, and 1 well for GFP transfection). Immediately after seeding the ES cells, the DNA/lipofectamine mix was added to the appropriate well and incubated for 6 hours. The transfection mix was then diluted out by adding 1 mL of ES media (now with penicillin/streptomycin, plasmocin, and LIF) to each well. The following morning the cells were given fresh ES media and allowed to grow undisturbed for an additional 48 hours. After the 48-hour growth step, the cells were washed with 1X PBS, trypsinized, centrifuged at 5000 RPM, and resuspended in 0.3 mL of 1% FBS/PBS, after which they were kept on ice and protected from light until analysis.

Flow cytometry was performed on a FACScalibur machine using CellQuest software (BD Biosciences), and analysis of the data was carried out using FlowJo version X software. The efficiency of repair of the I-SceI-induced chromosomal break was measured by the percentage of GFP-positive cells. Gating for ES cells was performed by sorting cells based on side-scatter height vs forward-scatter height and selecting the appropriate sized population of cells. GFP-positive ES cells were then selected by sorting cells by green fluorescence intensity (FL1-H) vs orange fluorescence intensity (FL2-H) and gating for cells that showed a significant increase in green fluorescence intensity compared to orange fluorescent intensity (and therefore were not autofluorescent). At least 25,000 cells were counted per experimental condition. The percent of GFP positive cells was normalized to transfection efficiency by measuring the percentage of GFP positive cells in the well that was transfected with the GFP expression vector and then dividing the percentage of GFP positive cells in the I-SceI transfected wells with this value.

**M. Western blot and cell fractionation**

Exponentially growing immortalized MEFs seeded 48 hours earlier were harvested by scraping in 3 mL of ice cold 1X PBS and kept on ice. To harvest any remaining cells, the plate was then washed with an additional 2 mL of ice-cold 1X PBS and the wash transferred to the 3 mL of cell/PBS suspension. The cells were then centrifuged at 1200 RPM for 3 minutes at 4˚C. After centrifugation, the cells were lysed in low-salt Nonidet-40 (NP40) lysis buffer (10 mM Hepes pH 7.6, 0.25 M NaCl, 0.1% NP40, 5 mM EDTA,
10% glycerol) supplemented with complete protease inhibitor cocktail (Roche), 1 mM dithiothreital (DTT), and 25 mM NaF, and incubated for 10 minutes on ice. The cell/lysis buffer mixture was then centrifuged at 13000 RPM for 10 minutes at 4°C and the supernatant (now referred to as the “cell lysate”) transferred to a separate tube for protein concentration measurement.

To perform cell fractionation, exponentially growing immortalized MEFs seeded 48 hours earlier were harvested, washed with 1X PBS, and centrifuged as described above. After centrifugation, the cells were lysed in 5 times the cell pellet volume of buffer A (10 mM Hepes pH 7.9, 10 mM KCl) supplemented with protease inhibitor cocktail (Roche) and incubated for 10 minutes on ice. Following the incubation, 1/16th lysate volume of 10% NP40 was added to the cell lysate and vortexed for 10 seconds. The lysates were then centrifuged at 5000 RPM for 2 minutes at 4°C to separate into nuclear (pellet) and cytoplasmic (supernatant) fractions.

The resulting supernatant (cytoplasmic fraction) was removed and 0.11 times the total cytoplasmic fraction volume of buffer B (0.3 M Hepes pH 7.9, 1.4 M KCl, 1 mM DTT) was added. The cytoplasmic fraction was then centrifuged at 13000 RPM for 10 minutes at 4°C and the resulting supernatant collected for protein concentration measurement.

To process the nuclear fraction, the cell pellet obtained from the 5000 RPM centrifugation step was resuspended in 2 times the cell pellet volume of buffer C (20 mM Hepes pH 7.9, 25% v/v glycerol, 0.42 M NaCl, 0.2 mM EDTA, protease inhibitor cocktail, and 1 mM DTT). The nuclear fraction was then vortexed for 10 minutes at 4°C and centrifuged at 13000 RPM for 10 minutes. The resulting supernatant (nuclear extract) was collected for protein concentration measurement.

Using the Bradford protein assay reagent (Bio-Rad), a Bradford assay was performed to determine the concentration of proteins in the cell lysates, cytoplasmic fractions, and nuclear extracts. 5X protein loading dye (0.313 M Tris-Cl pH6.8, 10% SDS, 50% glycerol, 25% 2-mercaptoethanol, and 0.05% bromophenol blue) was then added to the cell lysates before boiling the loading dye/lysate mixture for 3 minutes to denature the proteins. The lysates were then loaded onto 6.5% polyacrylamide gels and electrophoresed in tris-glycine SDS gel running buffer (250 mM trizma base, 1.9 M glycine, 1% SDS) at 130 V for approximately 1 hour and 45 minutes. Following electrophoresis, lysates were transferred onto an Amersham Protran 0.45 µm nitrocellulose membrane (GE Healthcare Life Sciences) in western
transfer buffer (25 mM tris-Cl pH 7.6, 190 mM glycine, 20% methanol, 0.04% SDS) at 22 V overnight at room temperature. The following day, the membrane was blocked in 10% milk in TBS-T (20 mM Tris-Cl pH 7.6, 0.137 M NaCl, 0.1% Tween 20) for 30 minutes at room temperature. The membrane was then washed once in 1X TBS-T for 5 minutes on a shaking platform, followed by staining with the appropriate primary antibody (see Table 2 for antibody concentrations) diluted in 2% milk/TBS-T for 2 hours at room temperature. Following primary antibody staining, the membrane was rinsed once with 1X TBS-T and then washed three times for 10 minutes each in 1X TBS-T on a shaking platform. The membrane was then incubated with either HRP-conjugated goat anti-mouse (Sigma) or HRP-conjugated donkey anti-rabbit (GE Healthcare Life Sciences) secondary antibodies diluted 1:10,000 in 2% milk/TBS-T. Once again, the membrane was rinsed once and then washed three times for 10 minutes each with 1X TBS-T on a shaking platform. After the wash steps, the membrane was incubated with either SuperSignal West Pico Chemiluminescent substrate (ThermoFischer Scientific) or SuperSignal West Dura Chemiluminescent substrate (ThermoFischer Scientific) for 3 minutes. The membranes were then dried by blotting with Whatman paper and then exposed on CL-XPosure film (ThermoFischer Scientific).

**N. Immunoprecipitation with Bard1**

For immunoprecipitation experiments, exponentially growing cells seeded 48-hours prior to collection were harvested in low salt lysis buffer supplemented with complete protease inhibitor cocktail, 1 mM dithiothreital (DTT), and 25 mM NaF, and the protein concentration of the resulting lysate determined by Bradford assay as described in Section M. Immunoprecipitation was then performed by incubating 600 µg of protein with the mouse Bard1-specific 1734 rabbit polyclonal antibody (1:50) (E. E. McCarthy et al., 2003) at 4°C on a rotator for 2 hours. The 600 µg of protein was diluted as needed with low salt lysis buffer so that all samples were in the same total volume. After the 2-hour incubation, 50 µL of protein A sepharose CL-4B beads (50% v/v in low salt lysis buffer, GE Healthcare Life Sciences) was added to the protein lysate/antibody mixture and incubated on a rotator at 4°C for an additional 30 minutes. The lysate/bead mixture was then centrifuged at 500 g for 3 minutes at 4°C and the resulting supernatant was discarded. The beads were then washed with 800 µL of low salt lysis buffer, incubated on a rotator at 4°C for 3 minutes, and centrifuged at 500 g for 3 minutes at 4°C. The resulting supernatant was again
discarded and the wash procedure repeated two more times. Following the final wash, approximately 25 µL of low salt lysis buffer was left on the beads and the rest discarded. Twenty-five µL of 2x protein loading dye was added to the beads and then boiled for 3 minutes to elute the protein. Immediately after boiling, the samples were electrophoresed on 6.5% polyacrylamide gels in tris-glycine SDS gel running buffer at 140V for approximately 1 hour and 45 minutes. The gel was then transferred overnight at 22 V at room temperature and the western blotting procedure was followed the next day as described in Section M.

O. Rad51 staining and foci quantification

Immortalized MEFs were seeded onto poly-L-lysine (Sigma) coated coverslips in 6-well plates. Forty-eight hours after seeding, the cells were treated with 10 Gy of ionizing radiation using an Atomic Energy of Canada Gammacell 40 Cesium Unit. After irradiation, the cells were returned to the 37°C incubator for one hour. The cells were then fixed by washing three times with 1X PBS and incubating with 3.7% paraformaldehyde(PFA)/PBS solution for 20 minutes at room temperature. The PFA was aspirated and the cells were washed four times with 1X PBS before permeabilization with 1% triton X-100/PBS for 5 minutes at room temperature. The cells were then washed an additional three times with 1X PBS and blocked in 5% bovine serum albumin (BSA)/PBS for 1 hour at 37°C. After blocking, the cells were incubated with the Rad51 primary antibody (rabbit polyclonal, Calbiochem AB-1, 1:200 dilution) diluted in 5% BSA/PBS in a humidified chamber for 1 hour 45 minutes at 37°C. The cells were then washed three times with 1X PBS and incubated with secondary antibody (goat anti-rabbit Alexa 488, ThermoFischer Scientific, 1:1000 dilution) diluted in 5% BSA/PBS in a humidified chamber for 45 minutes at 37°C. Following application of the secondary antibody, the cells were rinsed four times with 1X PBS and dehydrated using successive rinses of 70%, 90%, and 100% ethanol. The cells were then mounted onto a glass slide with Vectashield hard set mounting medium with 4’, 6-diamidino-2-phenylindole (DAPI; Vector Laboratories). The mounting media was allowed to dry for 20 minutes before sealing the coverslips to the glass slides with clear nail polish.

The cells were imaged on an Axio Imager Z2 fluorescent microscope with Coolcube1 camera (Zeiss) at 40x magnification. Automated Rad51 foci quantification was carried out using the Metafer 4
software (Metasystems). At least 300 cells were counted per trial.

**P. PCNA-Bard1 and PCNA-Brca1 staining and colocalization**

Immortalized MEFs were seeded onto poly-L-lysine (Sigma) coated coverslips in 6-well plates. 48 hours after seeding, the cells were treated with either 2 mM hydroxyurea (HU, Sigma) or PBS for 1.5 hours. After treatment, the cells were harvested by washing three times with ice cold CSK buffer (10 mM piperezine-N,N'-bis(2-ethanesulfonic acid) (PIPES), 100 mM NaCl, 300 mM sucrose, and 3 mM MgCl₂). Cells were then permeabilized with ice cold 0.5% triton/CSK for 5 minutes at 4°C. Permeabilization buffer was washed off by rinsing twice with ice-cold CSK buffer and twice with 1X PBS. Cells were then fixed by incubating with ice cold 100% methanol at −20°C for 10 minutes. After fixation, the cells were rinsed twice with 1X PBS and washed three times with 1X PBS on a platform shaker for 5 minutes each. Blocking was performed by incubating the cells in 5% BSA, 0.1% triton/PBS for 30 minutes on a platform shaker at room temperature. Cells were then stained with the following primary antibodies: PCNA (mouse monoclonal, Santa Cruz Biotechnology PC10, 1:200 dilution) and either Brca1 (rabbit polyclonal 57J, 1:250 dilution) (Shakya et al., 2011) or Bard1 (rabbit polyclonal 1734R, 1:500 dilution) (E. E. McCarthy et al., 2003) diluted in 1% BSA 0.1% triton/PBS for 1 hour at room temperature. Following primary antibody application, cells were rinsed twice with 0.1% triton/PBS and then washed three times with 0.1% triton/PBS for 5 minutes each on a platform shaker. Cells were then incubated with secondary antibodies (goat anti-rabbit Alexa Fluor 488, ThermoFischer Scientific, 1:1000 dilution; and goat anti-mouse Alexa Fluor 568, ThermoFischer Scientific, 1:400 dilution) diluted in 1% BSA, 0.1% triton/PBS for 30 minutes at room temperature while protected from light. Once again, the cells were rinsed twice with 0.1% triton/PBS and then washed three times with 0.1% triton/PBS for 5 minutes each on a platform shaker while protected from light. Following the wash steps, the cells were rinsed twice with 1X PBS and then dehydrated using successive rinses of 70%, 90%, and 100% ethanol. The cells were then mounted onto a glass slide with Vectashield hard set mounting medium with 4’, 6-diamidino-2-phenylindole (DAPI; Vector Laboratories). The mounting media was allowed to dry for 20 minutes before sealing the coverslips to the glass slides with clear nail polish.

The cells were imaged on an Eclipse 80i fluorescent microscope (Nikon) with CoolSNAP HQ2
camera (Photometrics) at 40x magnification. Blue, red, and green channel images were merged and analyzed using imageJ software. At least 200 cells were counted by hand per trial. A “Brca1-positive” cell was defined as a cell containing 5 or more Brca1 foci. A “Bard1-positive” cell was defined as a cell containing 5 or more Bard1 foci. A “PCNA-positive” cell was defined as a cell containing 5 or more PCNA foci. PCNA and Bard1 (or Brca1) were scored as “co-localizing” if over half of the Bard1 (or Brca1) foci overlapped with PCNA foci. Percent co-localization was defined as the number of co-localizing cells over total number of PCNA-positive cells (Figure 33).

**Table 2: Antibody dilutions**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Species</th>
<th>Source</th>
<th>Western blot</th>
<th>Immunocytochemistry</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bard1 (1734)</td>
<td>Rabbit polyclonal</td>
<td>(E. E. McCarthy et al., 2003)</td>
<td>1:2000</td>
<td>1:500</td>
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<tr>
<td>Brca1</td>
<td>Mouse monoclonal</td>
<td>(Fernandez-Capetillo &amp; Nussenzweig, 2013)</td>
<td>1:500</td>
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</tr>
<tr>
<td>Brca1 (57)</td>
<td>Rabbit polyclonal</td>
<td>(Shakya et al., 2011)</td>
<td>1:2000</td>
<td>1:250</td>
</tr>
<tr>
<td>Ctip (14-1)</td>
<td>Mouse monoclonal</td>
<td>(Yu &amp; Baer, 2000)</td>
<td>1:50</td>
<td></td>
</tr>
<tr>
<td>PCNA</td>
<td>Mouse monoclonal</td>
<td>Santa Cruz Biotechnology (PC10)</td>
<td>1:200</td>
<td></td>
</tr>
<tr>
<td>Rad51</td>
<td>Rabbit polyclonal</td>
<td>Calbiochem (Ab-1)</td>
<td>1:200</td>
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<tr>
<td>α-Tubulin</td>
<td>Mouse monoclonal</td>
<td>Calbiochem (DM1A)</td>
<td>1:10,000</td>
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</tr>
</tbody>
</table>

**Q. Analysis of metaphase spreads using Giemsa stain or Telomere fluorescent in situ hybridization (T-FISH)**

For T-FISH analysis, metaphase spreads were prepared from passage 3 (P3) or earlier primary MEFS. The day prior to drug treatment, the primary MEFs were plated on 0.2% gelatin-coated plates and allowed to attach overnight. For Giemsa stain analysis, metaphase spreads were prepared from immortalized MEFs plated one day prior to genotoxin exposure. For genotoxin exposure in both T-FISH and Giemsa stain analysis, cells were treated the day after plating with either 40 ng/mL mitomycin C
(MMC, Sigma) or mock treatment with 1X PBS for 16 hours (overnight). Four hours prior to the end of genotoxin treatment, Karyomax colcemid solution (Invitrogen) was added at a concentration of 0.1 µg/mL.

Following the colcemid treatment, the cell media was collected in order to preserve any mitotic cells that may have become detached. The cells were then washed with 1X PBS and trypsinized, saving the wash and trypsinized cells along with the cell media. The media/wash/cell mixture was then centrifuged at 1000 RPM for 5 minutes. The supernatant was aspirated, the resulting cell pellet was washed with 10 mL of 1X PBS, and then centrifuged again at 1000 RPM for 5 minutes. The cell pellet was then resuspended in 0.4% KCl (w/v) solution by adding the solution dropwise while gently vortexing. The cells were then allowed to swell by incubating in the KCl solution for 20 minutes at room temperature while gently inverting the tubes every 5 minutes. At the end of the incubation period, 10 drops of 3:1 methanol/glacial acetic acid fixative solution was added to the tube before centrifuging for 10 min at 800 RPM. The resulting supernatant was then removed and the cells were resuspended in 10 mL of fixative solution by adding the fixative dropwise while gently vortexing. Again, the cells were centrifuged at 800 RPM and the resulting supernatant was aspirated. The fixative wash and centrifugation steps were then repeated an additional three times, before resuspending the cells in 10 mL of fixative and incubating at – 20˚C overnight.

The next day, the fixative wash process was repeated three more times before resuspending the cells in a volume of 250µL – 1 mL depending on the confluency of the culture at the time of harvest. A volume of 10 µL of each cell suspension was dropped from a height of approximately 0.3 m onto glass slides. To facilitate the spreading of the cells across the slide, the slide was held over a 95˚ C steam bath for 5 seconds, approximately 10 seconds after dropping the cell suspension. The cells were allowed to dry overnight before staining with either Giemsa stain or telomere probe for T-FISH.

To stain the slides for Giemsa stain analysis, cells were incubated with Karyomax Giemsa stain (Gibco) diluted 1:10 in 1X Gurr Buffer (Gibco) for 3 minutes. The slides were then rinsed with ddH2O to remove excess stain. The metaphase spreads were then imaged using a Nikon Eclipse E600 light microscope using a Nikon DS-Fi1 camera at 100X magnification. Twenty-five metaphases were analyzed for gross structural abnormalities per condition.

To stain the slides for T-FISH analysis, they were first washed in 1x PBS for 15 minutes followed
by fixation in 4% formaldehyde/PBS for 2 minutes. The slides were then washed three times in 1X PBS for five minutes each. After the washes, the cells were digested for 10 minutes at 37°C in pre-warmed acidified pepsin (2500 units/mL Sigma and 10 M HCl). Two washes for 5 minutes in 1X PBS were then performed followed by a second fixation step in 4% formaldehyde/PBS for two minutes, after which an additional three washes for 5 min each in 1X PBS were performed. The slides were then dehydrated using successive washes in 70% ethanol, 90% ethanol, and 100% ethanol for five minutes each before allowing the slides to air dry for 20 minutes. Probe mix (10 mM Tris Cl pH 7.2, 2 mM MgCl₂, 0.75 mM citric acid, 7 mM Na₂HPO₄, 70% deionized formamide, 0.5 µg/µL telomere probe, and 0.25% blocking reagent for nucleic acid hybridization and detection (Sigma)) was added to the slides and incubated at 80°C for three minutes to denature the DNA. Following the denaturation step, the slides (with probe mix still present) were transferred to a humidified chamber and incubated at room temperature for 2 hours. The slides were then incubated with wash buffer (70% formamide, 10 mM Tris pH 7.2, 0.1% BSA) on a shaking incubator twice for 15 minutes each. Next, the slides were washed in 0.08% TBS-T (20 mM Tris-Cl pH 7.6, 0.137 M NaCl, 0.08% Tween 20) three times for five minutes each. After the washes, the slides were dehydrated using successive washes in 70% ethanol, 90% ethanol, and 100% ethanol for five minutes each before allowing to air dry for 20 minutes. Finally, glass coverslips were mounted onto the slides using Prolong Gold Antifade with DAPI (Thermo Fisher Scientific) and sealed with clear nail polish.

The T-FISH metaphase spreads were imaged on an Axio Imager Z2 fluorescent microscope with Coolcube1 camera (Zeiss). Metafer software version 3.10.6 (Metasystems) was used to automatically locate metaphases at 10x magnification and then automatically capture images at 63x magnification. The metaphases were then analyzed on Isis fluorescent imaging system software (Metasystems). At least 25 metaphases were counted per experimental condition.

**R. DNA Fiber Assay**

Forty-eight hours after seeding in 6-well plates, exponentially growing immortalized MEFs were pulse labeled with 200 µM 5-iodo-2'-deoxyuridine (IdU, Sigma) for 20 minutes at 37°C. After IdU treatment, the cells were washed three times with warm (37°C) 1X PBS and pulse labeled with 100 µM 5-chloro-2'-deoxyridine (CldU, Sigma) for 20 minutes at 37°C. The cells were again washed with warm 1X
PBS three times and then either harvested (untreated control) or treated with 2 mM hydroxyurea (HU, Sigma) for 1.5 hours followed by harvest (Figure 27A). To inhibit the Mre11 nuclease, cells were treated with 50 µM mirin (Sigma) during pulse labeling with IdU and CldU, as well as during the subsequent hydroxyurea treatment. To harvest the cells, 0.5 mL of 1X trypsin-EDTA (0.25% trypsin/2.21 mM EDTA in Hank’s Balanced Salt Solution without sodium bicarbonate, calcium, and magnesium; Cellgro) was added to each well of the 6-well plate and incubated at 37°C for 3 min. The trypsin was then neutralized with 2.5 mL immortalized MEF media (DMEM supplemented with 10% FBS, 100 µg/mL penicillin/streptomycin, and 2 mM L-glutamine), transferred to a 15-mL falcon tube, and centrifuged at 1200 RPM for 2 minutes. After centrifugation, the cell pellet was resuspended in 500 µL ice-cold 1X PBS and kept on ice thereafter to prevent further DNA synthesis or degradation. The concentration of cells in 1X PBS was then determined with a hemocytometer and adjusted to 0.3 x 10^6 cells/mL. Two microliters of the cell mixture was pipetted onto precleaned glass microscope slides and allowed to partially dry for 5 minutes. After partial drying, the cells were lysed using pre-warmed spreading buffer at 37°C (0.5% sodium dodecyl sulfate, 20 mM Tris-Cl pH 7.4, and 50 mM EDTA) for 10 minutes in a humidified chamber at room temperature. Spreading of the DNA was then achieved by tilting the slides at a 15° angle relative to horizontal and allowing the cell lysis buffer mixture to run down the slide for 3 minutes. Following spreading, the slides were dried for 20 minutes in a fume hood and then fixed by incubating in an ice cold (~20°C) 3:1 methanol:acetic acid mixture at room temperature for 2 minutes. The slides were once again allowed to dry for approximately 20 minutes and then denatured in a 2.5 M HCl solution for 45 minutes at room temperature. The slides were then rinsed 5 times with 1X PBS to remove all residual HCl. After rinsing, the slides were blocked for 1 hour at room temperature in 3% bovine serum albumin (BSA), 0.1% triton X-100/PBS. To stain the slides with primary antibody, the slides were incubated with rat anti-BrdU to detect CldU (Abcam ab6326, diluted 1:100) and mouse anti-BrdU to detect IdU (BD biosciences, BD 347580, 1:100 dilution) diluted in blocking solution for 1 hour at room temperature in a humidified chamber. The slides were then rinsed three times with 1X PBS and stained with anti-mouse Alexa Fluor 488 (ThermoFischer Scientific, 1:300 dilution) and anti-rat Alexa Fluor 594 (ThermoFischer Scientific, 1:300 dilution) diluted in blocking solution for 30 min at room temperature in a humidified chamber. Following secondary antibody staining, the slides were washed three times with 1X PBS, dried for 45
minutes in a fume hood, and then mounted in Prolong Gold Antifade (ThermoFischer Scientific). The slides were kept at 4°C until ready for imaging. Imaging of fibers was carried out on an Eclipse 80i fluorescent microscope (Nikon) with CoolSNAP HQ2 camera (Photometrics) at 40x magnification. Analysis was performed using imageJ software. At least 150 individual fibers were measured per experimental condition.

**S. Genotoxin sensitivity assays**

For all genotoxin sensitivity assays, immortalized MEFs were used to seed 6-well plates with 1000 cells/well for both drug-treated and control plates. Each experimental condition was plated in triplicate (3 wells per condition). In the case of mitomycin C treatment (MMC; Sigma) treatment, 48 hours after plating, the immortalized MEFs were exposed to varying doses of MMC (0 ng/mL, 50 ng/mL, 100 ng/mL, 200 ng/mL, 400 ng/mL, and 800 ng/mL) for 4 hours. In the case of camptothecin (CPT; Sigma) treatment, 48 hours after plating the immortalized MEFs were exposed to varying doses of CPT (0 µM, 0.05 µM, 0.1 µM, 0.2 µM, 0.4 µM, and 1.0 µM) for 1 hour. After drug treatment the cells were washed two times with 1X PBS, given fresh media, and allowed to grow until harvest 5-7 days post treatment.

In the case of PARP inhibitor (PARPi) treatment, at 24 hours after plating, immortalized MEFs were exposed to various concentrations of olaparib (SelleckChem; 0 µM, 0.064 µM, 0.16 µM, 0.4 µM, 1.0 µM, 2.5 µM, and 5.0 µM). The media containing olaparib was replaced with fresh media containing olaparib every 48 hours until cell harvest 6-8 days after initial drug treatment.

In the case of ionizing radiation (IR) treatment, immortalized MEFS were irradiated 48 hours after plating with varying doses of IR (0 Gy, 2 Gy, 4 Gy, 6 Gy, 8 Gy, and 10 Gy) with an Atomic Energy of Canada Gammacell 40 Cesium unit. After irradiation, the cells were allowed to grow undisturbed until harvest 5-7 days later.

In all cases, the immortalized MEFs were harvested approximately 7 – 9 days after seeding, when the colonies in the untreated control plate were judged to be large enough to count. For harvest, the cells were washed twice with 1X PBS and then stained with a 0.5% crystal violet, 50% methanol solution for 20 minutes. Excess stain was removed by washing the plates with H2O three times and drying overnight. The following day, the surviving colonies of cells were counted using a light box and
magnifying glass. A colony was only counted if it contained 50 or more cells.

**T. Alkaline comet assays**

One day prior to performing the assay, precleaned glass microscope slides (Fisher Scientific) were coated with two layers of molten 1% agarose/PBS. The slides were allowed to dry for at least 5 hours between the first and second agarose coat. Immortalized MEFs were seeded onto 12-well plates and then treated the following day with 2 mM hydroxyurea (HU, Sigma), 100 nM camptothecin (CPT, Sigma), PBS mock treatment, or DMSO mock treatment for 5 hours at 37°C. Immediately following the drug treatment, the cells were harvested using 0.25 mL of 1X trypsin-EDTA (0.25% trypsin/2.21 mM EDTA in Hank’s Balanced Salt Solution without sodium barconbanate, calcium, and magnesium; Cellgro) for 5 min at 37°C, inactivated with 1.5 mL immortalized MEF media media (DMEM supplemented with 10% FBS, 100 µg/mL penicillin/streptomycin, and 2 mM L-glutamine), and centrifuged at 1200 RPM for 5 minutes. The cells were then resuspended in 0.5 mL ice cold 1X PBS and kept on ice thereafter to prevent DNA repair. The cells were centrifuged again at 1200 RPM for 5 minutes at 4°C to remove any residual media and resuspended in 20 µL of ice cold 1X PBS. Ten µL of the cell/PBS mixture were then added to 75 µL of 0.5% low melting agarose/PBS and pipetted onto the agarose-coated slides. Spreading of the cell/low melting agarose mixture was facilitated by application of a glass coverslip. The low melting agarose was then allowed to solidify for 10 minutes at room temperature before removal of the coverslip.

After solidification of the low melting agarose, the slides were incubated with pH 10.0 lysis buffer (25 mM NaCl, 100 mM EDTA, 10 mM trisma base, and 1% triton X-100) at 4°C overnight while protected from light. The following the day, the slides were equilibrated in pre-chilled (4°C) electrophoresis buffer (300 mM NaOH, 1 mM EDTA) for 20 minutes. After equilibration, electrophoresis was performed at 0.6 V/cm (20 V) in a horizontal chamber (FischerScientific) for 20 minutes. The slides were then neutralized by washing with neutralization buffer (0.4 M Tris-Cl, pH 7.5) three times for five minutes each and then rinsed with ddH2O three times. Following the washing steps, the slides were fixed in ice cold 100% ethanol for 20 minutes at room temperature and then allowed to dry completely in a fume hood.

Following drying of the slides, the comets were stained by pipetting 90 µL of a fluorescent dye
(GelRed, Biotium, dilution 1:1000 in H2O) onto the slide and placing a coverslip over the dye. The comets were then imaged on an Eclipse 80i fluorescent microscope (Nikon) with CoolSNAP HQ2 camera (Photometrics) at 20x magnification. Comet tail moment values were determined using CometScore Software Version 1.5. At least 75 tails were analyzed per experimental condition. Apoptotic cells (small comet head and very large comet tail) were excluded from the analysis.
CHAPTER III

PHOSPHO-RECOGNITION BY THE BARD1 BRCT DOMAIN IS DISPENSABLE FOR BRCA1/BARD1-MEDIATED HOMOLOGY DIRECTED REPAIR
A. Introduction

Approximately 12% of women in the general population will develop breast cancer at some point during their lives (Siegel et al., 2017). In contrast, women who inherit a single pathogenic allele of the BRCA1 gene have a 50-80% lifetime risk of breast cancer (Antoniou et al., 2003; Roy et al., 2011). BRCA1 has been implicated in a myriad of cellular functions, including transcriptional regulation, chromatin remodeling, protein ubiquitination, cell cycle checkpoint activation, stalled fork protection, centrosome duplication, maintenance of genome stability, and homology directed repair (Jiang & Greenberg, 2015; Moynahan & Jasin, 2010; Nagaraju & Scully, 2007; Roy et al., 2011; Venkitaraman, 2014). Despite decades of BRCA1 research, it is not known which of these function(s) is required for the tumor suppressor activity of BRCA1, and how its loss accounts for the staggering increase in breast cancer risk. Two aspects of BRCA1 function, homology-directed repair (HDR) and stalled replication fork protection (SFP), have received special attention, in part because they are critical for genome stability and in part because they are also dependent on BRCA2. In particular, this chapter will focus on the role of BRCA1, and its associated protein BARD1, in HDR.

BRCA1 is required for homology-directed repair (HDR), a cellular pathway that repairs double-strand DNA breaks (DSBs) with high fidelity in S and G2 phase cells. Fluorescent staining studies demonstrated that BRCA1 colocalizes with other HDR components at the sites of DSBs, and subsequent DR-GFP reporter studies demonstrated that BRCA1-mutant cells display a profound deficiency in HDR (Jin et al., 1997; Moynahan et al., 1999; Scully, Chen, Ochs, et al., 1997; Scully, Chen, Plug, et al., 1997). Consequently, cells harboring pathogenic BRCA1 lesions have a reduced capacity for HDR and accumulate high levels of chromosomal abnormalities.

The BRCA1 gene encodes a polypeptide of 1863 amino acids that contains an N-terminal RING domain and two tandem C-terminal BRCT repeats (Figure 3). In vivo, BRCA1 exists as a heterodimer with the structurally related protein BARD1 (Baer & Ludwig, 2002; Jin et al., 1997; L. C. Wu et al., 1996). Most of the cellular pool of BRCA1 polypeptides exists in complex with BARD1 and multiple lines of evidence indicate that these proteins function as an obligate stoichiometric heterodimer (Joukov et al., 2006; Yu & Baer, 2000). Of note, cellular loss of BARD1 results in HDR deficiencies and chromosomal
abnormalities similar to the phenotype of BRCA1-mutant cells (Baer & Ludwig, 2002; Fabbro et al., 2002; Joukov et al., 2006; Laufer et al., 2007; E. E. McCarthy et al., 2003; Westermark et al., 2003).

Importantly, the tumor suppression activity of BRCA1 also appears to be mediated by the BRCA1/BARD1 heterodimer, since mammary-specific inactivation of either Brca1 or Bard1 elicits basal-like triple-negative breast carcinomas in mice that are phenotypically indistinguishable (Shakya et al., 2008). In addition, germline mutations of human BARD1 have been identified as the pathogenic lesion in some families with hereditary breast and ovarian cancer (De Brakeleer et al., 2016; De Brakeleer et al., 2010; Ratajska et al., 2012; Sabatier et al., 2010). Together, these results argue that BARD1 makes unknown, but essential, contributions to the tumor suppression activity of the BRCA1/BARD1 heterodimer.

Most of the pathogenic BRCA1 lesions associated with familial breast and ovarian cancer are frameshift or nonsense mutations that would eliminate the potential to encode an intact BRCT domain. However, in some families, tumor susceptibility can be attributed to a single amino acid substitution, often involving residues within the BRCT domain. Moreover, structural studies have shown that one of these residues (S1655) forms a hydrogen bond with the phosphate group of BRCA1 phospho-ligands, and that the pathogenic S1655F mutation disrupts the interaction of BRCA1 with its known BRCT phospho-ligands (Figure 4) (Botuyan et al., 2004; Clapperton et al., 2004; Shiozaki et al., 2004; Varma, Brown, Birrane, & Ladias, 2005; Williams et al., 2004). Previously, our laboratory observed that the corresponding mutation in murine Brca1 (S1598F) abrogates HDR and renders mice susceptible to basal-like triple-negative mammary tumors reminiscent of those that arise in human BRCA1 mutation carriers (Shakya et al., 2011). These observations indicate that BRCT phospho-recognition is required for BRCA1-mediated tumor suppression and suggest that HDR is a critical component of this process.

Structural studies show that the 3-dimensional fold of the BARD1 BRCT repeats contains a hydrophilic cleft that is strikingly similar to the BRCT phosphate-binding pockets of BRCA1 and MDC1 (Figure 5) (Birrane et al., 2007) (Edwards et al., 2008). Nonetheless, proteins that bind the BARD1 BRCT domain in a phospho-dependent manner have not as yet been reported. Instead, Li and Yu (2013) showed that this domain specifically recognizes poly(ADP-ribose) (PAR), a nucleic acid-like polymer that rapidly assembles at DNA breaks and stalled replication forks (M. Li & Yu, 2013). Moreover, they found that the BARD1/PAR interaction was ablated by missense mutations predicted to disrupt the phosphate-
binding cleft of the BARD1 BRCT domain. Interestingly, this interaction was specifically required for early recruitment of the BRCA1/BARD1 heterodimer to sites of DNA damage, whereas the BRCT domain of BRCA1 mediated a subsequent phase of γH2AX-dependent BRCA1/BARD1 recruitment (M. Li & Yu, 2013). However, it is unknown whether the ability of the BARD1 BRCT domain to specifically bind PAR and/or any as yet undiscovered phosphoprotein ligands (hereafter these properties of the BRCT domain will be described as "phospho-recognition") is required for BRCA1/BARD1 maintenance of genome stability and HDR. This chapter will describe the characterization of novel Bard1-BRCT mutant mice and cell lines, and address the role of the Bard1-BRCT domain in chromosomal stability and HDR.

B. Results

1. Generation and phenotype of Bard1 BRCT-mutant mice
i. Structure of the Bard1 knock-in constructs and the phenotype of Bard1-mutant mice

Although phosphoprotein ligands for the BRCT domain of BARD1 have not yet been identified, structural studies indicate that these sequences have the potential to form a phosphate-binding cleft similar to the BRCT domains of BRCA1 and MDC1 (Figure 5) (Birrane et al., 2007; Edwards et al., 2008), and biochemical analyses confirm that this cleft specifically recognizes the phosphate-rich PAR polymer (M. Li & Yu, 2013). Each of the four amino acids of human BRCA1 that form direct contacts with phosphoserine (S1655, G1656, T1700, K1702) is conserved in the BRCT sequences of human (S575, G576, T617, K619) and mouse (S563, G564, T605, K607) BARD1. Therefore, to evaluate the function of phospho-recognition by the BARD1 BRCT domain, our laboratory introduced the S563F and K607A missense mutations into the mouse Bard1 gene. The corresponding mutations of human BARD1 (S575F and K619A) have been shown to abrogate BARD1 recognition of PAR and to disrupt γH2AX-independent recruitment of the BRCA1/BARD1 heterodimer to sites of DNA damage (M. Li & Yu, 2013). Structural studies also predict that mutations of the mouse Bard1 residues S563 or K607 would disrupt the phospho-binding activity of its BRCT domain. Moreover, the Bard1-S563F mutation is structurally analogous to the pathogenic Brca1-S1598F mutation that ablates the HDR and tumor suppression activities of Brca1 (Shakya et al., 2011).
For my dissertation research, I studied the phenotypes of mice harboring the $Bard1^{S563F}$ and $Bard1^{K607A}$ alleles. These mutant alleles had been previously generated in our laboratory as follows. A S563F knock-in targeting vector containing mouse $Bard1$ genomic DNA was constructed by inserting a neomycin-resistance gene cassette flanked by loxP sites (loxP-neo-loxP) into intron 7 and the S563F missense mutation into exon 8 (Figure 6). Likewise, a K607A targeting construct was generated by inserting the loxP-neo-loxP cassette into intron 8 and the K607A missense mutation into exon 9 (Figure 7). These vectors were then electroporated into ES cells, properly recombined neomycin-resistant ES clones were identified, and the presence and correct integration of the desired mutations confirmed by nucleotide sequence and Southern blot analyses (data not shown). Two independent clones of both $Bard1^{S563F-neo/+}$ and $Bard1^{K607A-neo/+}$ ES cells were injected into blastocysts for the production of germline-transformed mice.

To excise the loxP-neo-loxP cassette from the targeted alleles, chimeric male $Bard1^{S563F-neo/+}$ and $Bard1^{K607A-neo/+}$ mice were mated with females carrying a ubiquitously expressed Cre transgene (E2A-Cre) to produce offspring with the desired $Bard1^{S563F}$ (Figure 6D) or $Bard1^{K607A}$ alleles (Figure 7D). For convenience, the $Bard1^{S563F}$ and $Bard1^{K607A}$ alleles will be abbreviated hereafter as $Bard1^{SF}$ and $Bard1^{KA}$, respectively, and the $Brca1^{S1598F}$ allele as $Brca1^{SF}$ (Shakya et al., 2011). Genomic DNA from $Bard1^{SF/+}$ and $Bard1^{KA/+}$ was then examined by nucleotide sequence and Southern blot analyses to confirm the presence of the BRCT mutation and proper excision of the neomycin cassette (Figure 8).

I began my dissertation research by analyzing the phenotypes of homozygous $Bard1^{SF/SF}$ and $Bard1^{KA/KA}$ mice. To assess the viability, I first conducted intercrosses of the heterozygous mutant animals ($Bard1^{SF/+} \times Bard1^{SF/+}$ and $Bard1^{KA/+} \times Bard1^{KA/+}$). $Bard1^{SF/SF}$ and $Bard1^{KA/KA}$ mice were born at the expected Mendelian ratios (~25%) and displayed no obvious developmental abnormalities (Figure 9). This is in sharp contrast to $Bard1$-null ($Bard1^{-/-}$) and $Brca1$-null ($Brca1^{-/-}$) animals, which display identical phenotypes of embryonic lethality between embryonic days E7.5 and E8.5 as a result of an impairment in cell proliferation (Hakem et al., 1996; Ludwig et al., 1997; E. E. McCarthy et al., 2003). The absence of a developmental phenotype in $Bard1^{SF/SF}$ and $Bard1^{KA/KA}$ mice also contrasted with previously-described mice harboring hypomorphic mutations that alter ($Brca1^{SF/SF}$) or remove ($Brca1^{tr/tr}$) the Brca1 BRCT domain (Ludwig et al., 2001; Shakya et al., 2011). While these hypomorphic Brca1 mice are viable, they
A. 

Bard\textsuperscript{1+} allele

Ex 7

probes: 5'

3'

B. 

targeting vector

HR

C. 

Bard\textsuperscript{563F-neo} allele

Ex 7

probes: 5'

3'

D. 

Bard\textsuperscript{563F} allele

Ex 7

probes: 5'

3'

E. 

5' probe: RV

RV

9.3 Kb wt

7.4 Kb targeted

3' probe:

Kpn1

Kpn1

9.4 Kb wt

5.9 Kb targeted

Kpn1 4.0 Kb floxed
Figure 6. Design of the mutant Bard1^{S563F} allele. A) Map of the wild type Bard1 locus (Bard1*) encompassing exons 7-9 and illustrating the position of the S563F mutation (shown in green). B) Map of the targeting vector, which contains the S563F missense mutation inserted into exon 8 (shown in red) and a neomycin-resistance gene cassette flanked by loxP sites (LNL) inserted into intron 7. The loxP sites are represented as yellow triangles and the neomycin-resistance cassette as a black line. The targeting vector also contains EcoRV and KpnI restriction sites (shown in orange) at 5' end of the LNL that are relevant for Southern blot analysis. C) Map of the targeted Bard1 allele (Bard1^{S563F-neo}) after integration of the targeting vector via homologous recombination (HR). D) Map of the targeted Bard1 allele after Cre-mediated recombination (Bard1^{S563F}). E) Two separate Southern blot strategies were designed to detect the properly targeted Bard1^{S563F} allele. In the first strategy (top), genomic DNA from mice or ES cells was digested with EcoRV and hybridized with a probe upstream of exon 7 ("5' probe", annealing location is shown in green in panels A, C, and D above). Bard1^{S563F-neo} or Bard1^{S563F} was detected as a 7.4 kb band whereas Bard1* was detected as a 9.3 kb band. In the second strategy (bottom), which can ascertain the presence or absence of the neomycin-resistance cassette (Bard1^{S563F-neo} vs. Bard1^{S563F}), genomic DNA was digested with KpnI. The digested DNA was then hybridized with a probe downstream of exon 9 ("3' probe", annealing location is shown in green in panels A, C, and D above), yielding bands of 9.4 kb for Bard1*, 5.9 kb for Bard1^{S563F-neo}, and 4.0 kb for Bard1^{S563F}. 
Figure 7. Design of the mutant Bard1<sup>K607A</sup> allele. A) Map of the wild type Bard1 locus (Bard1<sup>+</sup>) encompassing exons 7-9 and illustrating the position of the K607A mutation (shown in green). B) Map of the targeting vector, which contains the K607A missense mutation inserted into exon 9 (shown in red) and a neomycin-resistance gene cassette flanked by loxP sites (LNL) inserted into intron 8. The loxP sites are represented as yellow triangles and the neomycin-resistance cassette as a black line. The targeting vector also contains EcoRV and KpnI restriction sites (shown in orange) at 5’ end of the LNL that are relevant for Southern blot analysis. C) Map of the targeted Bard1 allele (Bard1<sup>K607A-neo</sup>) after integration of the targeting vector via homologous recombination (HR). D) Map of the targeted Bard1 allele after Cre-mediated recombination (Bard1<sup>K607A</sup>). E) Two separate Southern blot strategies were designed to detect the properly targeted Bard1<sup>K607A</sup> allele. In the first strategy (top), genomic DNA from mice or ES cells was digested with EcoRV and hybridized with a probe upstream of exon 7 (“5’ probe”, annealing location is shown in green in panels A, C, and D above). Bard1<sup>K607A-neo</sup> or Bard1<sup>K607A</sup> was detected as an 8.5 kb band whereas Bard1<sup>+</sup> was detected as a 9.3 kb band. In the second strategy (bottom), which can ascertain the presence or absence of the neomycin-resistance gene (Bard1<sup>K607A-neo</sup> vs. Bard1<sup>K607A</sup>), genomic DNA was digested with KpnI. The digested DNA was then hybridized with a probe downstream of exon 9 (“3’ probe”, annealing location is shown in green in panel A, C, and D above), yielding bands of 9.4 kb for Bard1<sup>+</sup>, 5.0 kb for Bard1<sup>K607A-neo</sup>, and 3.0 kb for Bard1<sup>K607A</sup>. 
Figure 8. Confirmation of the genotypes of \textit{Bard1}^{S563F+/+} and \textit{Bard1}^{K607A+/+} mice by Southern blot and sequence analysis. A) To excise the \textit{loxP}-\textit{neo}\textit{-loxP} cassette from the targeted allele, chimeric male \textit{Bard1}^{S563F-\textit{neo}+/+} mice were mated with females carrying a ubiquitously-expressed \textit{Cre} transgene (E2A-Cre). To identify pups with the desired \textit{Bard1}^{S563F} allele, genomic tail DNAs were either digested with \textit{EcoRV} and hybridized with the 5’ probe (left) or digested with \textit{KpnI} and hybridized with the 3’ probe (right). Using the 5’ probe, the \textit{Bard1}^{+} allele is detected as a 9.3 kb DNA fragment while the \textit{Bard1}^{S563F} allele is detected as a 7.4 kb fragment. With the 3’ probe, the \textit{Bard1}^{+} allele is detected as a 9.4 kb fragment and the \textit{Bard1}^{S563F} allele as a 4.0 kb fragment. Correctly-targeted \textit{Bard1}^{S563F+/+} mice are shown in lanes 2, 3, and 4. The details of the Southern blotting scheme are shown in Figure 6. B) To excise the \textit{loxP}-\textit{neo}\textit{-loxP} cassette from the targeted allele, chimeric male \textit{Bard1}^{K607A-\textit{neo}+/+} mice were mated with females carrying a ubiquitously-expressed \textit{Cre} transgene (E2A-Cre). To identify pups with the desired \textit{Bard1}^{K607A} allele, genomic tail DNAs were either digested with \textit{EcoRV} and hybridized with the 5’ probe (left) or digested with \textit{KpnI} and hybridized with the 3’ probe (right). Using the 5’ probe, \textit{Bard1}^{+} alleles are detected as a 9.3 kb fragment and \textit{Bard1}^{K607A} alleles as an 8.5 kb fragment. With the 3’ probe, \textit{Bard1}^{+} alleles are detected as a 9.4 kb fragment and \textit{Bard1}^{K607A} alleles as a 3.0 kb fragment. Correctly-targeted \textit{Bard1}^{K607A+/+} mice are shown in lanes 2, 3, 4, 5, and 6. The details of the Southern blotting scheme are shown in Figure 7. C) Sequence analysis of a \textit{Bard1}^{S563F+/+} mouse shows the desired AG to TT mutation (noted by the black bar and double peaks) that converts the serine 563 codon (AGT) into a phenylalanine codon (TTT). D) Sequence analysis of a \textit{Bard1}^{K607A+/+} mouse shows the desired AA to GC mutation (noted by the black bar and double peaks) that converts the lysine 607 codon (AAG) into an alanine codon (GCG).
### A.

<table>
<thead>
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<th>Expected</th>
<th>Observed</th>
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<tr>
<td>Bard1(^{SF/+})</td>
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<td>54.3%</td>
</tr>
<tr>
<td>Bard1(^{SF/SF})</td>
<td>25%</td>
<td>22.2%</td>
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n = 221

### B.

<table>
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<th>Observed</th>
</tr>
</thead>
<tbody>
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<td>25%</td>
<td>26.5%</td>
</tr>
<tr>
<td>Bard1(^{KA/+})</td>
<td>50%</td>
<td>51.6%</td>
</tr>
<tr>
<td>Bard1(^{KA/KA})</td>
<td>25%</td>
<td>21.9%</td>
</tr>
</tbody>
</table>

n = 223

**Figure 9.** Bard1\(^{SF/SF}\) and Bard1\(^{KA/KA}\) mice are born at the expected Mendelian ratios. **A)** Bard1\(^{SF/4}\) mice were intercrossed and the percentage of pups (total n = 221) with each genotype was determined and compared to the expected Mendelian ratio. **B)** Bard1\(^{KA/+}\) mice were intercrossed and the percentage of pups (total n = 223) with each genotype was determined and compared to the expected Mendelian ratio.
are born at sub-Mendelian ratios (5-20%, depending on genetic background). Also, Bard1\textsuperscript{SF/SF} and Bard1\textsuperscript{KA/KA} mice, unlike Brca1\textsuperscript{SF/SF} and Brca1\textsuperscript{tr/tr} mice, do not display other developmental abnormalities characteristic of mice homozygous for hypomorphic Brca1 mutations, such as growth retardation, kinked tails, and white spots on the belly and hind feet (Ludwig et al., 2001; Shakya et al., 2011). Together, these data imply that phospho-recognition by the Bard1 BRCT domain is largely dispensable for mouse development.

ii. Bard1\textsuperscript{SF/SF} and Bard1\textsuperscript{KA/KA} mice display a defect in spermatogenesis distinct from that of Brca1\textsuperscript{SF/SF} mice

While Bard1 homozygous mice initially appeared normal, we observed that Bard1\textsuperscript{SF/SF} and Bard1\textsuperscript{KA/KA} males, but not females, are sterile. Male sterility is a commonly observed trait of Brca1 hypomorphic mice, including the Brca1\textsuperscript{SF/SF} and Brca1\textsuperscript{tr/tr} mutants (Shakya et al., 2011) (Ludwig et al., 2001). Furthermore, sterility or reduced fertility is also frequently observed in male mice harboring mutations in other HDR and DNA damage response genes, such as Brca2, Fancd2, Palb2, and H2AX (Celeste et al., 2002; Houghtaling et al., 2003; Sharan et al., 2004; Simhadri et al., 2014).

To assess the fertility defect in males, testes from 6-week-old Bard1\textsuperscript{SF/SF}, Bard1\textsuperscript{KA/KA} and Brca1\textsuperscript{SF/SF} mice were subjected to histological analysis. As shown in Figure 10A, the testes of Bard1\textsuperscript{SF/SF}, Bard1\textsuperscript{KA/KA} and Brca1\textsuperscript{SF/SF} mice are markedly smaller than those of their wildtype and heterozygous-mutant littermates. As expected, in wild type mice, histological staining revealed normal spermatogenesis in the seminiferous tubules (Figure 10B and C). Briefly, spermatogenesis occurs in the seminiferous tubules beginning in the outermost region of the tubule with the diploid spermatogonia and proceeding through several intermediate differentiation steps (primary spermatocyte, secondary spermatocyte, round spermatid, elongated spermatid), eventually yielding mature haploid spermatozoa near the lumen of the tubule (J. M. Turner, 2007). In contrast, histological staining of Bard1\textsuperscript{SF/SF} and Bard1\textsuperscript{KA/KA} testes revealed a complete absence of mature spermatozoa in the seminiferous tubules (Figure 10B and C). Closer examination revealed a mosaic pattern in which Bard1\textsuperscript{SF/SF} and Bard1\textsuperscript{KA/KA} seminiferous tubules displayed either of two distinct abnormalities: that is, roughly half of the tubules were almost entirely devoid of germ cells, apart from a ring of spermatogonia adjacent to the basal membrane (indicated by
Figure 10. *Bard1*<sup>SK/SF</sup> and *Bard1*<sup>KAKA</sup> male mice display a fertility defect distinct from that of *Brc1*<sup>SK/SF</sup> male mice. A) The testes of six-week-old *Bard1*<sup>KAKA</sup>, *Bard1*<sup>SK/SF</sup>, and *Brc1*<sup>SK/SF</sup> mice are smaller than those of their wild type and heterozygous littermate controls. B) H&E-stained transverse sections of seminiferous tubules from the testes of six-week-old mice at 20X magnification. Unlike those of wild type mice, all seminiferous tubules of *Brc1*<sup>SK/SF</sup> mice were devoid of elongated spermatids and mature spermatozoa. In contrast, the seminiferous tubules of *Bard1*<sup>SK/SF</sup> and *Bard1*<sup>KAKA</sup> mice show two distinct patterns of maturation arrest. Approximately 50% of the tubules (arrows) were almost entirely devoid of germ cells, apart from a ring of spermatogonia adjacent to the basal membrane. The remaining tubules (asterisk) showed maturation arrest at the pachytene stage of spermatogenesis, with a complete absence of secondary spermatocytes, round spermatids, elongated spermatids, and spermatozoa. Scale bars = 100 micrometer. C) Seminiferous tubules of *Bard1*<sup>AA</sup> (a), *Brc1*<sup>SK/SF</sup> (d), *Bard1*<sup>KAKA</sup> (b, c), and *Bard1*<sup>SK/SF</sup> (e, f) testes from 6-week-old mice shown at higher (40x) magnification. (a) *Bard1*<sup>AA</sup> tubules display normal germ cell maturation in all seminiferous tubules with production of elongated spermatids (arrowheads). Conversely, *Brc1*<sup>SK/SF</sup> mice (d) show germ cell maturation in all seminiferous tubules only up to the round spermatid stage (arrowheads), some of which form multinucleated giant cells (arrow). Approximately half of the *Bard1*<sup>KAKA</sup> (b) and *Bard1*<sup>SK/SF</sup> (e) seminiferous tubules are largely acellular apart from a few spermatogonia (arrows) and Sertoli cells (arrowheads). The remaining *Bard1*<sup>KAKA</sup> (c) and *Bard1*<sup>SK/SF</sup> (f) tubules exhibit a block in germ cell maturation at the pachytene spermatocyte stage (arrow) with the characteristic coarse chromatin pattern representing the “thick threads” of chromosomal tetrads. Scale bars = 20 micrometer.
the arrow in Figure 10B), while the remaining tubules showed maturation arrest and accumulation of primary spermatocytes, characterized by scant cytoplasm and large, dark-staining nuclei (indicated by an asterisk in Figure 10B). Histological analysis suggests that the primary spermatocytes are specifically stalled during the pachytene stage of meiosis I, as the arrested cells display the characteristic coarse chromatin pattern representing the “thick threads” of chromosomal tetrads in pachytene. In any case, the \textit{Bard1}^{SF/SF} and \textit{Bard1}^{KA/KA} tubules displayed a complete absence of all subsequent stages of sperm development, including secondary spermatocytes, round spermatids, elongated spermatids, and mature spermatozoa, thus explaining the observed fertility defect. As the \textit{Bard1}^{SF/SF} and \textit{Bard1}^{KA/KA} mice aged, testes from mice older than one year became increasingly dominated by the first seminiferous tubule phenotype (i.e., entirely devoid of germ cells) indicative of stem cell exhaustion (data not shown).

The phenotype of the \textit{Bard1}^{SF/SF} and \textit{Bard1}^{KA/KA} testes was distinct from that of 6-week-old \textit{Brca1}^{SF/SF} mice in that \textit{Brca1}^{SF/SF} tubules displayed a uniform pattern of maturation arrest without the empty tubules observed in \textit{Bard1}^{SF/SF} and \textit{Bard1}^{KA/KA} testes (Figure 10B and C). Furthermore, the stage of arrest during spermatogenesis was different in \textit{Brca1}^{SF/SF} tubules compared to \textit{Bard1}^{SF/SF} and \textit{Bard1}^{KA/KA} tubules. Specifically, spermatogenesis in \textit{Brca1}^{SF/SF} tubules was able to proceed past the stage of the primary spermatocyte, where the arrest occurred in \textit{Bard1}^{SF/SF} and \textit{Bard1}^{KA/KA} tubules, to the stage of the round spermatid. Thus, \textit{Brca1}^{SF/SF} seminiferous tubules completely lacked elongated spermatids but contained all prior stages of germ cell development, including secondary spermatocytes and round spermatids. However, no elongated spermatids or mature spermatozoa were observed, accounting for the male sterility. These observations imply that Bard1 BRCT phospho-recognition mediates functions in spermatogenesis that are distinct from those of Brca1 BRCT phospho-recognition.

iii. \textit{Bard1}^{KA/KA} mice are not sensitive to ionizing radiation

Mice with HDR defects have previously been reported to be sensitive to ionizing radiation, including mice deficient for Abraxas, one of the phospho-dependent binding partners of the BRCA1 BRCT domain (Castillo et al., 2014). To determine whether phospho-recognition by the Bard-BRCT domain is required for \textit{in vivo} resistance to ionizing radiation, cohorts of 5-week-old \textit{Bard1}^{+/+}, \textit{Bard1}^{KA/+}, and \textit{Bard1}^{KA/KA} mice were exposed to a sub-lethal dose (7.5 Gy) of ionizing radiation. The survival of the mice
was then monitored for 60 days after radiation treatment. At the end of the monitoring period, 14 of 16 Bard1+/+ mice were still alive compared with 17 of 18 Bard1K607A/+ and 11 of 11 Bard1KA/KA mice (Figure 11). Therefore, there was no statistical difference in survival between the homozygous, heterozygous, and wild type Bard1 mice. These data suggest that phospho-recognition by the Bard-BRCT domain is dispensable for in vivo resistance to ionizing radiation.

2. Generation and characterization of Bard1-SF and Bard1-KA cell lines

i. Generation of Bard1-SF and Bard1-KA ES cells and MEFs

To evaluate the function of Bard1 BRCT phospho-recognition at the cellular level, heterozygous mutant mice (e.g., Bard1SF/+) were intercrossed, and isogenic panels of mouse embryonic fibroblasts (MEFs) were derived from wildtype, heterozygous-mutant, and homozygous-mutant embryos at embryonic day E13.5. The cell lines were then immortalized with SV40 large-T antigen and their genotypes verified by PCR (Figure 12A and B). A comprehensive list of the Bard1 cell lines described in this thesis can be found in Table 3. Additionally, Brca1 SF/+ mice were intercrossed to create isogenic panels of immortalized MEFs as described above, and the genotypes of these lines were verified as shown in Figure 12C. A comprehensive list of the Brca1 cell lines described in this thesis can be found in Table 4. Additionally, embryonic stem (ES) cell lines were derived by intercrossing heterozygous Bard1 mice and harvesting embryos at embryonic day E3.5. Genotypes of the ES cell lines were also confirmed by PCR (data not shown).

ii. Expression and subcellular localization of Brca1 and Bard1 in Bard1SF/SF and Bard1KA/KA cells

To determine whether the Bard1SF and Bard1KA mutant proteins were expressed, lysates from wild type, heterozygous, and homozygous Bard1-BRCT mutant MEF lines were fractionated by SDS-PAGE. Immunoblotting with a Bard1-specific antiserum revealed that Bard1SF/SF and Bard1KA/KA cells expressed the mutant Bard1 protein at levels comparable to wild type and heterozygous MEFs, albeit with modest inter-clonal variation of steady-state Bard1 levels that did not correlate with mutation status (Figure 13). Immunoblotting with a Brca1-specific antiserum also revealed that Brca1 expression was unaffected by the Bard1 mutations in Bard1SF/SF and Bard1KA/KA cell lines, as their Brca1 levels were
Figure 11. *Bard1*<sup>KA/KA</sup> mice are not sensitive to ionizing radiation. The Kaplan-Meier survival curves of *Bard1<sup>+/+</sup>* (blue curve, n = 16), *Bard1<sup>KA/+</sup>* (red curve, n = 18), and *Bard1*<sup>KA/KA</sup> (green curve, n = 11) mice. The mice were exposed to 7.5 Gy of radiation and monitored for survival for 6 weeks post treatment. All mice in the *Bard1*<sup>KA/KA</sup> cohort were still alive after 6 weeks, compared to 14 of 16 *Bard1<sup>+/+</sup>* mice and 17 of 18 *Bard1<sup>KA/+</sup>* mice.
Figure 12. Creation of \textit{Bard1}^{SFSF}, \textit{Bard1}^{KAKA} and \textit{Brcal}^{SFSF} mouse embryonic fibroblast (MEF) lines. \textbf{A)} PCR analyses of genomic DNAs harvested from the embryos used to generate \textit{Bard1}^{SFSF} isogenic cell lines. PCR amplification of the \textit{Bard1}^{SFSF} allele generates a 521 base-pair fragment, while amplification of the \textit{Bard1}^{+} allele yields a 426 base-pair fragment. \textbf{B)} PCR analysis of genomic DNAs harvested from embryos used to generate \textit{Bard1}^{KAKA} isogenic cell lines. PCR amplification of the \textit{Bard1}^{KAKA} allele generates a 596 base-pair fragment, while amplification of the \textit{Bard1}^{+} allele yields a 500 base-pair fragment. \textbf{C)} PCR analysis of genomic DNAs harvested from embryos used to generate \textit{Brcal}^{SFSF} isogenic cell lines. Two separate PCR reactions were used to differentiate the \textit{Brcal}^{S1598F} allele (459 base-pair fragment, top reaction) and the \textit{Brcal}^{+} allele (396 base-pair fragment, bottom reaction). The harvest date, embryo number, and clone ID of each \textit{Bard1} and \textit{Brcal} MEF line is listed in Tables 3 and 4, respectively.
Table 3. *Bard1<sup>SF</sup> and Bard1<sup>KA</sup> isogenic MEF lines*

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PCR confirmation of genotypes from all cell lines are shown in figure 12A and B.
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PCR confirmation of genotypes from all cell lines are shown in Figure 12C
Figure 13. Expression and subcellular localization of Brca1 and Bard1 polypeptides in immortalized Bard1^{SFSF} and Bard1^{KA/KA} MEFs. A) Isogenic Bard1^{+/+}, Bard1^{SFSF} and Bard1^{SFSF} immortalized MEFs were subjected to subcellular fractionation and immunoblotted for Brca1, Ctip, Bard1, and tubulin. Although there is modest variability in the expression levels of Brca1 and Bard1 in different MEF clones, these variations do not correlate with the presence of the Bard1^{SFSF} allele. In all clones, Brca1, Bard1, and Ctip polypeptides are properly localized in the nuclear fraction and are not found in the cytoplasmic fraction. B) Isogenic Bard1^{+/+}, Bard1^{KA/KA} and Bard1^{KA/KA} immortalized MEFs were subjected to subcellular fractionation and immunoblotted for Brca1, Ctip, Bard1, and tubulin. While there is modest variability in the expression levels of Brca1 and Bard1 in different MEF clones, these variations do not correlate with the presence of the Bard1^{KA} allele. In all clones, Brca1, Bard1, and Ctip polypeptides are properly localized in the nuclear fraction and are not found in the cytoplasmic fraction.
comparable to wild type MEFs. Given that Brca1 and Bard1 are dependent on their interaction with each other for stability, the comparable expression levels imply that the Bard1\(^{KA}\) and Bard1\(^{SF}\) mutations have no effect on their interaction with Brca1 or the stability of the Brca1/Bard1 heterodimer. Furthermore, Western analysis of cytoplasmic and nuclear subcellular fractions demonstrated that Brca1 and Bard1 are both properly localized to the nucleus in Bard1\(^{KA/KA}\) and Bard1\(^{SF/SF}\) cells. Once again, this result suggests that the interaction of Brca1 and Bard1 is preserved in the Bard1\(^{KA}\) and Bard1\(^{SF}\) mutant proteins, as the association of Bard1 with Brca1 masks a nuclear export signal found in Brca1 (Fabbro et al., 2002).

iii. Brca1/Bard1 heterodimerization is preserved in Bard1\(^{SF/SF}\) and Bard1\(^{KA/KA}\) cells

Since formation of the BRCA1/BARD1 heterodimer is facilitated by sequences encompassing their respective RING domains, mutations in the Bard1-BRCT domain are not predicted to affect the interaction of Brca1 and Bard1 (Brzovic et al., 2001; L. C. Wu et al., 1996). Nonetheless, to ascertain whether the mutant Bard1 polypeptides retain the ability to heterodimerize with Brca1, cell lysates from Bard1\(^{KA/KA}\) and Bard1\(^{SF/SF}\) mutants were immunoprecipitated with a mouse Bard1-specific antiserum (IP: Bard1) or the corresponding pre-immune serum (IP: Pre), fractionated by PAGE, and immunoblotted with monoclonal antibodies that recognize Brca1 or its associated protein Ctip. As shown in Figure 14, Brca1 was efficiently co-immunoprecipitated with Bard1 from the lysates of both wildtype (Bard1\(^{+/+}\)) and mutant (Bard1\(^{SF/SF}\) and Bard1\(^{KA/KA}\)) MEFs, demonstrating that phospho-recognition by the Bard1 BRCT domain is not required for formation of the Brca1/Bard1 heterodimer.

While the BARD1 and BRCA1 BRCT domains share significant sequence and structural homology, they are not predicted to bind the same phospho-ligands due to important differences in their hydrophobic binding pockets (Figure 5B) (Birrane et al., 2007; Edwards et al., 2008). As such, the phospho-recognition function of the Bard1 BRCT domain is not believed to interact directly with any of the known BRCA1 phospho-ligands, including Ctip. Indeed, the efficient co-immunoprecipitation of Ctip with Bard1 from Bard1\(^{SF/SF}\) and Bard1\(^{KA/KA}\) cell lysates demonstrates that the in vivo association of Ctip with the Brca1/Bard1 heterodimer is not dependent on phospho-recognition by the Bard1 BRCT domain (Figure 14).
Figure 14. Co-immunoprecipitation of Brca1 with Bard1-specific antiserum in Bard1^SF/SF and Bard1^KA/KA immortalized MEFs. In vivo formation of the Brca1/Bard1 heterodimer is unaffected by the Bard1 BRCT phospho-recognition mutations. A) Whole cell lysates from isogenic Bard1^+/+, Bard1^SF/SF and Bard1^SF/SF immortalized MEFs were immunoprecipitated with a Bard1-specific antiserum (IP: Bard1) or the corresponding pre-immune serum (IP: Pre) and then immunoblotted with either Brca1- or Ctip-specific monoclonal antibodies (right). The input (left) represents 12.5% of the protein amount used for immunoprecipitation. B) Whole cell lysates from isogenic Bard1^+/+, Bard1^KA/KA and Bard1^KA/KA immortalized MEFs were immunoprecipitated with a Bard1-specific antiserum (IP: Bard1) or pre-immune serum (IP: Pre) and then immunoblotted with Brca1- or Ctip-specific monoclonal antibodies (right). The input (left) represents 12.5% of the protein amount used for immunoprecipitation.
3. *Bard1*<sup>SF/SF</sup> and *Bard1*<sup>KA/KA</sup> cells display a pattern of genotoxin sensitivity distinct from that of *Brca1*<sup>SF/SF</sup> cells

i. *Bard1*<sup>SF/SF</sup> and *Bard1*<sup>KA/KA</sup> cells are highly sensitive to MMC

To determine whether Bard1 BRCT phospho-recognition affects the cellular response to DNA damage, we used clonogenic survival assays to evaluate the sensitivity of Bard1-mutant immortalized MEFs to various genotoxic agents. In these clonogenic survival assays, isogenic wild type, heterozygous, and homozygous MEFs harboring phospho-recognition mutations in the Bard1-BRCT domain were exposed to varying concentrations of a DNA-damaging agent, after which the cells were then allowed to recover and the surviving colonies quantified.

A hallmark of BRCA1-mutant cells is their exquisite sensitivity to DNA interstrand crosslinking reagents (ICL), such as cisplatin and Mitomycin C (MMC) (Moynahan, Cui, et al., 2001). Indeed, cisplatin is a component of standard chemotherapeutic regimens for certain malignancies, including BRCA1-mutant ovarian tumors. Crosslinking reagents damage DNA by preventing the Watson and Crick DNA strands from separating, resulting in stalled replication forks and DNA double strand breaks, and thereby activating both the SFP and HDR pathways. Indeed, cells that are mutant for other SFP and HDR factors, such as RAD51, BRCA2, and FancD2, are highly sensitive to ICL reagents (N. Liu et al., 1998; Schlacher et al., 2012). However, recent evidence suggests that the ability of BRCA1 to repair ICLs is independent of its function in HDR (Bunting et al., 2012).

To determine whether phospho-recognition by the Bard1 BRCT domain is required for resistance to DNA interstrand crosslinking reagents, *Bard1*<sup>SF/SF</sup> and *Bard1*<sup>KA/KA</sup> immortalized MEFs were exposed to varying concentrations of MMC for 4 hours and then allowed to recover for 7-10 days post treatment. As shown in Figure 15, two independent clones each of *Bard1*<sup>SF/SF</sup> and *Bard1*<sup>KA/KA</sup> MEFs were hypersensitive to MMC compared to isogenic wild type and heterozygous cell lines. Additionally, as had been previously reported by Shakya *et al.*, *Brca1*<sup>SF/SF</sup> MEFs are also hypersensitive to MMC compared to their wild type control (Shakya et al., 2011). Taken together, *Bard1*<sup>SF/SF</sup> and *Bard1*<sup>KA/KA</sup> MEFs are hypersensitive to the DNA inter-strand crosslinking agent mitomycin C (MMC), to an extent that is comparable to or even greater than that of *Brca1*<sup>SF/SF</sup> MEFs.
Figure 15. **Bard1^{SF/SF}** and **Bard1^{KA/KA}** cells are sensitive to the DNA interstrand crosslinking reagent mitomycin C (MMC).  

**A)** Isogenic **Bard1^{+/+}, Bard1^{SF/+}** and **Bard1^{SF/SF}** immortalized MEFs, as well as isogenic **Brcal^{+/-}** and **Brcal^{SF/SF}** MEFs, were exposed to varying concentrations of MMC for four hours and then allowed to recover for 7-10 days before counting the surviving colonies. **Survival** is quantified as the percentage of colonies on the treated plates relative to the untreated plates. Each experimental condition was plated in triplicate, and error bars represent standard error of the mean.  

**B)** Isogenic **Bard1^{+/+}** and **Bard1^{KA/KA}** immortalized MEFs, as well as isogenic **Brcal^{+/-}** and **Brcal^{SF/SF}** MEFs, were exposed to varying concentrations of MMC for four hours and then allowed to recover for 7-10 days before counting the surviving colonies. **Survival** is quantified as the percentage of colonies on the treated plates relative to the untreated plates. Each experimental condition was plated in triplicate, and error bars represent standard error of the mean.
ii. \textit{Bard1}^{SF/SF} and \textit{Bard1}^{KA/KA} MEFs display an intermediate hypersensitivity to PARP inhibition.

BRCA1-mutant cells and tumors are exquisitely sensitive to poly(ADP-ribose) polymerase inhibitors (PARPi) (Bryant et al., 2005) (Farmer et al., 2005) (Fong et al., 2009). This extreme hypersensitivity may reflect the combined deficiencies of these cells for both HDR and SFP. PARPi, which is required for single-strand break repair (SSB), was originally proposed to exploit the HDR defect of BRCA1/2-mutant cells through a synthetic lethal interaction. Thus, since BRCA1-deficient cells are already deficient in DSB repair by HDR, inhibition of SSB repair by PARPi would lead to a toxic accumulation of DNA damage as unrepaired SSBs are converted into DSBs during DNA replication. Supporting this hypothesis is the clinical observation that BRCA1 tumors often evolve resistance to PARPi treatment by restoring HDR function (Lord & Ashworth, 2013). However, PARPi treatment is also thought to stall replication forks by generating DNA-protein adducts (Helleday, 2011). Indeed, recent reports suggest that restoration of the SFP pathway in BRCA1-deficient cells is an alternative mechanism for PARPi resistance (Ray Chaudhuri et al., 2016; Taglialatela et al., 2017).

To determine whether Bard1 BRCT phospho-recognition is required for PARPi resistance, \textit{Bard1}^{SF/SF} and \textit{Bard1}^{KA/KA} MEFs were exposed to continuous treatment with the PARP inhibitor olaparib for 7-10 days. Compared to their isogenic wild type and heterozygous controls, two independent clones each of \textit{Bard1}^{SF/SF} and \textit{Bard1}^{KA/KA} MEFs displayed hypersensitivity to the PARP inhibitor (Figure 16). However, the degree of hypersensitivity of the Bard1-mutant MEFs is reproducibly less than that observed with \textit{Brca1}^{SF/SF} MEFs (Figure 16), suggesting that Bard1 BRCT phospho-recognition is partially required for resistance to PARP inhibitors.

iii. \textit{Bard1}^{SF/SF} and \textit{Bard1}^{KA/KA} MEFs are not hypersensitive to ionizing radiation

Ionizing radiation (IR) induces many different types of DNA damage, including, most notably, DNA double strand breaks. These DSBs may subsequently be repaired by HDR, non-homologous end joining (NHEJ), or microhomology mediated end joining (MMEJ). Cells deficient in BRCA1 and other HDR factors typically display a moderate hypersensitivity to IR (Abbott et al., 1999; N. Liu et al., 1998). As shown in Figure 17, Bard1 and Brca1 mutant MEFs were exposed to varying doses of ionizing radiation and allowed to recover for 7 days post treatment. As expected, \textit{Brca1}^{SF/SF} MEFs displayed
Figure 16. **Bard1^{SF/SF}** and **Bard1^{KA/KA}** cells display intermediate sensitivity to the PARP inhibitor olaparib. 

A) Isogenic **Bard1^{+/+}, Bard1^{SF/F}** and **Bard1^{SF/SF}** immortalized MEFs, as well as isogenic **Brcal^{+/+}** and **Brcal^{SF/SF}** MEFs, were continuously exposed to varying concentrations of olaparib for 7-10 days before counting the surviving colonies. Survival is quantified as the percentage of colonies on the treated plates relative to the untreated plates. Each experimental condition was plated in triplicate, and error bars represent standard error of the mean.

B) Isogenic **Bard1^{+/+}** and **Bard1^{KA/KA}** immortalized MEFs, as well as isogenic **Brcal^{+/+}** and **Brcal^{SF/SF}** MEFs, were continuously exposed to varying concentrations of olaparib for 7-10 days before counting the surviving colonies. Survival is quantified as the percentage of colonies on the treated plates relative to the untreated plates. Each experimental condition was plated in triplicate, and error bars represent standard error of the mean.
Figure 17. \textit{Bard1}^{SF/SF} and \textit{Bard1}^{KA/KA} cells are not sensitive to ionizing radiation (IR). \textbf{A)} Isogenic \textit{Bard1}^{+/+}, \textit{Bard1}^{SF/+} and \textit{Bard1}^{SF/SF} immortalized MEFs, as well as isogenic \textit{Bra1}^{+/+} and \textit{Bra1}^{SF/SF} MEFs, were exposed to varying dosages of IR and allowed to recover for 7-10 days before counting the surviving colonies. Survival is quantified as the percentage of colonies on the treated plates relative to the untreated plates. Each experimental condition was plated in triplicate, and error bars represent standard error of the mean. \textbf{B)} Isogenic \textit{Bard1}^{+/+}, \textit{Bard1}^{KA/+} and \textit{Bard1}^{KA/KA} immortalized MEFs, as well as isogenic \textit{Bra1}^{+/+} and \textit{Bra1}^{SF/SF} MEFs, were exposed to varying dosages of IR and allowed to recover for 7-10 days before counting the surviving colonies. Survival is quantified as the percentage of colonies on the treated plates relative to the untreated plates. Each experimental condition was plated in triplicate, and error bars represent standard error of the mean.
moderate hypersensitivity to IR compared to their wild type controls. However, $Bard1^{SF/SF}$ and $Bard1^{KA/KA}$ MEFs showed no difference in survival when compared to their wild type and heterozygous controls. Therefore, phospho-recognition by the Bard1 BRCT domain is dispensable for resistance to IR. This observation is consistent with the IR resistance of $Bard1^{KA/KA}$ mice shown in Figure 11.

iv. $Bard1^{SF/SF}$ and $Bard1^{KA/KA}$ MEFs are not hypersensitive to camptothecin

Camptothecin generates DNA damage by stabilizing the covalent linkage of type I topoisomerase to DNA ends. Under normal cellular conditions, type I topoisomerases relieve the supercoils generated from DNA replication by inducing transient single-stranded breaks in S-phase cells. By stabilizing the covalent linkage of topoisomerase to DNA ends, camptothecin prevents the proper re-ligation of these ssDNA breaks, which can be subsequently converted to dsDNA breaks upon DNA replication (Helleday, 2011; Pommier et al., 2003; Strumberg et al., 2000). Indeed, mutations in HDR components like BRCA1, BRCA2, and CtIP have been shown to sensitize cells to camptothecin treatment (Scully, 2011) (Sartori et al., 2007).

To determine whether Bard1-BRCT phospho-recognition is required for resistance to camptothecin, $Bard1^{SF/SF}$ and $Bard1^{KA/KA}$ immortalized MEFs were exposed to varying concentrations of camptothecin for 1 hour and then allowed to recover for 7-10 days. As shown in Figure 18, two independent clones of both $Bard1^{SF/SF}$ and $Bard1^{KA/KA}$ MEFs displayed no hypersensitivity to camptothecin compared to their isogenic wild type and heterozygous controls. On the other hand, $Brca1^{SF/SF}$ MEFs were moderately hypersensitive to camptothecin compared to a $Brca1^{+/+}$ cell line. Therefore, phospho-recognition by the Bard1-BRCT domain is dispensable for resistance to camptothecin.

In summary, cells harboring the $Bard1^{SF/SF}$ or $Bard1^{KA/KA}$ mutations displayed identical patterns of sensitivity or resistance to genotoxic stress, which supports the structural predictions that both of these mutations will result in identical functional consequences (i.e., disruption of BRCT phosphoprotein recognition and PAR binding). Although each of the agents tested can, to some extent, induce a variety of genotoxic lesions, the Bard1 mutant cells appear to be more hypersensitive to drugs that induce stalled DNA replication forks (MMC and olaparib), rather than those that generate primarily double-stand DNA
Figure 18. Bard1SF/SF and Bard1KA/KA cells are not sensitive to the topoisomerase I inhibitor camptothecin (CPT). A) Isogenic Bard1+/+, Bard1SF/+ and Bard1SF/SF immortalized MEFs, as well as isogenic Brca1+/+ and Brca1SF/SF MEFs, were exposed to varying concentrations of CPT for one hour and then allowed to recover for 7-10 days before counting the surviving colonies. Survival is quantified as the percentage of colonies on the treated plates relative to the untreated plates. Each experimental condition was plated in triplicate, and error bars represent standard error of the mean. B) Isogenic Bard1+/+, Bard1KA/KA and Bard1KA/KA immortalized MEFs, as well as isogenic Brca1+/+ and Brca1SF/SF MEFs, were exposed to varying concentrations of CPT for one hour and then allowed to recover for 7-10 days before counting the surviving colonies. Survival is quantified as the percentage of colonies on the treated plates relative to the untreated plates. Each experimental condition was plated in triplicate, and error bars represent standard error of the mean.
breaks (e.g., IR). On the other hand, \( Brca1^{SF/SF} \) cells are hypersensitive to all the genotoxins tested (MMC, olaparib, IR, and CPT), suggesting defects in both SFP and HDR.

4. Bard1\(^{SF/SF} \) and Bard1\(^{KA/KA} \) cells exhibit chromosomal instability in response to genotoxic stress

i. Giemsa karyotype analysis suggest chromosomal instability in MMC-treated Bard1\(^{SF/SF} \) immortalized MEFs

The hypersensitivity of the Bard1 and Brca1 BRCT domain mutants to certain genotoxins (Section 3) suggests the presence DNA repair deficiencies in these cell lines. Indeed, deficiencies in DNA repair may lead to gross chromosomal rearrangements, which can be detected through cytogenetic analysis. Relative to wild type cells, Brca1- and Bard1-null cells accumulate increased levels of both spontaneous and genotoxic-induced chromosomal abnormalities (E. E. McCarthy et al., 2003) (Xu et al., 1999) (A. McCarthy et al., 2007) (X. Liu et al., 2007) (Shakya et al., 2008) (Shakya et al., 2011). Since genome instability is a hallmark of cancer in general (Hanahan & Weinberg, 2011), the chromosomal instability of BRCA1/BARD1-mutant cells may be a critical factor in the formation of hereditary breast cancer.

Thus, to determine whether phospho-recognition by the Bard1-BRCT domain is required for chromosomal stability, two independent clones of Bard1\(^{SF/SF} \) immortalized MEFs, along with an isogenic Bard1\(^{+/+} \) MEF clone, were cultured in the presence or absence of 40 ng/mL MMC for 16 hours. Metaphases stained with Giemsa were then examined for structural chromosome abnormalities. In parallel, immortalized lines of Brca1\(^{SF/SF} \) and Brca1\(^{+/+} \) MEFs were also subjected to the same analysis.

By examining the prevalence of chromosomal aberrations in untreated cell lines, we could determine whether the Bard1- or Brca1-BRCT mutant MEF lines spontaneously accumulate chromosomal rearrangements (Figure 19A and C). The Bard1\(^{+/+} \) clone displayed abnormalities in 44% of mock-treated karyotypes examined. In comparison, we observed a similar percentage of abnormal karyotypes in two independent Bard1\(^{SF/SF} \) MEF clones, with abnormalities in 44% (clone A) and 48% (clone C) of metaphases, respectively. The burden of abnormalities appeared to be modestly higher in the two Bard1\(^{SF/SF} \) cell lines (0.80 and 0.88 aberrations per metaphase) compared to the Bard1\(^{+/+} \) cell line (0.68 aberrations per metaphase), but the difference is not statistically significant. As such, these data suggest
A. (-) MMC

B. (+) MMC

C.

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<td>Bard1**/SF/SF (C)</td>
<td>–</td>
<td>25</td>
<td>48</td>
<td>0.88</td>
<td>14</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>25</td>
<td>72</td>
<td>1.44</td>
<td>27</td>
<td>9</td>
</tr>
<tr>
<td>Brca1**/+</td>
<td>–</td>
<td>25</td>
<td>52</td>
<td>0.64</td>
<td>7</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>25</td>
<td>56</td>
<td>1.12</td>
<td>21</td>
<td>7</td>
</tr>
<tr>
<td>Brca1**/SF/SF</td>
<td>–</td>
<td>25</td>
<td>56</td>
<td>0.96</td>
<td>11</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>25</td>
<td>72</td>
<td>1.68</td>
<td>29</td>
<td>13</td>
</tr>
</tbody>
</table>
Figure 19. Giemsa-stain karyotyping suggests MMC-induced chromosomal instability in Bard1<sup>SP/SP</sup> and Brca1<sup>SP/SP</sup> immortalized MEFs. MMC-treated, but not untreated, Bard1<sup>SP/SP</sup> cells display increased chromosomal instability relative to isogenic Bard1<sup>+/+</sup> cells. Isogenic Bard1<sup>+/+</sup> and Bard1<sup>SP/SP</sup> immortalized MEFs, as well as isogenic Brca1<sup>+/+</sup> and Brca1<sup>SP/SP</sup> MEFs, were subjected to Giemsa stain karyotype analysis after mock treatment with PBS (A) or 40 ng/mL MMC for 16 hours (B). Karyotypes were analyzed for gross structural abnormalities under a light microscope at 63X magnification. C) Table showing the breakdown of abnormalities seen in the karyotypes analyzed in panels A and B.
that the \textit{Bard1}^{SF/SF} cells do not accumulate spontaneous chromosomal abnormalities at a markedly heightened rate. Similarly, the Brca1 wild type cell line showed abnormalities in 52\% of untreated karyotypes as compared with 56\% in the \textit{Brca1}^{SF/SF} cell line. While at first glance there does not appear to be much difference between the Brca1\textsuperscript{+/+} and Brca1\textsuperscript{SF/SF} cell lines, closer examination revealed that the Brca1\textsuperscript{SF/SF} cells carry a greater burden of chromosomal abnormalities, with 36\% of Brca1\textsuperscript{SF/SF} metaphases displaying two or more abnormalities (0.96 aberrations per metaphase) compared to just 14\% of Brca1\textsuperscript{+/+} metaphases (0.64 aberrations per metaphase). Therefore, the \textit{Brca1}^{SF/SF} mutation may predispose to spontaneous genome instability, but again the results do not achieve statistical significance. Moreover, the interpretation of this experiment is limited by the relatively high burden of spontaneous abnormalities in all cell lines (ranging from 44\%-56\%). This high background level of genome instability may be a product of the MEF immortalization process, in which expression of the SV40 large-T antigen abrogates several cellular activities, including the p53 pathway.

In contrast to untreated cells, both \textit{Bard1}^{SF/SF} clones displayed an increase in MMC-induced chromosomal rearrangements compared to their isogenic \textit{Bard1}^{+/+} control (Figure 19A and C). Sixty-eight percent of karyotypes from \textit{Bard1}^{SF/SF} clone A and 72\% from \textit{Bard1}^{SF/SF} clone C displayed abnormalities compared with 48\% from \textit{Bard1}^{+/+} clone E. Moreover, the total burden of chromosomal rearrangements was higher in the \textit{Bard1}^{SF/SF} mutants, as 56\% (clone A) and 48\% (clone C) of metaphases displayed two or more aberrations (1.68 and 1.44 aberrations per metaphase) compared to 32\% in the Bard1 wild type clone (0.88 aberrations per metaphase). Comparing the treated and untreated \textit{Bard1}^{SF/SF} cells, the increase in aberrations was largely accounted for by a greater number of chromosome and chromatid breaks, as the number of exchanges/fusions stayed relatively constant in the two conditions. \textit{Brca1}^{SF/SF} cells also displayed an increase in the percentage of abnormal metaphases after MMC treatment (72\%, 1.68 aberrations per metaphase) compared to \textit{Brca1}^{+/+} cells (56\%, 1.12 aberrations per metaphase). Once again, the \textit{Brca1}^{SF/SF} clone carried a greater burden of abnormalities with 48\% of cells displaying two or more aberrations compared with 40\% in the \textit{Brca1}^{+/+} clone. However, likely due to the low number of metaphases analyzed, we were not able to demonstrate statistical significance in these observations.

Together, these data suggest that phospho-recognition by the Bard1 BRCT domain may be dispensable for suppression of spontaneous chromosomal rearrangements but required for suppression
of MMC-induced rearrangements. Consistent with the results in Shakya et al. (2011), Brca1 BRCT phospho-recognition appears to be required for suppression of both spontaneous and MMC-induced chromosomal rearrangements. However, the interpretation of these experiments is limited by the high background of chromosomal aberrations in the untreated condition. As a result, rather than carrying out further trials in immortalized MEFs to demonstrate statistical significance, we decided to instead examine primary MEFs.

ii. Telomere fluorescent in situ hybridization (T-FISH) reveals MMC-induced chromosomal instability in Bard1\textsuperscript{SF/SF} and Bard1\textsuperscript{KA/KA} primary MEFs

As the interpretation of the karyotyping in Figure 19 was limited by the high background chromosomal instability present in wild type immortalized MEFs, we performed another experiment in low passage primary MEFs, which, unlike immortalized MEFs, still retain a functional p53 pathway. As such, primary MEFs should also have a lower baseline level of chromosome instability in the wild type clones. Additionally, since aberrations observed in the experiment above included many chromosome/chromatid breaks, we examined the metaphases using telomere fluorescent in situ hybridization (T-FISH) rather than conventional Giemsa-stained cytogenetics, as T-FISH is more sensitive to chromosome/chromatid breaks.

Three independent clones of early passage Bard1\textsuperscript{SF/SF} primary MEFs, along with two isogenic Bard1\textsuperscript{+/+} primary MEF clones, were cultured in the presence or absence of 40 ng/mL MMC for 16 hours and the resulting metaphases analyzed by T-FISH. As shown in Figure 20A, in contrast to immortalized MEFs, the two Bard1\textsuperscript{+/+} clones displayed low levels of spontaneous chromosomal rearrangements, with only 4.6% of metaphases from clone E and 1.8% of metaphases from clone I harboring abnormalities. These wild type clones also displayed a low burden of chromosomal abnormalities, with an average of 0.04 abnormalities per metaphase in clone E and 0.01 abnormalities per metaphase in clone I. Thus, the use of primary MEFs solved the problem of high background abnormalities in wild type cells observed in the immortalized MEFs shown in Figure 19A. The three independent Bard1\textsuperscript{SF/SF} clones did not display a significant increase in spontaneous chromosomal rearrangements compared to the isogenic wild type controls (Figure 20A, Table 5A). Bard1\textsuperscript{SF/SF} clones A, C, and H showed abnormalities in 7.1%, 1.5%, and
Figure 20. T-FISH karyotyping reveals MMC-induced chromosomal instability in Bard1<sup>SF/SF</sup> primary MEFs. A) Isogenic Bard1<sup>SF/SF</sup> and Bard1<sup>+/+</sup> primary MEFs were cultured in the presence or absence of 40 ng/mL MMC for 16 hours. Structural chromosome abnormalities were then quantified by T-FISH. The data from two independent experiments are pooled and presented as a dot plot. The mean number of aberrations per cell is denoted by a horizontal red line, and the error bars represent the standard error of the mean. P values were calculated by one-way ANOVA (* = p<0.05, **** = p<0.0001). A detailed breakdown of the chromosomal aberrations observed is presented in Table 5A. B) A dot plot representation of the pooled data from all the independent Bard1<sup>+/+</sup> (clones E and I) and Bard1<sup>SF/SF</sup> (clones A, C, and H) clones shown in (A). The mean number of aberrations per cell is denoted by a horizontal red line, and the error bars represent the standard error of the mean. P values were calculated by unpaired Student’s t test (** = p<0.01). A detailed breakdown of the chromosomal aberrations is presented in Table 5B.
Table 5A. Spontaneous and MMC-induced chromosomal aberrations in *Bard1<sup>+/+</sup>* and *Bard1<sup>SF/SF</sup>* primary MEFs

<table>
<thead>
<tr>
<th>Genotype (clone)</th>
<th>MMC</th>
<th>Total metaphase counted</th>
<th>Total abnormal metaphase</th>
<th>% abnormal metaphase</th>
<th>Aberrations / metaphase</th>
<th>Total breaks</th>
<th>Total fusions</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bard1&lt;sup&gt;+/+&lt;/sup&gt;</em> (E)</td>
<td>−</td>
<td>65</td>
<td>3</td>
<td>4.61%</td>
<td>0.05</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>42</td>
<td>15</td>
<td>35.71%</td>
<td>0.62</td>
<td>15</td>
<td>11</td>
</tr>
<tr>
<td><em>Bard1&lt;sup&gt;+/+&lt;/sup&gt;</em> (I)</td>
<td>−</td>
<td>55</td>
<td>1</td>
<td>1.81%</td>
<td>0.02</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>68</td>
<td>19</td>
<td>27.94%</td>
<td>0.72</td>
<td>11</td>
<td>38</td>
</tr>
<tr>
<td><em>Bard1&lt;sup&gt;SF/SF&lt;/sup&gt;</em> (A)</td>
<td>−</td>
<td>70</td>
<td>5</td>
<td>7.14%</td>
<td>0.07</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>70</td>
<td>39</td>
<td>55.71%</td>
<td>1.50</td>
<td>85</td>
<td>20</td>
</tr>
<tr>
<td><em>Bard1&lt;sup&gt;SF/SF&lt;/sup&gt;</em> (C)</td>
<td>−</td>
<td>65</td>
<td>1</td>
<td>1.54%</td>
<td>0.02</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>48</td>
<td>25</td>
<td>72.82%</td>
<td>2.33</td>
<td>51</td>
<td>61</td>
</tr>
<tr>
<td><em>Bard1&lt;sup&gt;SF/SF&lt;/sup&gt;</em> (H)</td>
<td>−</td>
<td>65</td>
<td>4</td>
<td>6.15%</td>
<td>0.14</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>63</td>
<td>34</td>
<td>53.97%</td>
<td>1.21</td>
<td>44</td>
<td>33</td>
</tr>
</tbody>
</table>

Data represents metaphases counted in two independent experiments. Percent abnormal metaphase, aberrations per metaphase, and breakdown of aberration type are shown in the table above. The data is represented graphically in Figure 20A.

Table 5B. Spontaneous and MMC-induced chromosomal aberrations in pooled analysis of *Bard1<sup>+/+</sup>* and *Bard1<sup>SF/SF</sup>* primary MEFs

<table>
<thead>
<tr>
<th>Genotype</th>
<th>MMC</th>
<th>Total metaphase counted</th>
<th>Total abnormal metaphase</th>
<th>% abnormal metaphase</th>
<th>Aberrations / metaphase</th>
<th>Total breaks</th>
<th>Total fusions</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bard1&lt;sup&gt;+/+&lt;/sup&gt;</em></td>
<td>−</td>
<td>120</td>
<td>4</td>
<td>3.33%</td>
<td>0.03</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>110</td>
<td>34</td>
<td>30.91%</td>
<td>0.68</td>
<td>26</td>
<td>49</td>
</tr>
<tr>
<td><em>Bard1&lt;sup&gt;SF/SF&lt;/sup&gt;</em></td>
<td>−</td>
<td>200</td>
<td>10</td>
<td>5.00%</td>
<td>0.08</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>181</td>
<td>108</td>
<td>59.67%</td>
<td>1.62</td>
<td>180</td>
<td>114</td>
</tr>
</tbody>
</table>

Data represents metaphases counted in two independent experiments. *Bard1<sup>+/+</sup>* data was pooled from two wild type clones (E and I) and *Bard1<sup>SF/SF</sup>* data was pooled from three mutant clones (A, C, and H) shown above. The data is represented graphically in Figure 20B.
6.1% of metaphases, respectively, as well as a low burden of chromosomal aberrations with averages of 0.07, 0.01, and 0.13 abnormalities per metaphase. If the results from the two \( \text{Bard}^+/+ \) clones (E and I) and the three \( \text{Bard}^{SF/SF} \) clones are pooled (A, C, and H), no statistically significant difference is observed between wild type and mutant cells with regards to either the percent of abnormal metaphases (3.3% vs 5.0%) or the average abnormalities per metaphase (0.03 vs 0.07) (Figure 20B, Table 5B).

However, when treated with MMC, the three independent \( \text{Bard}^{SF/SF} \) clones showed marked increases in the percentage of abnormal metaphases (55.7%, 72.9%, and 54.0%) compared to the wild type clones (35.7% and 27.9%) (Figure 20A, Table 5A). Furthermore, three \( \text{Bard}^{SF/SF} \) clones displayed a higher burden of abnormalities as reflected by the average number of abnormalities per metaphase (1.5, 2.3, and 1.2 abnormalities per metaphase) compared to the two wild type clones (0.62 and 0.72 abnormalities per metaphase). This result is statistically significant when each of the wild type clones (E and I) was compared to two of the \( \text{Bard}^{SF/SF} \) clones (A and C), but not to clone H. However, if the results from the two independent wild type clones (E and I) and the three independent \( \text{Bard}^{SF/SF} \) clones (A, C, and H) are separately pooled, then statistically significant differences in the percentage of abnormal metaphases (30.9% in wild type vs. 59.7% in \( \text{Bard}^{SF/SF} \)) and average abnormalities per metaphase (0.68 in \( \text{Bard}^+/+ \) vs 1.62 in \( \text{Bard}^{SF/SF} \)) are observed (Figure 20B, Table 5B).

Similar to the \( \text{Bard}^{SF/SF} \) cell lines, three independent \( \text{Bard}^{KA/KA} \) primary MEF lines (clones J, L, and M) did not display an increase in spontaneous chromosomal rearrangements when compared to two wild type isogenic controls (I and N) in either the percentage of abnormal metaphases (1.6% and 0% in \( \text{Bard}^+/+ \) vs 8.3%, 4.7%, and 6.9% in \( \text{Bard}^{KA/KA} \)) or the average aberrations per metaphase (0.03 and 0.00 in \( \text{Bard}^+/+ \) vs 0.08, 0.063, and 0.103 in \( \text{Bard}^{KA/KA} \)) (Figure 21A, Table 6A). However, as was the case with \( \text{Bard}^{SF/SF} \) lines, the \( \text{Bard}^{KA/KA} \) cells displayed a significant increase in chromosomal instability when treated with mitomycin C as reflected in both the percentage of abnormal metaphases (20.3% and 21.1% in \( \text{Bard}^+/+ \) vs 50.6%, 51.4%, and 27.5% in \( \text{Bard}^{KA/KA} \)) and the average number of aberrations per metaphase (0.29 and 0.37 in \( \text{Bard}^+/+ \) vs 1.14, 1.12, and 0.63 in \( \text{Bard}^{KA/KA} \)) (Figure 21A, Table 6A). When comparing the average number of aberrations per metaphase, \( \text{Bard}^{KA/KA} \) clone M (0.63 aberrations per metaphase) was not statistically significant compared to the corresponding wild type clones. However, if the two wild type clones (I and N) are pooled together and the three \( \text{Bard}^{KA/KA} \)
Figure 21. T-FISH karyotyping reveals MMC-induced chromosomal instability in Bard1^{KA/KA} primary MEFs. A) Isogenic Bard1^{KA/KA} and Bard1^{+/-} primary MEF lines were cultured in the presence or absence of 40 ng/mL MMC for 16 hours. Structural chromosome abnormalities were then quantified by T-FISH. The data from two independent experiments are pooled and presented as a dot plot. The mean number of aberrations per cell is denoted by a horizontal red line, and the error bars represent the standard error of the mean. P values were calculated by one-way ANOVA (*** = p<0.001, **** = p<0.0001). A detailed breakdown of the chromosomal aberrations observed is presented in Table 6A. B) A dot plot representation of the pooled data from all the independent Bard1^{+/-} (clones I and N) and Bard1^{KA/KA} (clones J, L, and M) clones shown in (A). The mean number of aberrations per cell is denoted by a horizontal red line, and the error bars represent the standard error of the mean. P values were calculated by unpaired Student's t test (*** = p<0.001). A detailed breakdown of the chromosomal aberrations is presented in Table 6B.
Table 6A. Spontaneous and MMC-induced chromosomal aberrations in *Bard1*+/+ and *Bard1*KA/KA primary MEFs

<table>
<thead>
<tr>
<th>Genotype (clone)</th>
<th>MMC</th>
<th>Total metaphase counted</th>
<th>Total abnormal metaphase</th>
<th>% abnormal metaphase</th>
<th>Aberrations/metaphase</th>
<th>Total breaks</th>
<th>Total fusions</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bard1</em>+/+ (I)</td>
<td>−</td>
<td>60</td>
<td>1</td>
<td>1.67%</td>
<td>0.03</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>59</td>
<td>12</td>
<td>20.34%</td>
<td>0.29</td>
<td>12</td>
<td>5</td>
</tr>
<tr>
<td><em>Bard1</em>+/+ (N)</td>
<td>−</td>
<td>35</td>
<td>5</td>
<td>0.00%</td>
<td>0.00</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>19</td>
<td>4</td>
<td>21.05%</td>
<td>0.37</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td><em>Bard1</em>KA/KA (J)</td>
<td>−</td>
<td>60</td>
<td>5</td>
<td>3.60%</td>
<td>0.08</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>77</td>
<td>39</td>
<td>50.65%</td>
<td>1.14</td>
<td>43</td>
<td>45</td>
</tr>
<tr>
<td><em>Bard1</em>KA/KA (L)</td>
<td>−</td>
<td>64</td>
<td>3</td>
<td>4.69%</td>
<td>0.06</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>72</td>
<td>37</td>
<td>72.82%</td>
<td>2.33</td>
<td>37</td>
<td>44</td>
</tr>
<tr>
<td><em>Bard1</em>KA/KA (M)</td>
<td>−</td>
<td>58</td>
<td>4</td>
<td>6.90%</td>
<td>0.10</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>40</td>
<td>11</td>
<td>27.50%</td>
<td>10.63</td>
<td>19</td>
<td>6</td>
</tr>
</tbody>
</table>

Data represents metaphases counted in two independent experiments. Percent abnormal metaphase, aberrations per metaphase, and breakdown of aberration type are shown in the table above. The data is represented graphically in Figure 21A.

Table 6A. Spontaneous and MMC-induced chromosomal aberrations in pooled analysis of *Bard1*++ and *Bard1*SR/SF cell lines

<table>
<thead>
<tr>
<th>Genotype</th>
<th>MMC</th>
<th>Total metaphase counted</th>
<th>Total abnormal metaphase</th>
<th>% abnormal metaphase</th>
<th>Aberrations/metaphase</th>
<th>Total breaks</th>
<th>Total fusions</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bard1</em>+/+</td>
<td>−</td>
<td>95</td>
<td>4</td>
<td>3.33%</td>
<td>0.02</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>78</td>
<td>16</td>
<td>30.77%</td>
<td>0.31</td>
<td>14</td>
<td>10</td>
</tr>
<tr>
<td><em>Bard1</em>KA/KA</td>
<td>−</td>
<td>182</td>
<td>12</td>
<td>6.59%</td>
<td>0.08</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>189</td>
<td>87</td>
<td>46.03%</td>
<td>1.03</td>
<td>99</td>
<td>95</td>
</tr>
</tbody>
</table>

Data represents metaphases counted in two independent experiments. *Bard1*++ data was pooled from two wild type clones (I and N) and *Bard1*KA/KA data was pooled from mutant clones (J, L, and M) shown above. The data is represented graphically in Figure 21B.
clones (J, L, and M) pooled together, a significant difference between the $Bard1^{+/+}$ and $Bard1^{KA/KA}$ cells in the average number of abnormalities per metaphase after MMC treatment (0.31 in $Bard1^{+/+}$ vs 1.03 in $Bard1^{KA/KA}$) is observed (Figure 21B, Table 6B).

In contrast to the $Bard1^{SF/SF}$ and $Bard1^{KA/KA}$ cell lines, untreated $Brca1^{SF/SF}$ primary MEFs displayed a substantial increase in chromosomal aberrations compared to their wild type isogenic controls, thus confirming the data obtained by giemsa-stained karyotypes of immortalized $Brca1^{SF/SF}$ MEFs in Figure 19A and by Shakya et al. (Shakya et al., 2011). Pooled data from $Brca1^{+/+}$ clones (Q and T) displayed spontaneous chromosomal rearrangements in 3.8% of metaphases with an average of 0.05 aberrations per metaphase compared to 33.8% abnormal metaphases and 0.60 aberrations per metaphase in the pooled $Brca1^{SF/SF}$ clones (P and R) (Figure 22). Furthermore, there was a drastic increase in MMC-induced aberrations in $Brca1^{SF/SF}$ cells compared to wild type cells, with 85.7% abnormal metaphases and 3.7 abnormalities per metaphase in the $Brca1^{SF/SF}$ clones compared with 27.5% abnormal metaphases and 0.8 abnormalities per metaphase in the $Brca1^{+/+}$ clones (Figure 22).

In summary, the data suggest that phospho-recognition by the Bard1 BRCT domain is not required for suppression of spontaneous chromosomal rearrangements, as the baseline level of aberrations in wild type primary MEFs is similar to that of $Bard1^{SF/SF}$ and $Bard1^{KA/KA}$ cells. However, in accord with the MMC hypersensitivity shown in Figure 15, $Bard1^{SF/SF}$ and $Bard1^{KA/KA}$ cells displayed significant increases in chromosomal aberrations, implying that Bard1 BRCT phospho-recognition is required for suppression of MMC-induced chromosomal instability. Furthermore, our data confirms previous studies that the phospho-recognition ability of the BRCA1 BRCT domain is required for suppression of both spontaneous and MMC-induced chromosomal instability (Shakya et al., 2011). While direct comparisons between the Bard1-mutant and Brca1-mutant MEF lines is fraught by their different genetic backgrounds (Bard1 lines were derived from C57Bl/6J mice, while the Brca1 lines were obtained from mice on a mixed m129 and c57Bl/6J background), $Brca1^{SF/SF}$ cells appear to be more prone to MMC-induced chromosomal instability than the Bard1 BRCT-mutant cells.
Figure 22. T-FISH karyotyping reveals both spontaneous and MMC-induced chromosomal instability in Brca1^{SP/SF} primary MEFs. A) Isogenic Brca1^{SP/SF} and Brca1^{+/+} primary MEF lines were cultured in the presence or absence of 40 ng/mL MMC for 16 hours. Structural chromosome abnormalities were then quantified by T-FISH. The data from four independent experiments with two Brca1^{+/+} (clones Q and T) and two Brca1^{SP/SF} (clones P and R) clones are pooled and presented as a dot plot. The mean number of aberrations per cell is denoted by a horizontal red line, and the error bars represent the standard error of the mean. P values were calculated by unpaired Student's t test (** = p<0.001). B) A detailed breakdown of the chromosomal aberrations.
5. **Bard1**$^{SF/SF}$ and **Bard1**$^{KA/KA}$ cells are proficient for HDR

i. **Bard1**$^{SF/SF}$ and **Bard1**$^{KA/KA}$ cells are proficient for Rad51 focus formation

Multiple lines of evidence implicate BRCA1 as a key component in homology-directed repair (HDR) of double-strand DNA breaks (Moynahan et al., 1999; Scully, Chen, Ochs, et al., 1997; Scully, Chen, Plug, et al., 1997). Like BRCA1-null cells, BARD1-null cells are deficient in HDR (Baer & Ludwig, 2002; Fabbro et al., 2002; Joukov et al., 2006; Laufer et al., 2007; E. E. McCarthy et al., 2003; Westermark et al., 2003). Moreover, the phospho-recognition property of the Brca1 BRCT domain is essential for HDR, as **Brca1**$^{SF/SF}$ cells have been shown to possess a clear HDR defect (Shakya et al., 2011). Therefore, on the basis of these findings, we hypothesized that Bard1 BRCT phospho-recognition would also be required for BRCA1/BARD1-mediated HDR.

To test this hypothesis, we first examined the ability of **Bard1**$^{KA/KA}$ and **Bard1**$^{SF/SF}$ cell lines to form Rad51 foci after treatment with ionizing radiation. During HDR, Rad51 polypeptides are assembled onto the resected ends of DSBs to form ssDNA/Rad51 nucleoprotein filaments. In cells treated with ionizing radiation (IR), this process is manifested cytologically by the appearance of Rad51 nuclear foci. Cells with defects in HDR, including **Brca1**$^{SF/SF}$ cells, do not form Rad51 foci as efficiently as HDR-competent cells (Shakya et al., 2011). To ascertain whether ssDNA/Rad51 filaments are assembled efficiently in response to DNA damage in the Bard1-mutant MEFs, we examined Rad51 focus formation by immunofluorescent microscopy. Immortalized MEFs were treated with 10 Gy of IR, harvested one hour later, and stained with a Rad51 specific antibody. The number of cells with ≥10 Rad51 foci was quantified to detect the presence or absence of an HDR defect. As shown in Figure 23A, isogenic **Bard1**$^{+/+}$, **Bard1**$^{SF/+}$, and two independent **Bard1**$^{SF/SF}$ clones displayed a significant increase in Rad51 foci after IR treatment. Furthermore, the percentage of cells with ≥10 Rad51 foci were comparable between the **Bard1**$^{+/+}$ clone (I – 52.6%) and the two **Bard1**$^{SF/SF}$ clones (A – 57.0% and C – 51.3%). In accord with previous studies, Rad51 focus formation was markedly reduced in **Brca1**$^{SF/SF}$ cells relative to **Brca1**$^{+/+}$ control cells. Specifically, 53.6% of **Brca1**$^{+/+}$ cells had ≥10 Rad51 foci compared to only 31.4% of **Brca1**$^{SF/SF}$ cells (Figure 23A).

Similar to the **Bard1**$^{SF/SF}$ cells, the levels of Rad51 focus formation in **Bard1**$^{KA/KA}$ cells were indistinguishable from those of their isogenic wild type control, with 49.6% (**Bard1**$^{KA/KA}$ clone J) and 50.0%
A. % cells with ≥10 Rad51 foci

-IR | +IR
---|---
Bard1 +/+ (E) |  | **
Bard1 SF/+ (D) |  | 
Bard1 SF/SF (C) |  | 
Bard1 SF/SF (A) |  | 
Brca1 SF/SF (P) |  | 
Brca1 +/+ (Q) |  | 

B. % cells with ≥10 Rad51 foci

-IR | +IR
---|---
Bard1 +/+ (I) |  | **
Bard1 KA/+ (K) |  | 
Bard1 KA/KA (J) |  | 
Bard1 KA/KA (L) |  | 
Brca1 SF/SF (P) |  | 
Brca1 +/+ (Q) |  | 

C. Rad51/DAPI

<table>
<thead>
<tr>
<th>Genotype (clone)</th>
<th>Bard1 +/+ (E)</th>
<th>Bard1 SF/SF (A)</th>
<th>Bard1 KA/KA (J)</th>
<th>Brca1 +/+ (Q)</th>
<th>Brca1 SF/SF (P)</th>
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</thead>
<tbody>
<tr>
<td>-IR</td>
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<tr>
<td>+IR</td>
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-IR: Irradiation
+IR: + Irradiation
Figure 23. Bard1^{SF/SF} and Bard1^{KA/KA} cells are competent for ionizing radiation-induced Rad51 focus formation. 
A) Isogenic Bard1^{+/+}, Bard1^{SF/+} and Bard1^{SF/SF} immortalized MEFs, as well as isogenic Brca1^{+/+} and Brca1^{SF/SF} MEFs, were exposed to 10 Gy of ionizing radiation (IR) and allowed to recover one hour before fixation. The percentage of cells with ≥10 Rad51 foci was quantified by immunofluorescent microscopy. At least 300 cells were counted per condition. The bar graph represents the averages of three independent experiments, and the error bars represent the standard error the mean. Statistical analysis of the Brca1 clones was conducted using unpaired Student's T-Test \( ** = p<0.001 \). 
B) Isogenic Bard1^{+/+}, Bard1^{KA/+} and Bard1^{KA/KA} immortalized MEFs, as well as isogenic Brca1^{+/+} and Brca1^{SF/SF} MEFs, were exposed to 10 Gy of IR and allowed to recover one hour before fixation. The percentage of cells with ≥10 Rad51 foci was quantified by immunofluorescent microscopy. At least 300 cells were counted per condition. The bar graph represents the averages of three independent experiments, and the error bars represent the standard error the mean. Statistical analysis of the Brca1 clones was conducted using unpaired Student's T-Test \( ** = p<0.001 \). 
C) Representative images of untreated and IR-treated cells from the experiments shown in A and B.
(Bard1<sup>KA/KA</sup> clone L) of cells displaying more than 10 Rad51 foci compared 50.6% of cells in the Bard1<sup>+/+</sup> clone (I) (Figure 23B). Once again, a Brca1<sup>SF/SF</sup> cell line treated in parallel with the Bard1<sup>KA/KA</sup> cell lines displayed a defect in Rad51 focus formation compared to its isogenic wild type control (32.6% - Brca1<sup>SF/SF</sup> vs. 54.1% - Brca1<sup>+/+</sup>) (Figure 23B).

Moreover, the Rad51 foci levels in Bard1<sup>SF/SF</sup> and Bard1<sup>KA/KA</sup> cells were also similar to those of wild type controls beyond one-hour post treatment. As shown in Figure 24, we performed a time-course experiment in which cells were treated with 10 Gy of ionizing radiation and then harvested at 1, 2, 4, 6, 8, and 10 hours post treatment. Upon quantifying the percentage of cells with ≥10 Rad51 foci, it was observed that Rad51 foci levels remained elevated hours after IR induction and only approached baseline levels near the end of the time course in both wildtype and Bard1-mutant MEFs. This observation indicates that the Bard1 mutations do not significantly affect the kinetics of Rad51 focus resolution. As expected, Brca1<sup>SF/SF</sup> cells displayed a decreased ability to form Rad51 foci across all time points (Figure 24A and B). Therefore, phospho-recognition by the Bard1 BRCT domain is not required for Rad51 focus formation after IR treatment. These data suggest that the Bard1 BRCT domain is dispensable for BRCA1/BARD1-mediated HDR.

ii. Bard1<sup>SF/SF</sup> and Bard1<sup>KA/KA</sup> cells display gene-targeting efficiencies similar to those of wild type cells

To further investigate the role of the Bard1 BRCT domain in HDR, we compared gene-targeting efficiencies in cells that do or do not harbor the Bard1 BRCT mutations. Although the precise mechanisms by which gene targeting through homologous recombination (HR) occurs are unclear, it is believed to entail many of the same components as homology-directed repair (HDR) of DSBs. In support of this notion, BRCA1-mutant cells display severe defects in gene targeting compared to wild type cells (Moynahan et al., 1999).

To quantitate gene targeting into the Pim1 locus on mouse chromosome 17, Bard1<sup>SF/SF</sup>, Bard1<sup>KA/KA</sup>, or Bard1<sup>+/+</sup> embryonic stem (ES) cells were electroporated with the Xhol-linearized p59xDR-GFP6 targeting vector. The p59xDR-GFP6 targeting vector is a plasmid that contains the DR-GFP reporter construct (described in the next section), along with a promoterless hygromycin resistance gene
Figure 24. The kinetics of ionizing radiation-induced Rad51 focus formation is unaffected in Bard1<sup>SF/SF</sup> and Bard1<sup>KA/KA</sup> cells.  A) Isogenic Bard1<sup>+/−</sup>, Bard1<sup>SF/SF</sup> and Bard1<sup>SF/SF</sup> immortalized MEFs, as well as isogenic Brca1<sup>−/−</sup> and Brca1<sup>SF/SF</sup> MEFs, were treated with 10 Gy of ionizing radiation (IR) and fixed at various times post-IR treatment. The percentage of cells with ≥10 Rad51 foci was quantified by immunofluorescent microscopy. At least 300 cells were counted per condition. B) Isogenic Bard1<sup>+/−</sup>, Bard1<sup>KA/KA</sup> and Bard1<sup>KA/KA</sup> immortalized MEFs, as well as isogenic Brca1<sup>−/−</sup> and Brca1<sup>SF/SF</sup> MEFs, were treated with 10 Gy of IR and fixed at varying times post-IR treatment. The percentage of cells with ≥10 Rad51 foci was quantified by immunofluorescent microscopy. At least 300 cells were counted per condition.
flanked by *Pim1* genomic DNA sequences (Moynahan, Pierce, et al., 2001; Pierce & Jasin, 2001). The ES cells were then selected with hygromycin and proper homologous integration into the *Pim1* locus was assessed by Southern blot analysis of the individual hygromycin-resistant subclones. As shown in Figures 25A and B, the p59xDR-GFP6 plasmid was properly targeted to the *Pim1* locus in 47 of 48 (97.9%) of *Bard1*\(^{SF/SF}\) clones compared to 40 of 47 (85.1%) of isogenic *Bard1*\(^{+/+}\) clones. Similarly, correct targeting was observed in 29 of 30 (96.7%) of *Bard1*\(^{KA/KA}\) clones compared to 22 of 25 (88.0%) of isogenic *Bard1*\(^{+/+}\) clones (Figure 25C and D). Therefore, the *Bard1*\(^{SF/SF}\) and *Bard1*\(^{KA/KA}\) ES cells do not display a defect in gene targeting and, in fact, appear to be slightly more efficient than their isogenic wild type controls.

**iii. *Bard1*\(^{SF/SF}\) and *Bard1*\(^{KA/KA}\) cells do not display a defect in double-strand break repair by HDR**

Using the DR-GFP reporter system, we next determined whether phospho-recognition by the Bard1 BRCT domain is required for HDR of an induced double-strand break (DSB) *in vivo*. The DR-GFP reporter is a DNA construct that consists of two non-functional GFP genes (Figure 26A). One of the GFP genes harbors both an in-frame stop codon and a restriction site for the I-SceI endonuclease, whereas the coding sequences of the second GFP gene is truncated at both the N and C-termini (iGFP). A DNA DSB can be induced within the first GFP gene by transfection of the I-SceI restriction enzyme into cells possessing the DR-GFP reporter. In cells that are competent for HDR, the DSB can be repaired using iGFP as a template for homologous recombination, resulting in the formation of a functional GFP gene. Successful HDR repair can then be quantified by detection of GFP-positive cells using fluorescent activated cell sorting (FACS). Previous studies using the DR-GFP reporter have shown that Brca1-null, Bard1-null, and *Brca1*\(^{SF/SF}\) cells all display a marked decrease in GFP-positive cells compared to wild type controls (Laufer et al., 2007; Moynahan et al., 1999; Moynahan, Cui, et al., 2001; Shakya et al., 2011). To measure repair of an I-SceI-induced chromosomal DSB within the DR-GFP reporter, the *Bard1*\(^{SF/SF}\), *Bard1*\(^{KA/KA}\) and their isogenic *Bard1*\(^{+/+}\) controls (targeted at the *Pim1* locus as described in the previous Section 5-ii) were transiently transfected with either an expression vector encoding the I-SceI meganuclease or an empty vector (EV), and the proportions of GFP-positive ES cells were quantified by flow cytometry. In parallel, we also analyzed an isogenic pair of wildtype (*Brca1*\(^{+/+}\)) and Brca1-mutant
Figure 25. Gene targeting efficiency at the Pim1 locus of Bard1^{SF/SF} and Bard1^{KA/KA} ES cells. Mutation of Bard1 BRCT phospho-recognition does not impair gene-targeting efficiency. A) Isogenic Bard1^{+/+} and Bard1^{SF/SF} ES cells were electroporated with linearized p59xDR-GFP6 targeting vector, selected for hygromycin resistance, and evaluated for incorporation of the reporter into the Pim1 locus by Southern blot analysis. Properly targeted clones show a 2.4 kb band whereas the wild type Pim1 locus yields a 3.6 kb band. B) A table showing the number of correctly targeted clones over the number of clones analyzed in isogenic Bard1^{+/+} and Bard1^{SF/SF} ES cells. C) Isogenic Bard1^{+/+} and Bard1^{KA/KA} ES cells were electroporated with linearized p59xDR-GFP6 targeting vector, selected for hygromycin resistance, and evaluated for incorporation of the reporter into the Pim1 locus by Southern blot analysis. Properly targeted clones show a 2.4 kb band whereas the wild type Pim1 locus yields a 3.6 kb band. D) A table showing the number of correctly targeted clones to the Pim1 locus over the number of clones analyzed in isogenic Bard1^{+/+} and Bard1^{KA/KA} ES cells.
Figure 26. *Bard1*^{1SR/1SF} and *Bard1*^{1KA/1KA} cells do not have a defect in HDR. A) Schematic of the DR-GFP reporter. The reporter consists of two nonfunctional GFP genes, one that harbors both an in-frame stop codon and a restriction site for the I-SceI endonuclease and one that has an N- and C-terminally truncated reading frame (iGFP). Upon induction of a DSB by transfection of the I-SceI endonuclease, cells that are competent for HDR may repair the DSB by using N- and C-terminal truncated GFP (iGFP) as a template for homologous recombination, resulting in the formation of a functional GFP gene. B) Three independent subclones each of isogenic *Bard1*^{1+/+} and *Bard1*^{1SR/1SF} cells containing the DR-GFP reporter were transfected with an I-SceI expression plasmid or the empty vector (EV) (left). Additionally, an isogenic pair of *Brcal*^{1+/+} and *Brcal*^{1SR/1SF} ES subclones containing the integrated DR-GFP reporter at the same site of the Pim1 locus were examined in parallel (right). The percentage of GFP-positive cells was then quantified by flow cytometry. As expected, I-SceI-expressing *Brcal*^{1SR/1SF} cells displayed a marked decrease in GFP+ cells compared to their isogenic *Brcal*^{1+/+} control. At least 25,000 cells were counted per experimental condition, and the percentage of GFP-positive cells was normalized to the transfection efficiency as determined by percentage of GFP-positive cells in clones transfected with a GFP expression vector. Each ES subclone was analyzed in triplicate with three independent transfections. Error bars represent standard error of the mean. C) Three independent subclones each of isogenic *Bard1*^{1+/+} and *Bard1*^{1KA/1KA} cells containing the DR-GFP reporter were transfected with an I-SceI expression plasmid or the empty vector (EV) (left). Additionally, an isogenic pair of *Brcal*^{1+/+} and *Brcal*^{1SR/1SF} ES subclones containing the integrated DR-GFP reporter at the same site of the Pim1 locus were examined in parallel (right). The percentage of GFP-positive cells was then quantified via flow cytometry. As expected, I-SceI-expressing *Brcal*^{1SR/1SF} cells displayed a marked decrease in GFP+ cells compared to their isogenic *Brcal*^{1+/+} control. In contrast, the proportion of GFP-positive cells observed in I-SceI-expressing *Bard1*^{1KA/1KA} subclones was comparable to that observed in *Bard1*^{1+/+} subclones, indicating efficient HDR. At least 25,000 cells were counted per experimental condition, and the percentage of GFP-positive cells was normalized to the transfection efficiency as determined by percentage of GFP-positive cells in clones transfected with a GFP expression vector. Each ES subclone was analyzed in triplicate with three independent transfections. Error bars represent standard error of the mean.
(Brca1<sup>SF/SF</sup>) ES cell lines harboring DR-GFP integrated into the same position of the Pim1 locus (Shakya et al., 2011). The percentage of GFP-positive cells was normalized to transfection efficiency as determined by the percentage of GFP-positive cells after transfection with a GFP expression vector. In all cell lines transfected with the empty vector (EV), the percent of GFP-positive cells was very low (<0.1%), indicating that spontaneous gene conversion within the HDR reporter is exceedingly rare (Figure 26B and C). In contrast, the percent of GFP-positive cells in wild type subclones was markedly elevated upon expression of I-SceI, indicating that HDR within the reporter is induced by DSB formation (Figure 26B and C) (Pierce & Jasin, 2001). As expected, when transfected with I-SceI, the proportion of GFP-positive cells was markedly reduced (approximately 9-fold) in Brca1<sup>SF/SF</sup> cells relative Brca1<sup>+</sup>/<sup>+</sup> cells (Figure 26B and C) (Shakya et al., 2011). In contrast, no significant difference was observed upon analysis of multiple independent isogenic subclones of wildtype (Bard1<sup>+</sup>/<sup>+</sup>) and Bard1-mutant (Bard1<sup>SF/SF</sup> and Bard1<sup>KA/KA</sup>) ES cells (Figure 26B and C). These results, together with the analysis of Rad51 focus formation (Figures 23 and 24) and gene targeting efficiency (Figure 25), indicate that HDR is dependent on the phospho-recognition potential of the Brca1 BRCT domain, but not that of the Bard1 BRCT domain.

C. Discussion

BRCA1 polypeptides exist primarily in a nuclear complex with the BARD1 tumor suppressor, and the resulting BRCA1/BARD1 heterodimer mediates many of the functions ascribed to BRCA1, including HDR and tumor suppression (L. C. Wu et al., 1996) (Laufer et al., 2007) (Westermark et al., 2003) (Shakya et al., 2008). Like BRCA1, BARD1 harbors two tandem C-terminal BRCT repeats that form a phosphate binding cleft. Although BRCA1 uses its BRCT domain to recognize particular phosphoproteins, several of which have been implicated in HDR, no phosphoprotein ligands have yet been identified for the BARD1 BRCT domain. Nonetheless, the BARD1 BRCT domain can bind in a phospho-dependent manner to poly(ADP-ribose) (PAR), a nucleic acid polymer that is rapidly assembled at sites of DNA breaks and stalled replication forks (M. Li & Yu, 2013), but the functional relevance of BARD1 BRCT phospho-recognition has remained unclear.

To address this question, we examined novel mouse strains harboring either of two missense mutations predicted to disrupt the BRCT phosphate-binding cleft of Bard1 (Bard1<sup>SF</sup> and Bard1<sup>KA</sup>),
including one that is structurally analogous to the Brca1-S1598F mutation. Homozygous Bard1\textsuperscript{SF/SF} and Bard1\textsuperscript{KA/KA} mice are born at the expected Mendelian ratios (Figure 9) and, apart from male infertility, they appear to develop normally. As such, Bard1\textsuperscript{SF/SF} and Bard1\textsuperscript{KA/KA} mice are considerably more robust than Brca1\textsuperscript{SF/SF} mice, which are born at reduced Mendelian ratios (dependent on the genetic background) and exhibit, in addition to male infertility, growth retardation and mild developmental defects (Shakya et al., 2011). Since cells harvested from Bard1\textsuperscript{SF/SF} and Bard1\textsuperscript{KA/KA} mice are competent for homology-directed repair (HDR) while Brca1\textsuperscript{SF/SF} cells display a clear HDR defect, this implies that the loss of HDR function is, at least in part, responsible for the developmental defects observed in Brca1-mutant mice. Indeed, it has been proposed that loss of HDR function leads to an accumulation of DNA damage during the rapid cell divisions that occur during embryogenesis, and that the resultant DNA damage induces premature cellular senescence, which can be partially bypassed by deletion of p53 or p16 (Hakem et al., 1996) (Cressman et al., 1999) (Xu et al., 2001).

Nevertheless, the Bard1 mutations elicit a more severe defect with respect to male germ cell development than the Brca1\textsuperscript{SF/SF} mutation, with the Bard1 mutations resulting in maturation arrest at significantly earlier stages of spermatogenesis (Figure 10). As mentioned above, spermatogenesis occurs in the seminiferous tubules beginning with the diploid germ cells (spermatogonia) which undergo a series of mitotic divisions. The developing germ cells then enter meiosis and proceed through several intermediate differentiation steps in the following order: primary spermatocyte, secondary spermatocyte, round spermatid, elongated spermatid before finally becoming mature haploid spermatozoa (de Rooij, 2001; J. M. Turner, 2007) (Kotaja & Sassone-Corsi, 2007) (Ahmed & de Rooij, 2009). Specifically, spermatogenesis in Bard1\textsuperscript{SF/SF} and Bard1\textsuperscript{KA/KA} seminiferous tubules appears to arrest at the stage of the primary spermatocytes, as shown by the accumulation of cells with large, dark staining nuclei and scant cytoplasm, compared to arrest at the round spermatid stage in Brca1\textsuperscript{SF/SF} tubules (Figure 10 B and C). Together, these observations indicate that the developmental functions of BRCT phospho-recognition by BRCA1 and BARD1 are at least partly distinct.

Clearly, the testes of Bard1-BRCT and Brca1-BRCT mutant mice display maturation arrests at distinct stages of spermatogenesis. In the Bard1-BRCT mutant mice arrest occurs at the level of primary spermatocytes, which would normally undergo meiosis I, during which the homologous chromosomes
align, DNA double-strand breaks are induced by the Spo11 enzyme, and crossing over (also known as
synapsis) occurs to facilitate the exchange of DNA sequences between homologous chromosomes
before their eventual separation. However, in the Brca1-BRCT mutant mice arrest take place at the level
of round spermatids, at a point in which both meiotic divisions should be complete (de Rooij, 2001; J. M.
Turner, 2007) (Kotaja & Sassone-Corsi, 2007) (Ahmed & de Rooij, 2009). Therefore, the Bard1SF/SF and
Bard1KA/KA mutants, in contrast to the Brca1SF/SF mutants, appear unable to complete meiosis as reflected
by their different stages of arrest and their distinct correlation with meiotic events.

Taking a closer look at the stages of meiosis I, where the Bard1-BRCT mutants appear to be
arrested as primary spermatocytes, prophase of meiosis I can be broken down into four distinct stages
that correspond with events in homologous recombination. These stages are: leptotene (when the DNA
DSBs are generated), zygotene (when the homologous chromosomes align), pachytene (when crossing
over facilitated by Rad51 occurs), and diplotene (when the synapsis between homologous chromosomes
begins to dissolve). Histological analysis suggests that the primary spermatocytes are specifically stalled
during the pachytene stage of meiosis I, as the arrested cells display the characteristic coarse chromatin
pattern representing the “thick threads” of chromosomal tetrads in pachytene. However, further staining
experiments are necessary to fully support this assertion. Interestingly, pachytene arrest has also been
observed in other Brca1 hypomorphic mutations, including Brca1Δ11, which deletes exon 11 and the
majority of the coding sequence of Brca1 (Xu, Aprelikova, Moens, Deng, & Furth, 2003).

Given the role of homologous recombination in the production of mature germ cells during
meiosis, it is tempting to speculate that a deficiency in HDR is responsible for the sterility defect seen in
Brca1 pathway mutant mice. However, since homologous recombination occurs in both male and female
gametogenesis and the sterility defect is only observed in males, a deficiency in HDR cannot completely
explain the phenotype. Furthermore, our data demonstrating the absence of an HDR defect in Bard1SF/SF
and Bard1KA/KA mutant cells argues that the sterility defect observed in our Bard1-mutant mice is
independent of HDR function. While HDR and homologous recombination in meiosis involve many of the
same components, the two processes differ considerably. Thus, it is feasible that some aspects of
protein function required for meiosis may be dispensable for HDR and vice versa. It is also possible that
there are HDR-independent and meiosis-independent functions of the BRCA1/BARD1 heterodimer that are required for production of mature sperm.

Taking into consideration the likely pachytene arrest and male-specific infertility of the Bard1-BRCT mutants, it is possible that the defect in spermatogenesis stems from a failure of meiotic sex chromosome inactivation (MSCI). MSCI is a process whereby the unsynapsed regions of the X and Y chromosomes in developing male gametes are transcriptionally silenced during prophase of meiosis I. The transcriptionally silenced, highly condensed, X and Y chromosomes are visible as a compact structure termed the XY body or sex body beginning in zygotene and persisting into meiosis II. Indeed, failure to silence the Y chromosome is believed to lead to inappropriate expression of Y chromosome genes that are toxic to the spermatocyte during pachytene (J. M. Turner, 2007). Brca1 is believed to facilitate MSCI, as Brca1 is recruited to the X and Y chromosomes during zygotene, resulting in the recruitment of the ATR kinase, phosphorylation of histone H2AX, chromatin compaction, and finally transcriptional silencing (J. M. Turner et al., 2004). The Brca1 hypomorphic mutant Brca1<sup>α11/α11</sup>, which also displays pachytene arrest of spermatocytes, has been shown to have a defect in MSCI (Xu et al., 2003). Surprisingly, another Brca1-BRCT phospho-recognition mutant (Brca1<sup>M1717R</sup>), which also has a male fertility defect similar to Brca1<sup>SF/SF</sup> mice, is competent for XY body formation (Baer Lab, unpublished data). This suggests that the phospho-recognition function of the Brca1 BRCT domain is not required for MSCI. Interestingly, deletion of poly(ADP-ribose) polymerase 2 (PARP2), an enzyme that synthesizes PAR polymers, leads to defects in XY body formation (Dantzer et al., 2006). Therefore, it is tempting to speculate that the PAR-binding function of the Bard1-BRCT domain is responsible for the initial recruitment of Brca1 to the X and Y chromosomes during MSCI and that failure of this recruitment is responsible for the male fertility defects observed in the Bard1<sup>SF/SF</sup> and Bard1<sup>KA/KA</sup> mice. However, further studies are necessary to confirm this hypothesis.

Given the well-established roles of BRCA1 and BARD1 in HDR, we initially hypothesized that phospho-recognition by the BARD1 BRCT domain is required for HDR (Baer & Ludwig, 2002; Fabbro et al., 2002; Joukov et al., 2006; Laufer et al., 2007; E. E. McCarthy et al., 2003; Moynahan et al., 1999; Scully, Chen, Ochs, et al., 1997; Scully, Chen, Plug, et al., 1997; Westermark et al., 2003). To test this hypothesis, we created isogenic panels of MEF and ES cell lines, representing a clean genetic system
compared to siRNA-mediated knockout/reconstitution experiments. Surprisingly, Bard1\textsuperscript{SF/SF} and Bard1\textsuperscript{KA/KA} cells showed no discernable defects in three separate assays designed to detect HDR deficiencies. First, the Bard1-BRCT mutants were competent for Rad51 focus formation (Figures 23 and 24), a key step in the repair of DSBs by HDR. As expected, Brca1\textsuperscript{SF/SF} cells did not form Rad51 foci as efficiently as their wild type controls or Bard1\textsuperscript{SF/SF} and Bard1\textsuperscript{KA/KA} cell lines. Second, we tested the gene targeting efficiency of our Bard1 cell lines (Figure 25). While the connection between gene targeting and HDR is still unclear, many HDR components, including BRCA1, are believed to be vital to both processes (Moynahan et al., 1999). Both Bard1-BRCT mutants displayed gene targeting efficiencies comparable to, or even slightly greater than, isogenic Bard1\textsuperscript{+/+} ES cell lines. Finally, we used the DR-GFP reporter system to measure in vivo repair of a DSB by HDR (Figure 26). Neither Bard1\textsuperscript{SF/SF} or Bard1\textsuperscript{KA/KA} ES cells displayed a decrease in GFP-positive cells when compared to their isogenic wild type control, while Brca1\textsuperscript{SF/SF} cells were clearly defective for HDR in the same assay (Shakya et al., 2011). Taken together, these data strongly suggest that phospho-recognition by the Bard1 BRCT domain is dispensable for BRCA1/BARD1-mediated HDR.

The lack of a role for the Bard1-BRCT domain in HDR is in line with previous experiments utilizing the DR-GFP reporter and involving reconstitution of a Bard1-null mouse carcinoma line with human BARD1-BRCT phospho-recognition mutants (S575F, K619A) (Laufer et al., 2007). Our data, performed in mouse ES cells harvested from transgenic Bard1-BRCT mutants, represents a clean genetic system and confirms the previous result. Furthermore, recent data suggests that, apart from stabilizing the BRCA1 protein, BARD1 may not have a unique role in HDR. This data derives from the analysis of “RING-less” BRCA1 polypeptides harboring an N-terminal truncation that disrupts BRCA1 binding to BARD1 while preserving BRCA1 stability through deletion of a nuclear export signal required for BRCA1 proteolysis (M. Li et al., 2016). As a result, these mutant cells express the RING-less BRCA1 polypeptide but have essentially no detectable levels of BARD1. Surprisingly, these cells were competent for HDR as confirmed by Rad51 focus formation after IR treatment, but still displayed genome instability associated with replication fork defects. Together these data imply that the Bard1-BRCT domain, and possibly the entire Bard1 protein, is not required for BRCA1/BARD1-mediated HDR.
Despite the absence of an HDR defect in the Bard1 BRCT phospho-recognition mutants, the BARD1SF/SF and BARD1KA/KA cell lines displayed a distinct pattern of genotoxin sensitivity, as indicated by clonogenic survival assays with immortalized MEFs (Figures 15-18). Although each of the genotoxins investigated (MMC, PARPi, IR, camptothecin) can cause multiple types of DNA damage which activate a number of DNA repair pathways, broadly the Bard1-BRCT mutants appear to be more sensitive to DNA damaging agents that had the capacity to stall replication forks (MMC, PARPi) and less sensitive to agents that primarily generate DSBs (IR). The resistance of Bard1SF/SF and Bard1KA/KA cells to agents that generate DSBs fits with the data showing that these same mutations do not result in an HDR defect. Instead, these data raise the possibility that phospho-recognition by the Bard1-BRCT domain may be required for stalled replication fork protection.

While sensitivity to MMC and PARPi are thought to stem, at least in part, from defects in HDR, there is ample evidence that sensitivity to these agents is also partially HDR-independent in BRCA1-mutant cells. Specifically, rescue of HDR function in BRCA1-mutant cells by deletion of the non-homologous end joining (NHEJ) protein 53BP1 failed to rescue MMC sensitivity (Bunting et al., 2012), implying that sensitivity to DNA inter-strand crosslinking reagents is HDR-independent. Inter-strand crosslinks (ICLs) are repaired during replication through a series of steps involving the Fanconi Anemia (FA) proteins. Repair of the lesion is thought to be stimulated by replication fork stalling and some of the FA proteins have also been implicated in the SFP pathway (Raschle et al., 2008) (Schlacher et al., 2012). However, repair of ICLs is a special case of replication fork stalling, as it requires removal of a physical lesion. As such, BRCA1 has been shown to facilitate helicase unloading and FA protein ubiquitylation which stimulate excision of the ICL (Long, Joukov, Budzowska, & Walter, 2014). Thus, it is possible that phospho-recognition by the BARD1 BRCT domain is required for an ICL repair-specific function, in addition to its role in the canonical SFP pathway. Further experiments will be necessary to fully investigate this possibility.

Furthermore, while double-mutant Brca1−/− 53BP1−/− cells are not as sensitive as Brca1−/− cells to PARPi, the 53BP1 deletion did not completely restore their PARPi resistance to wild type levels (Bunting et al., 2012). This result also suggests that the PARPi sensitivity of BRCA1-mutant cells is not completely mediated by loss of its HDR function. Indeed, supporting this are recent observations that restoration of
the SFP pathway in HDR-deficient cells can mediate partial resistance to PARPi treatment, implying that SFP defects are, at least partly responsible for PARPi sensitivity (Ray Chaudhuri et al., 2016) (Taglialatela et al., 2017).

In addition to MMC sensitivity, \textit{Bard1}^{SF/SF} and \textit{Bard1}^{KA/KA} cells are also prone to MMC-induced chromosomal rearrangements. This suggests that the inability to repair MMC-induced crosslinks leads directly to chromosomal instability. Consistent with their hypersensitivity to MMC, rescue of HDR in Brca1-mutant cells did not restore resistance to MMC-induced genome stability (Bunting et al., 2012). Together, these results support the conclusion that MMC hypersensitivity and MMC-induced genome instability are independent of HDR function. Furthermore, cells expressing the “RING-less” Brca1 polypeptide, which fails to bind Bard1, show an increase in cisplatin-induced (another DNA crosslinking reagent) chromosomal aberrations compared to wild type cells (M. Li et al., 2016). This suggests that BARD1, and specifically the phospho-recognition function of its BRCT domain, make a unique contribution to the DNA crosslink repair functions of the BRCA1/BARD1 heterodimer.

On the other hand, \textit{Bard1}^{SF/SF} and \textit{Bard1}^{KA/KA} primary MEFs did not display spontaneous chromosomal abnormalities when compared to their isogenic wild type controls (Figures 20 and 21). In contrast, as had been previously reported, significant spontaneous genomic instability was observed in \textit{BraC1}^{SF/SF} cells (Figure 22) (Shakya et al., 2011). This suggests that the suppression of spontaneous chromosomal rearrangements is dependent upon HDR function. In line with this reasoning, rescue of HDR function in BRCA1-mutant cells by 53BP1 loss suppressed spontaneous chromosomal rearrangements, as did cells expressing the “RING-less” BRCA1 polypeptide, which lack BARD1 expression but appear to be competent for HDR (Bunting et al., 2012) (M. Li et al., 2016).

Taken together, the hypersensitivity to MMC and PARPi, the susceptibility to MMC-induced genome instability, and the male sterility phenotype imply that the BARD1-BRCT domain makes unique contributions towards BRCA1/BARD1-mediated functions, rather than simply stabilizing BRCA1. Additionally, the pattern of genotoxin sensitivity implies that phospho-recognition by the Bard1 BRCT domain may be required for protection of stalled replication forks. This hypothesis will be investigated in the next chapter.
CHAPTER IV

PHOSPHO-RECOGNITION BY THE BARD1 BRCT DOMAIN IS REQUIRED FOR BRCA1/BARD1-
MEDIATED STALLED REPLICATION FORK PROTECTION BUT DISPENSABLE FOR TUMOR
SUPPRESSION
A. Introduction

Homology-directed repair (HDR) and stalled fork protection (SFP) are thought to be critical for BRCA1-mediated genome stability and tumor suppression (Kolinjivadi, Sannino, de Antoni, Techer, et al., 2017; Moynahan et al., 1999; Schlacher et al., 2012). However, given the many functions of BRCA1, it is difficult to determine the exact contributions that HDR or SFP make to these aspects of BRCA1 function. Further complicating matters is the significant overlap between the molecular components that serve in these two pathways (Kolinjivadi, Sannino, de Antoni, Techer, et al., 2017), making separation-of-function mutants valuable tools to investigate the relative contributions of HDR and SFP to genome stability and tumor suppression.

Stalled fork protection promotes genome stability by preventing the degradation and subsequent collapse of replication forks that have encountered a replication block and are unable to proceed with DNA replication (Figure 2) (Ciccia & Elledge, 2010; Cortez, 2015; Errico & Costanzo, 2012; Zeman & Cimprich, 2014). Since collapsed replication can lead to aberrant chromosomal rearrangements, it is not surprising that a deficiency in the SFP pathway is associated with genomic instability (Schlacher et al., 2011; Schlacher et al., 2012). Replication fork stalling is induced by replication stress, which can arise from many endogenous and exogenous sources. Exogenous sources include chemotherapeutic agents that deplete nucleotide pools or induce the formation of protein-DNA adducts or DNA inter-strand crosslinks. Replication stress can also occur from endogenous sources, such as oncogene activation, collision of the replication fork with transcriptional machinery, or encounters with DNA adducts created by byproducts of cellular metabolism (Ciccia & Elledge, 2010; Cortez, 2015; Errico & Costanzo, 2012; Zeman & Cimprich, 2014). Given the many sources of replication stress, replication fork stalling is likely a fairly common cellular event, thereby highlighting the importance of the SFP pathway in maintaining genome stability.

BRCA1 and BRCA2 are both required for an intact SFP pathway, and given that SFP is one of the activities common to both BRCA1 and BRCA2, SFP may be important for their tumor suppressor functions (Lomonosov et al., 2003; Schlacher et al., 2011; Schlacher et al., 2012; Ying et al., 2012). BRCA2 has been shown to protect stalled forks from degradation by MRE11 by promoting the assembly
of RAD51 onto DNA (Schlacher et al., 2011). While BRCA1-deficient cells show a defect in SFP, it is not clear mechanistically how BRCA1 contributes to the protection of stalled forks. Despite this, the role of BRCA1 in SFP has been firmly established, as BRCA1 colocalizes with the DNA replication machinery upon application of fork stalling reagents like hydroxyurea (Dungrawala et al., 2015; Scully, Chen, Ochs, et al., 1997).

BRCA1 binds with BARD1 to form an obligate stoichiometric heterodimer (Jin et al., 1997; L. C. Wu et al., 1996). Indeed, many of the functions of BRCA1, including tumor suppression and HDR, are mediated by the BRCA1/BARD1 heterodimer (Baer & Ludwig, 2002; Joukov et al., 2006; Yu & Baer, 2000). Indeed, conditional Bard1-null mice have been shown to develop mammary tumors at the same rate and of the same phenotype as those that arise in conditional Brca1-null mice (Shakya et al., 2008). Furthermore, Bard1-null cells display chromosome instability and HDR defects reminiscent of Brca1-null cells (Baer & Ludwig, 2002; Fabbro et al., 2002; Joukov et al., 2006; Laufer et al., 2007; E. E. McCarthy et al., 2003; Westermark et al., 2003).

Given that BRCA1 and BARD1 are dependent upon their heterodimerization for their stability, experiments using cells and mouse models that result in the complete inactivation of either Brca1 or Bard1 are largely uninformative in sorting out which functions of the heterodimer are mediated by BRCA1 or BARD1 individually. Furthermore, since null mutations disrupt all functions associated with the heterodimer, they cannot reveal which protein domains of BRCA1 and BARD1 are responsible for individual BRCA1/BARD1 functions like tumor suppression, HDR, or SFP. Illustrative of this point, while Bard1-null cells are defective for HDR, our data show that Bard1 BRCT phospho-recognition is dispensable for HDR (Chapter 3). On the other hand, the phospho-recognition by the Brca1-BRCT domain is required for both HDR and tumor suppression (Shakya et al., 2011). However, whether the Brca1<sup>SF/SF</sup> mutation leads to SFP defects has not been examined.

Furthermore, whether BARD1 plays a role in the SFP pathway is currently unknown. Despite this, there are some lines of evidence suggesting that BARD1 also functions in SFP. Like BRCA1, BARD1 colocalizes with the replication fork machinery after induction of replication stress by hydroxyurea (Scully, Chen, Ochs, et al., 1997). More recently, BARD1 has been shown to be highly enriched at stalled replication forks using a biochemical method called isolation of proteins on nascent DNA (iPOND)
Moreover, the sensitivity of the $Bard1^{SF/SF}$ and $Bard1^{KA/KA}$ mutants to agents that are known to stall replication forks (Chapter 3, MMC and PARPi) imply that phospho-recognition by the Bard1 BRCT domain is required for SFP. However, once again, it is unknown whether BARD1 is actually required for BRCA1/BARD1-mediated SFP, or if its role is largely to stabilize BRCA1.

Germline mutations of $BRCA1$ account for a significant proportion of the familial cases of breast and ovarian cancer (Easton, 1999). Furthermore, most BRCA1-mutant breast tumors display a “triple-negative” immunophenotype, meaning they lack expression of the estrogen receptor (ER), progesterone receptor (PR), and HER2/Neu growth factor receptor. The triple-negative immunophenotype corresponds to a “basal-like” molecular subtype as determined by gene expression profiling (Perou et al., 2000; Sorlie et al., 2001). Clinically, patients with ER-positive or HER2-positive breast cancers can be treated with tamoxifen and trastuzumab, respectively, two targeted therapies that have been shown to improve patient survival and decrease disease recurrence (Early Breast Cancer Trialists' Collaborative, 2005; Engel & Kaklamani, 2007; Piccart-Gebhart et al., 2005). However, since most BRCA1-mutant tumors do not express these markers, they are typically resistant to these targeted therapies and must be treated with standard chemotherapeutic agents (Foulkes et al., 2010). In part due to the lack of targeted therapy options, triple-negative and basal-like breast cancers are associated with poor clinical outcomes (Hennigs et al., 2016; Liedtke et al., 2008).

Given that a mutation in $BRCA1$ increases a woman’s lifetime risk of breast cancer approximately 10-30 fold, there is keen interest in determining how loss of $BRCA1$ and its associated functions lead to tumorigenesis (Antoniou et al., 2003; Roy et al., 2011). Indeed, determining the mechanism of tumor formation in BRCA1 mutants may help to reveal universally applicable concepts regarding malignant transformation. Additionally, mechanistic insights into BRCA1-mutant cancers may yield new targets for rational drug design that could be used in cancer treatment or even prevention. As proof of this concept, poly(ADP-ribose) polymerase inhibitors (PARPi) have arisen as an effective treatment for BRCA1/2-mutant tumors in recent years (Fong et al., 2009). Their deployment in the clinical setting can be traced back to basic science research that revealed deficiencies in homology-directed repair (HDR) of DNA double-strand breaks (DSB) in BRCA1-mutant cells (Moynahan et al., 1999). It was hypothesized that PARPi treatment, which inhibits the repair of single-strand DNA (ssDNA) breaks, would result in a
synthetic lethal interaction in cells with an HDR defect. Specifically, the inhibition of PARP would lead to an accumulation of ssDNA breaks, which would then be converted to DSBs during DNA replication leading to a toxic overload of DNA damage in HDR-deficient cells (Bryant et al., 2005; Farmer et al., 2005). Interestingly, sensitivity to PARPi has since been shown to stem, at least in part, from their ability to stall replication forks, indicating the stalled fork protection (SFP) pathway may also be important for BRCA1/2-mediated resistance to PARP inhibition (Pommier et al., 2016). Despite the clinical benefit offered by PARPi treatment, BRCA1/2-mutant tumors rapidly evolve resistance, often through restoration of the SFP or HDR pathway (Lord & Ashworth, 2013; Ray Chaudhuri et al., 2016; Taglialatela et al., 2017). Therefore, the identification of new drug targets, as well as how to combat therapy resistance, remains a high priority of BRCA1/2 research.

BRCA1 has been implicated in a wide spectrum of cellular activities, thus complicating the search for BRCA1 functions that are actually required for tumor suppression. As alluded to in the previous discussion of PARPi usage, the functions of BRCA1 in SFP and HDR are proposed to be important for tumor suppression. Supporting this hypothesis, loss of SFP and HDR functions have been shown to lead to genome instability, a hallmark of cancer that may also be a key initiating event in tumorigenesis (Moynahan et al., 1999; Schlacher et al., 2011; Schlacher et al., 2012). Furthermore, BRCA2 has also been implicated in both HDR and SFP, suggesting the possibility that BRCA1 and BRCA2 promote tumor suppression by common mechanisms (Couch et al., 2015; Schlacher et al., 2011).

Despite the evidence implicating SFP and HDR in the maintenance of genome stability, it has never been formally proven whether these aspects of BRCA1 function are required, either individually or together, for BRCA1-mediated tumor suppression. Additionally, since the SFP and HDR pathways share common components, it is difficult to assess the unique contributions of each pathway to tumor suppression (Kolinjivadi, Sannino, de Antoni, Techer, et al., 2017). Indeed, while mammary-specific conditional knockout mouse models have been instrumental in establishing BRCA1 and BARD1 as tumor suppressors (Shakya et al., 2008), they have been largely uninformative regarding the specific contributions of SFP and HDR to tumor suppression. Nonetheless, mechanistic insights can be obtained by incorporating mutations that target specific aspects of Brca1/Bard1 function into mouse models of hereditary breast cancer. As proof of this principle, our laboratory previously showed that a separation-of-
function mutation \((Brca1^{I26A})\) that ablates the E3 ubiquitin ligase activity of Brca1 without disrupting its interaction with Bard1 did not impair tumor suppression in mice (Shakya et al., 2011). Therefore, the E3 ubiquitin ligase activity appears to be dispensable for the tumor suppression activity of the BRCA1/BARD1 heterodimer. In contrast, the phspho-recognition function of the BRCA1 BRCT domain is required for both HDR and tumor suppression, implying that the HDR functions of BRCA1 contribute to its tumor suppression activity (Shakya et al., 2011). However, as discussed above, the contribution of the BRCA1 BRCT domain is to SFP is unknown, therefore it is unclear whether loss of HDR alone, or loss of HDR and SFP together is required for tumor formation.

In this chapter, we evaluate the roles of the Bard1 BRCT domain and the Brca1 BRCT domain in SFP. Additionally, we also evaluate whether Bard1\(^{SF/SF}\) and Bard1\(^{KA/KA}\) mice are tumor prone.

B. Results

1. The BRCT domains of Brca1 and Bard1 are required for stalled fork protection
   i. Bard1 BRCT phspho-recognition is required to protect stalled forks from nucleolytic degradation.

   Since BRCA1 is required for stalled fork protection (SFP), and SFP is critical for genome integrity, we next examined SFP in Bard1\(^{SF/SF}\) and Bard1\(^{KA/KA}\) cells (Schlacher et al., 2011; Schlacher et al., 2012). To this end, we performed DNA fiber analysis with mutant and wild type immortalized MEFs. Isogenic cell lines were subjected to two sequential 20-minute pulses with the nucleoside analogs 5'-iodo-2-deoxyuridine (IdU) and 5'-chloro-2-deoxyuridine (CldU) (Figure 27A). The track lengths of single DNA fibers were then measured immediately following CldU treatment (−HU condition) or after a 90-minute treatment with hydroxyurea (HU, +HU condition) at a dose known to stall replication forks (Schlacher et al., 2011). As a reversible inhibitor of ribonucleotide reductase, HU can induce replication fork stalling by depleting cellular nucleotide pools. Mre11-dependent fork degradation in BRCA1/2-mutant cells occurs in a directional manner such that the most recently synthesized DNA of the nascent strands is degraded first (Schlacher et al., 2011). Therefore, SFP was assessed by calculating the ratio of the lengths of adjacent
Figure 27. DNA fiber analysis reveals Mre11-mediated degradation of stalled replication forks in Bard1\superscript{SF/SF} cells. A) A schematic of the DNA fiber assay. Cycling immortalized MEFs were exposed to sequential 20-minute pulses with the nucleoside analogs 5'-iodo-2-deoxyuridine (IdU) and 5'-chloro-2-deoxyuridine (CldU). Cells were then either harvested (−HU control) or treated with 2 mM hydroxyurea (HU) for 1.5 hours and then harvested. To inhibit Mre11 nuclease activity, cells were exposed to 50 μM mirin throughout both nucleoside analogue pulses as well as the subsequent HU treatment. As Mre11-dependent fork degradation in BRCA1/2-mutant cells occurs in a directional manner such that the most recently synthesized DNA of the nascent strands is degraded first, the CldU and IdU tract lengths were measured and the CldU/IdU ratio calculated to ascertain fork degradation. B) DNA fiber analysis of two independent Bard1\superscript{SF/SF} MEF clones (G and H) and an isogenic Bard1\superscript{+/+} clone (I). For each condition, the CldU/IdU ratios of at least 150 individual DNA fibers are presented as a dot plot, and the median CldU/IdU ratio is denoted by a horizontal red line. Statistical analyses were conducted using the Mann-Whitney rank sum test (***, p<0.0001). The experiment presented here is representative of three independent experiments, each yielding the same statistically significant biological results.
CldU and ldU replication tracts. In the presence of an SFP defect, the CldU tract would be degraded first by Mre11, resulting in a decreased CldU/ldU ratio.

As expected, the median CldU/ldU ratios approximate unity in the untreated Bard1\(^{+/+}\) (clone I, 1.03) and the two untreated Bard1\(^{SF/SF}\) (G, 1.05 and H, 1.01) isogenic cell lines (Figure 27B). Furthermore, when the wild type cell line was exposed to 2 mM HU for 1.5 hours, there was no significant change in the median CldU/ldU ratios (1.05 +HU, 1.03 untreated), indicating an intact SFP pathway. In contrast, CldU/ldU ratios were significantly reduced after HU treatment in the two independent Bard1\(^{SF/SF}\) clones (0.80 and 0.77) compared to both untreated Bard1\(^{SF/SF}\) cells and HU-treated Bard1\(^{+/+}\) cells. Thus, Bard1 BRCT phospho-recognition is required for the stability of HU-stalled forks. Importantly, the CldU/ldU ratios of the two Bard1\(^{SF/SF}\) clones were fully restored by culturing these HU-treated cells in the presence of mirin, an inhibitor of Mre11 nuclease activity (1.01 in clone G and 0.98 in clone H) (Dupre et al., 2008). Thus, the DNA degradation occurring during HU treatment of Bard1\(^{SF/SF}\) cells is mediated by the MRE11 nuclease.

Similarly, the median CldU/ldU ratios of untreated isogenic clones of Bard1\(^{+/+}\) (clone N, 1.03) and Bard1\(^{KA/KA}\) (J, 0.88 and L, 0.89) were close to 1 (Figure 28B). As expected, upon treatment of the Bard1\(^{+/+}\) clone with HU, there was no significant reduction in the median CldU/ldU ratio (1.00) compared to the untreated Bard1\(^{+/+}\) cells (1.03). Consistent with the results from Bard1\(^{SF/SF}\) cells, upon HU exposure, Bard1\(^{KA/KA}\) clones J and L showed significant reductions in the median CldU/ldU ratio (0.45 and 0.47, respectively), which were largely restored by treatment with mirin (0.89 and 0.85). Taken together, these data demonstrate that phospho-recognition by the Bard1 BRCT domain is required for protection of stalled replication forks from Mre11 degradation.

ii. Brca1\(^{SF/SF}\) cells also fail to protect stalled replication forks

Although stalled fork protection (SFP) is clearly dependent on BRCA1, the functional domains of BRCA1 that mediate SFP have not yet been defined (Schlacher et al., 2012). Although Brca1\(^{SF/SF}\) cells are defective for HDR (Shakya et al., 2011), their ability to protect stalled forks is not known. Therefore, to ascertain whether the BRCT phospho-recognition activity of BRCA1 contributes to SFP, isogenic clones of Brca1\(^{SF/SF}\) (P and R) and Brca1\(^{+/+}\) (Q and T) immortalized MEFs were subjected to DNA fiber
Figure 28. DNA fiber analysis reveals Mre11-mediated degradation of stalled replication forks in Bard1<sup>KA/KA</sup> cells. **A** A schematic of the DNA fiber assay. Cycling immortalized MEFs were exposed to sequential 20-minute pulses of the nucleoside analogs 5'-ido-2-deoxyuridine (IdU) and 5'-chloro-2-deoxyuridine (CldU). Cells were then either harvested (−HU control) or treated with 2 mM hydroxyurea (HU) for 1.5 hours and then harvested. To inhibit Mre11 nuclease activity, cells were exposed to 50 μM mirin throughout both nucleoside analogue pulses as well as the subsequent HU treatment. As Mre11-dependent fork degradation in BRCA1/2-mutant cells occurs in a directional manner such that the most recently synthesized DNA of the nascent strands is degraded first, the CldU and IdU tract lengths were measured and the CldU/IdU ratio calculated to ascertain fork degradation. **B** DNA fiber analysis of two independent Bard1<sup>KA/KA</sup> MEF clones (J and L) and an isogenic Bard1<sup>+/+</sup> clone (N). For each condition, the CldU/IdU ratios of at least 150 individual DNA fibers are presented as a dot plot, and the median CldU/IdU ratio is denoted by a horizontal red line. Statistical analyses were conducted using the Mann-Whitney rank sum test (**** p<0.0001). The experiment presented here is representative of three independent experiments, each yielding the same statistically significant biological results.
analysis after sequential pulse labeling with IdU and CldU. As shown in Figure 29B, the median CldU/IdU ratios were close to 1 in both untreated Brca1^{+/+} (0.92 and 0.93) and untreated Brca1^{SF/SF} (0.92 and 0.97) clones. Likewise, the median CldU/IdU ratios of the HU-treated Brca1^{+/+} clones also approached unity (0.93 and 0.93). In contrast, however, both Brca1^{SF/SF} clones displayed a significant decrease in their median CldU/IdU ratios upon HU exposure (0.54 and 0.57), suggesting a defect in SFP. Once again, these stalled replication forks were shown to be degraded in an Mre11-dependent fashion, as application of the Mre11 inhibitor mirin to HU-treated Brca1^{SF/SF} cells rescued the median CldU/IdU ratio (0.89 and 0.91). Thus, the phospho-recognition property of the Brca1 BRCT domain is required for not only HDR (Shakya et al., 2011), but also for SFP.

2. Treatment with agents that create replication stress induce DNA damage in Bard1 and Brca1 BRCT-mutant cells

i. Bard1^{SF/SF}, Bard1^{KA/KA}, and Brca1^{SF/SF} cells display an increase in DNA damage after HU treatment

While the DNA fiber assay demonstrated that stalled replication forks are susceptible to Mre11-mediated degradation in Bard1^{SF/SF}, Bard1^{KA/KA}, and Brca1^{SF/SF} cells, the assay does not reveal whether this degradation translates into an increased burden of DNA damage and genome instability. Therefore, we used the alkaline comet assay to determine whether replication stress induces increased levels of DNA damage in Bard1^{SF/SF} and Bard1^{KA/KA} cells. In the alkaline comet assay, which detects single-strand and double-strand DNA breaks, cells are treated with a DNA damaging agent, embedded in agarose, and incubated in an alkaline denaturing buffer before being subjected to gel electrophoresis. Single-strand and double-strand DNA breaks will cause the damaged DNA to migrate though the gel faster than undamaged DNA. Upon staining with a fluorescent nucleic acid dye (GelRed); the damaged DNA can be visualized as a “comet tail” trailing the undamaged DNA (i.e., the “comet head”). The amount of DNA damage can then be quantified from the tail moment, a measurement of the length and amount of DNA present in the comet tail.

Thus, isogenic clones of Bard1^{+/+} (E and I) and Bard1^{SF/SF} (A, C, and H) immortalized MEFs, in parallel with an isogenic pair of Brca1^{+/+} (Q) and Brca1^{SF/SF} (P) clones, were exposed to 2 mM HU for 5
Figure 29. DNA fiber analysis reveals Mre11-mediated degradation of stalled replication forks in Brca1SF/SF cells. A) A schematic of the DNA fiber assay. Cycling immortalized MEFs were exposed to sequential 20-minute pulses of the nucleoside analogs 5'-ido-2-deoxyuridine (IdU) and 5'-chloro-2-deoxyuridine (CldU). Cells were then either harvested (−HU control) or treated with 2 mM hydroxyurea (HU) for 1.5 hours and then harvested. To inhibit Mre11 nuclease activity, cells were exposed to 50 μM mirin throughout both nucleoside analogue pulses as well as the subsequent HU treatment. As Mre11-dependent fork degradation in BRCA1/2-mutant cells occurs in a directional manner such that the most recently synthesized DNA of the nascent strands is degraded first, the CldU and IdU tract lengths were measured and the CldU/IdU ratio calculated to ascertain fork degradation. B) DNA fiber analysis of two independent Brca1SF/SF clones (P and R) and two isogenic Brca1+/+ clones (Q and W). For each condition, the CldU/IdU ratios of at least 150 individual DNA fibers are presented as a dot plot, and the median CldU/IdU ratio is denoted by a horizontal red line. Statistical analyses were conducted using the Mann-Whitney rank sum test (**** p<0.0001). The experiment presented here is representative of three independent experiments, each yielding the same statistically-significant biological results.
hours (Figure 30A). After HU treatment, the amount of DNA damage was quantified through measurement of the tail moment. As shown in Figure 30A, all three Bard1<sup>SF/SF</sup> cell lines displayed a significant increase in DNA damage after HU exposure when compared with their isogenic wild type controls. Specifically, the Bard1<sup>SF/SF</sup> clones (A, C, and H) displayed average tail moments of 10.1, 10.6, and 7.33, respectively, compared to averages of 2.43 and 2.57 in the two Bard1<sup>+/+</sup> clones. Additionally, the Brca1<sup>SF/SF</sup> cell line also displayed an increase in average tail moment (7.10) compared to its isogenic wild type control (3.10).

Similarly, isogenic clones of Bard1<sup>+/+</sup> (I and N) and Bard1<sup>KA/KA</sup> (J, L, and M) immortalized MEFs, in parallel with an isogenic pair of Brca1<sup>+/+</sup> (Q) and Brca1<sup>SF/SF</sup> (P) cell lines, were treated and analyzed as described above. As shown in Figure 30B, the three Bard1<sup>KA/KA</sup> clones all displayed higher levels of DNA damage after HU exposure as reflected by a significant increase in average tail moment (11.5, 10.2, and 9.17) when compared to the two Bard1<sup>+/+</sup> clones (2.96 and 3.47). Once again, the Brca1<sup>SF/SF</sup> cell line also displayed an increase in average tail moment over its Brca1<sup>+/+</sup> isogenic control (10.5 vs 4.11). Therefore, these data indicate that the SFP defects of Bard1<sup>SF/SF</sup> and Bard1<sup>KA/KA</sup> cells lead to DNA damage upon induction of replication stress.

ii. Bard1<sup>SF/SF</sup>, Bard1<sup>KA/KA</sup>, and Brca1<sup>SF/SF</sup> cells also display an increase in DNA damage after camptothecin treatment

To confirm that Bard1<sup>SF/SF</sup> and Bard1<sup>KA/KA</sup> cells are susceptible to replication stress-induced DNA damage, we performed the alkaline comet assay with camptothecin (CPT), a topoisomerase I (TopI) inhibitor that can block progression of the replication fork by stabilizing TopI-DNA adducts. Thus, the mechanism of fork stalling by camptothecin is very different from that of hydroxyurea, which creates replication stress without a physical block by the depletion of nucleotide pools. Indeed, CPT has been shown to lead to MRE11-mediated fork degradation in cell lines deficient for SFP (Seiler, Conti, Syed, Aladjem, & Pommier, 2007; Taglialatela et al., 2017).

Bard1<sup>+/+</sup> (E and I) and Bard1<sup>SF/SF</sup> (A, C, and H) isogenic cell lines were treated with 100 nM CPT for 5 hours to induce replication stress. An isogenic pair of Brca1<sup>+/+</sup> (Q) and Brca1<sup>SF/SF</sup> (P) immortalized MEF lines were also subjected to the same treatment in parallel. After treatment, the cells were
Figure 30. Alkaline comet assays of hydroxyurea-treated Bard1^{SF/SF}, Bard1^{K/AK}, and Brca1^{SF/SF} MEFs. DNA damage is specifically induced in Bard1 and Brca1 BRCT mutant cells by hydroxyurea (HU). A) Three independent Bard1^{SF/SF} MEF clones (A, C, and H) and two Bard1^{SF/SF} clones (E and I), as well as an isogenic pair of Brca1^{SF/SF} (P) and Brca1^{SF/SF} (Q) clones, were exposed to 2 mM HU for 5 hours. Cells were then harvested, embedded in agarose, denatured in alkaline buffer, electrophoresed, and incubated in a DNA-staining dye. For each condition, the comet tails of at least 75 cells were analyzed using CometScore Software Version 1.5, and the individual tail moments are presented as dot plots. The mean tail moment is denoted by a horizontal red line, and the error bars represent the standard error of the mean. Statistical analyses were conducted using one-way ANOVA (Bard1 clones) or unpaired Student's t test (Brca1 clones) (**p<0.0001). B) Three independent Bard1^{K/AK} clones (J, L, and M) and two Bard1^{SF/SF} clones (I and N), as well as an isogenic pair of Brca1^{SF/SF} (P) and Brca1^{SF/SF} (Q) clones, were exposed to HU and processed as described above. For each condition, the comet tails of at least 75 cells were analyzed using CometScore Software Version 1.5, and the individual tail moments are presented as dot plots. The mean tail moment is denoted by a horizontal red line, and the error bars represent the standard error of the mean. Statistical analyses were conducted using one-way ANOVA (Bard1 clones) or unpaired Student's t test (Brca1 clones) (**p<0.0001). C) Representative images from the experiments quantified in A and B.
embedded in agarose, incubated in denaturing buffer, electrophoresed, and stained with a DNA dye. As shown in Figure 31A, the average tail moments of the \textit{Bard1}^{SF/SF} cell lines (6.18, 5.84, and 4.78) were significantly higher than those of the two \textit{Bard1}^{+/+} cell lines (1.65 and 1.70). Additionally, the \textit{Brca1}^{SF/SF} cell line also displayed a significant increase in average tail moment compared to its isogenic wild type control (6.00 vs. 1.59).

Three \textit{Bard1}^{KA/KA} clones (J, L, and M) and their isogenic wild type controls (I and N) were also treated with CPT under the same conditions as above, along with an isogenic pair of \textit{Brca1}^{+/+} (Q) and \textit{Brca1}^{SF/SF} (P) immortalized MEF lines. Similar to those of \textit{Bard1}^{SF/SF} cells, the average tail moments of \textit{Bard1}^{KA/KA} clones (4.40, 4.83, and 4.87) were markedly increased compared to those of the \textit{Bard1}^{+/+} clones (1.79 and 1.05). The \textit{Brca1}^{SF/SF} cell line also displayed an increase in DNA damage, as its average tail moment (6.70) was significantly larger than that of its isogenic wild type control (1.95).

Therefore, the phospho-recognition functions of the both the Bard1 and Brca1 BRCT domains are required for resistance to camptothecin-mediated replication stress. Indeed, if the results from both the hydroxyurea and camptothecin alkaline comet assays are taken into consideration, then both Bard1 and Brca1 BRCT phospho-recognition are required for resistance to multiple types of replication stress.

3. Localization of the BRCA1/BARD1 heterodimer to stalled forks is impaired in \textit{Bard1}^{SF/SF} and \textit{Bard1}^{KA/KA} cells

i. Bard1 foci formation in \textit{Bard1}^{KA/KA} cells

A potential function for BRCA1 at stalled replication forks first emerged from studies of the nuclear distribution of BRCA1 and BARD1 polypeptides in S phase cells (Nagaraju & Scully, 2007; Scully, Chen, Ochs, et al., 1997). In particular, Scully et al. used immunofluorescent microscopy to show that, in unperturbed S phase cells, a fraction of the BRCA1/BARD1 pool resides in discrete nuclear foci that are clearly distinct from sites of DNA replication marked by PCNA immunostaining (Scully, Chen, Ochs, et al., 1997). However, upon HU treatment these S phase foci dissipate, and focal BRCA1/BARD1 immunostaining reappears within the PCNA-staining DNA replication structures of late S phase cells.

Recently, the notion that BRCA1 and BARD1 are recruited to stalled forks was established biochemically by Dungrawala et al., who used iPOND technology to demonstrate that BRCA1 and BARD1 specifically...
Figure 31. Alkaline comet assays of camptothecin-treated Bard1^{SP/SF}, Bard1^{KA/KA}, and Brca1^{SP/SF} MEFs. DNA damage is specifically induced in Bard1 and Brca1 BRCT mutant cells by camptothecin (CPT). A) Three independent Bard1^{SP/SF} clones (A, C, and H) and two Bard1^{+/+} clones (E and I), as well as an isogenic pair of Brca1^{SP/SF} (P) and Brca1^{+/+} (Q) clones, were treated with 100 nM CPT for 5 hours. Cells were then harvested, embedded in agarose, denatured in alkaline buffer, electrophoresed, and incubated in a DNA-staining dye. For each condition, the comet tails of at least 75 cells were analyzed using CometScore Software Version 1.5, and the individual tail moments are presented as dot plots. The mean tail moment is denoted by a horizontal red line, and the error bars represent the standard error of the mean. Statistical analyses were conducted using one-way ANOVA (Bard1 clones) or unpaired Student's t test (Brca1 clones) (**p<0.0001). B) Three independent Bard1^{KA/KA} clones (J, L, and M) and two Bard1^{+/+} clones (I and N), as well as an isogenic pair of Brca1^{SP/SF} (P) and Brca1^{+/+} (Q) clones, were treated with CPT and processed as described above. For each condition, the comet tails of at least 75 cells were analyzed using CometScore Software Version 1.5, and the individual tail moments are presented as dot plots. The mean tail moment is denoted by a horizontal red line, and the error bars represent the standard error of the mean. Statistical analyses were conducted using one-way ANOVA (Bard1 clones) or unpaired Student's t test (Brca1 clones) (**p<0.0001). C) Representative images from the experiments quantified in A and B.
associate with HU-stalled, but not unstressed, DNA replication forks (Dun格rawala et al., 2015). To ascertain whether the SFP defect of *Bard1*<sup>SF/SF</sup> and *Bard1*<sup>KA/KA</sup> cells reflects a failure of mutant BRCA1/BARD1 heterodimers to mobilize at stalled forks, we compared HU-induced recruitment of Brca1 and Bard1 polypeptides to PCNA-staining replication foci in isogenic wildtype and mutant MEFs. Thus, cells cultured for 90-minutes in the presence or absence of HU were harvested, co-stained with PCNA- and Bard1-specific antibodies, and visualized by immunofluorescent microscopy.

First, we analyzed the capacity of *Bard1*<sup>KA/KA</sup> cells to form Bard1 foci under both HU-treated and untreated conditions. Scully *et al.* (1997) reported that Brca1 and Bard1 form foci in S phase cells under both unperturbed and HU-treated conditions, however the total number of cells expressing Brca1 or Bard1 foci decreases substantially in HU-treated cells (Scully, Chen, Ochs, et al., 1997). The function of the BRCA1/BARD1 nuclear foci of unperturbed cells is currently unknown, but may represent recruitment of the heterodimer to spontaneous sites of DNA damage, or, alternatively, the sequestration of these proteins into discrete domains to prevent their interference with normal replication.

Thus, to see whether Bard1 foci dynamics are preserved in *Bard1*<sup>KA/KA</sup> cells, we cultured mutant cell lines and their isogenic wild type controls for 1.5 hours in the presence or absence of 2mM HU and quantified the percentage of cells that displayed five or more Bard1 nuclear foci (Figure 33A). As previously reported by Scully *et al.*, treatment with hydroxyurea led to a decrease in percentage of cells displaying Bard1 foci when compared to the untreated cells (Figure 32) (Scully, Chen, Ochs, et al., 1997). This decrease was observed in the both the *Bard1*<sup>KA/KA</sup> clones (J and L) and their wild type control (I). Furthermore, Bard1 foci levels in *Bard1*<sup>KA/KA</sup> clones were comparable to their wild type control in both untreated (53.0% and 55.5% in *Bard1*<sup>KA/KA</sup> clones vs. 53.5% in the *Bard1*<sup>+/+</sup> clone) and HU-treated (29.0% and 27.2% in *Bard1*<sup>KA/KA</sup> clones vs. 24.7% in the *Bard1*<sup>+/+</sup> control) conditions (Figure 32). Therefore, it is unlikely that Bard1-BRCT phospho-recognition is required for formation of Bard1 foci during S phase.

**ii. *Bard1*<sup>SF/SF</sup> and *Bard1*<sup>KA/KA</sup> cells show a defect in the recruitment of Bard1 to PCNA foci in HU-treated cells**

A recent study demonstrated that the PAR-binding ability of the Bard1-BRCT domain is required for γH2AX-independent recruitment of the BRCA1/BARD1 heterodimer to DNA damage sites induced by
Figure 32. Bard1 focus formation in Bard1^KA/KA cells. Bard1^KA/KA cells do not show a defect in Bard1 foci formation. Two independent Bard1^KA/KA clones (J and L) and an isogenic wild type control (I) were cultured for 1.5 hours in the presence or absence of 2 mM HU. The percentage of cells harboring ≥5 Bard1 foci was determined. At least 200 cells were examined for each condition. The histogram presents the average of three independent experiments and the error bars represent the standard error of the mean. Statistical analyses were performed using one-way ANOVA.
laser microirradiation (M. Li & Yu, 2013). Given that PAR chains are also rapidly assembled at stalled replication forks, and that SFP is dependent on PARP1 activity (Ying et al., 2012), we hypothesized that Bard1 BRCT phospho-recognition may be required for proper recruitment of the BRCA1/BARD1 heterodimer to stalled replication forks. To test this hypothesis, we examined the ability of Bard1 to localize to PCNA-staining replication structures after HU treatment in wild type and Bard1 BRCT-mutant cells (Figure 33A). To quantify this localization event, we first identified cells that exhibit the late S phase pattern of nodular PCNA staining (PCNA+ cells, Figure 33B). Of this PCNA+ population, cells could be further subdivided into three separate populations: cells that did not display Bard1 nuclear foci (Bard1− PCNA+), cells that displayed Bard1 foci that colocalized with PCNA (Bard1+ PCNA+ colocalized), and cells that displayed Bard1 foci that were spatially independent of PCNA (Bard1+ PCNA+ non-colocalized). We then defined the percent colocalization in PCNA+ cells as the number of Bard1+ PCNA+ colocalized cells divided by the total number of nodular-staining PCNA+ cells (i.e., the sum of the Bard1− PCNA+, Bard1+ PCNA+ non-colocalized, and Bard1+ PCNA+ colocalized populations). Therefore, we interpreted the Bard1− PCNA+ and Bard1+ PCNA+ non-colocalized cells as populations in which Bard1 had failed to mobilize to the stalled replication fork.

Thus, two independent Bard1SF/SF clones (A and C) and an isogenic Bard1+/+ clone (E) were incubated with or without 2 mM HU for 1.5 hours (Figure 33A). As was observed by Scully et al., Bard1 and PCNA rarely colocalized in untreated cells (Scully, Chen, Ochs, et al., 1997). This was true of the two Bard1SF/SF clones (A, 3.3%; C, 2.6% colocalization) as well as the isogenic Bard1+/+ control clone (E, 7.8% colocalization) (Figure 34A). As expected, upon HU treatment, the proportion of PCNA-staining late S-phase nuclei that harbor co-localizing PCNA and Bard1 foci increased approximately 10-fold in wild type cells (81.8% colocalization in +HU vs 7.8% colocalization in −HU condition) (Figure 34A), once again confirming the observations of Scully et al., (Scully, Chen, Ochs, et al., 1997). Significantly, however, we observed a marked reduction in the proportion of nuclei with co-localizing PCNA and Bard1 foci in the HU-treated Bard1SF/SF clones (22.2% and 24.5% colocalization compared to 81.8% colocalization in Bard1+/+) (Figure 34A). These results suggest that the recruitment of Bard1 to stalled forks is impaired by the Bard1SF/SF mutation.
A.

Control (−HU) → PBS mock treatment 1.5 hr → Fix cells

0 hr (+HU) → 2 mM HU treatment 1.5 hr → Fix cells

B.

\[
\text{\% colocalization} = \frac{\text{Brca1}^+ \text{ PCNA}^+ \text{ colocalizing}}{\text{PCNA}^+}
\]
Figure 33. **Brd1/Brc1 and PCNA co-staining experiments.** A) A schematic of the Brca1/Brd1 and PCNA co-staining experiments. Immortalized MEFs were cultured for 1.5 hours in the presence or absence of 2 mM hydroxyurea (HU). The cells were then harvested and co-stained for PCNA and either Brca1 or Bard1. **B) Diagram of PCNA+ cell populations.** To assess co-localization between PCNA (labeled with a red secondary antibody) and either Brca1 or Bard1 (labeled with a green secondary antibody), late S phase PCNA+ cells were identified. These PCNA+ cells were defined as any cell displaying ≥5 PCNA foci in the nodular pattern commonly observed in late S phase cells (Scully *et al.*, 1997). Three distinct populations of PCNA+ cells were readily identified: 1) cells that display ≥5 PCNA foci but no Brca1 (or Bard1) foci (Brca1−PCNA+); 2) cells that displayed both ≥5 Brca1 (or Bard1) foci and ≥5 PCNA foci in which greater than half of the Brca1 (or Bard1) foci colocalized with PCNA foci (Brca1+ PCNA+ colocalizing); and 3) cells that displayed both ≥5 Brca1 (or Bard1) foci and ≥5 PCNA foci that were spatially non-overlapping (Brca1+ PCNA+ non-colocalizing). To calculate the percent colocalization, the number of Brca1/Brd1+PCNA+ colocalizing cells was divided by the total number of PCNA+ cells (Brca1/Brd1+ PCNA+ + Brca1/Brd+ PCNA+ colocalizing + Brca1/ Bard+ PCNA+ non-colocalizing).
Figure 34. Co-localization between Bard1 and PCNA foci in \textit{Bard1}^{SF/SF} and \textit{Bard1}^{KA/KA} cells. A significant decrease in the co-localization of Bard1 and PCNA foci is observed in HU-treated \textit{Bard1}^{SF/SF} and \textit{Bard1}^{KA/KA} cells relative to HU-treated \textit{Bard1}^{+/+} cells, suggesting a recruitment defect to stalled replication forks. \textbf{A)} Two independent \textit{Bard1}^{SF/SF} MEF clones (A and C) and an isogenic \textit{Bard1}^{+/+} clone (E) were cultured for 1.5 hours in the presence or absence of 2 mM hydroxyurea (HU). At least 200 cells were examined for each condition, and the percentage of Bard1/PCNA co-localizing cells was determined as described in Figure X. The histogram presents the average of three independent experiments and the error bars represent the standard error of the mean. Statistical analyses were performed using one-way ANOVA (**** \( p < 0.0001 \)). \textbf{B)} Two independent \textit{Bard1}^{KA/KA} clones (J and L) and an isogenic \textit{Bard1}^{+/+} clone (I) were cultured for 1.5 hours in the presence or absence of 2 mM hydroxyurea (HU). At least 200 cells were examined for each condition, and the percentage of Bard1/PCNA co-localizing cells was determined as described in Figure X. The histogram presents the average of three independent experiments and error bars represent the standard error of the mean. Statistical analyses were performed using one-way ANOVA (** \( p < 0.0001 \), **** \( p < 0.0001 \)).
Figure 34. Colocalization between Bard1 and PCNA foci in *Bard1^{SP/SP}* and *Bard1^{KA/KA}* cells. C) Representative images from the Bard1 and PCNA co-staining experiments quantified in panels A and B.
To confirm this result for the Bard1\textsuperscript{KA} mutant, we subjected two independent Bard1\textsuperscript{KA/KA} clones (J and L) along with an isogenic Bard1\textsuperscript{+/+} clone (I) to the same analysis. Once again, colocalization between Bard1 and PCNA was exceedingly rare in untreated cells in both the Bard1\textsuperscript{KA/KA} clones (2.0% and 1.6% colocalization) and the Bard1\textsuperscript{+/+} clone (2.3% colocalization) (Figure 34B). Upon HU treatment of the Bard1\textsuperscript{+/+} clone, colocalization of Bard1 with PCNA was observed in the overwhelming majority of PCNA\textsuperscript{+} cells (87.3% colocalization). In sharp contrast, in the HU-treated Bard1\textsuperscript{KA/KA} clones Bard1 colocalized with PCNA-staining replication structures in only a fraction of PCNA\textsuperscript{+} cells (42.3% and 35.2% colocalization in PCNA\textsuperscript{+}) (Figure 34B). Therefore, the Bard1\textsuperscript{KA} mutation also impairs the recruitment of Bard1 to HU-stalled replication forks. Together, these results indicate that Bard1 BRCT phospho-recognition is required for recruitment of Bard1 to stalled replication forks.

iii. Brca1 focus formation in Bard1\textsuperscript{KA/KA} cells

Since Bard1 and Brca1 form an obligate heterodimer and colocalize in nuclear foci, we hypothesized that Brca1 would also display a recruitment defect to PCNA-staining replication structures (Baer & Ludwig, 2002; Jin et al., 1997; L. C. Wu et al., 1996). We first examined the formation of Brca1 nuclear foci in HU- and mock-treated clones of both Bard1\textsuperscript{+/+} and Bard1\textsuperscript{KA/KA} cells (Figure 35). As had been observed with Bard1 foci in Figure 32, the percentage of cells harboring ≥5 Brca1 foci decreased upon HU treatment in the two independent Bard1\textsuperscript{KA/KA} clones (L: 55.3% in untreated vs. 31.3% in HU-treated; M: 64.2% in untreated vs. 22.8% in HU-treated) and their isogenic Bard1\textsuperscript{+/+} control (I: 57.8% in untreated vs. 22.5% in HU-treated) (Figure 35B). Furthermore, consistent with the Bard1 focus formation data, there was no difference in the percentage of cells harboring five or more Brca1 foci between the Bard1\textsuperscript{KA/KA} and Bard1\textsuperscript{+/+} clones in the untreated (55.3% and 64.2% in Bard1\textsuperscript{KA/KA} vs. 57.8% in Bard1\textsuperscript{+/+}) or HU-treated (31.3% and 22.8% in Bard1\textsuperscript{KA/KA} vs. 22.5% in Bard1\textsuperscript{+/+}) conditions. Therefore, Bard1 BRCT phospho-recognition does not appear to be required for the formation of Brca1/Bard1 foci under either untreated or HU-treated conditions.
Figure 35. Brca1 focus formation in Bard1<sup>KA/KA</sup> cells. Bard1<sup>KA/KA</sup> cells do not show a defect in Brca1 foci formation. Two independent Bard1<sup>KA/KA</sup> clones (J and L) and an isogenic Bard1<sup>+/+</sup> clone (I) were cultured for 1.5 hours in the presence or absence of 2 mM HU. At least 200 cells were examined for each condition, and the percentage of cells harboring ≥5 Brca1 foci was determined. The histogram presents the average of three independent experiments and the error bars represent the standard error of the mean. Statistical analyses were performed using one-way ANOVA.
iv. **Bard1**<sup>SF/SF</sup> and **Bard1**<sup>KA/KA</sup> cells show a defect in the recruitment of Brca1 to PCNA foci in HU-treated cells

Next, we sought to confirm whether the defect in Bard1 recruitment to HU-stalled replication forks observed in **Bard1**<sup>SF/SF</sup> and **Bard1**<sup>KA/KA</sup> cell is accompanied by a corresponding defect in Brca1 recruitment. Therefore, we examined the ability of Brca1 to localize to PCNA-staining replication structures of late S phase in HU-treated wild type and Bard1 BRCT-mutant cells. As above, we defined the percent colocalization as the number of Brca1<sup>+</sup> PCNA<sup>+</sup> colocalized cells divided by the total number of PCNA<sup>+</sup> cells (i.e., the sum of Brca1<sup>−</sup> PCNA<sup>+</sup>, Brca1<sup>+</sup> PCNA<sup>+</sup> non-colocalized, and Brca1<sup>+</sup> PCNA<sup>+</sup> colocalized cells) (Figure 33B). Consistent with our analysis of Bard1-PCNA colocalization, we interpreted the Brca1<sup>−</sup> PCNA<sup>+</sup> and Bard1<sup>+</sup> PCNA<sup>+</sup> non-colocalized cells as populations where Brca1 had failed to localize at stalled replication forks.

As such, we cultured two independent **Bard1**<sup>SF/SF</sup> clones (A and C) along with their isogenic **Bard1**<sup>+/+</sup> control (E) in the presence or absence of 2 mM HU for 1.5 hours (Figure 33A). Brca1 localization to PCNA-staining replication structures was highly uncommon in untreated cells in both the isogenic **Bard1**<sup>SF/SF</sup> mutants (4.6% and 6.0% colocalization in PCNA<sup>+</sup> cells) and the **Bard1**<sup>+/+</sup> isogenic clone (9.5% colocalization in PCNA<sup>+</sup> cells) clones (Figure 36A). Upon HU treatment, Brca1 was efficiently recruited to PCNA-staining replication structures in the wild type cell line (66.1% colocalization in PCNA<sup>+</sup> cells). However, in both **Bard1**<sup>SF/SF</sup> clones, localization of Brca1 to PCNA-staining replication structures was significantly disrupted compared to the wild type cell line, as only 23.8% and 21.6% colocalization was observed in PCNA<sup>+</sup> cells in **Bard1**<sup>SF/SF</sup> clones A and C, respectively.

Consistent with what was observed in **Bard1**<sup>SF/SF</sup> clones, colocalization between Brca1 and PCNA was exceedingly rare in untreated **Bard1**<sup>KA/KA</sup> cells (clone J: 2.1% and clone L: 1.9% colocalization in PCNA<sup>+</sup> cells) and an isogenic **Bard1**<sup>+/+</sup> cell line (clone I: 2.1% colocalization in PCNA<sup>+</sup> cells) (Figure 36B). Upon treatment with HU, Brca1 localized to PCNA-staining replication structures in the vast majority of PCNA<sup>+</sup> cells in the **Bard1**<sup>+/+</sup> clone (94.7% colocalization). In sharp contrast, the two **Bard1**<sup>KA/KA</sup> clones displayed a defect in the recruitment of Brca1 to stalled replication forks as Brca1 localization to PCNA-staining replication structures was only observed in 30.4% and 41.3% of PCNA<sup>+</sup> cells. Therefore, the recruitment of Brca1 to HU-stalled replication forks is specifically impaired in both **Bard1**<sup>SF/SF</sup> and
Figure 36. Co-localization between Brca1 and PCNA foci in Bard1<sup>SS/SS</sup> and Bard1<sup>KA/KA</sup> cells. A significant decrease in the co-localization of Brca1 and PCNA foci is observed in HU-treated Bard1<sup>SS/SS</sup> and Bard1<sup>KA/KA</sup> cells relative to HU-treated Bard1<sup>+/+</sup> cells, suggesting a recruitment defect to stalled replication forks. A) Two independent Bard1<sup>SS/SS</sup> MEF clones (A and C) and an isogenic Bard1<sup>+/+</sup> clone (E) were cultured for 1.5 hours in the presence or absence of 2mM hydroxyurea (HU). At least 200 cells were examined for each condition, and the percentage of Brca1/PCNA co-localizing cells was determined as described in Figure X. The histogram presents the average of three independent experiments and the error bars represent the standard error of the mean. Statistical analyses were performed using one-way ANOVA (*** p<0.0001). B) Two independent Bard1<sup>KA/KA</sup> clones (J and L) and an isogenic Bard1<sup>+/+</sup> clone (I) were cultured for 1.5 hours in the presence or absence of 2 mM HU. At least 200 cells were examined for each condition, and the percentage of Brca1/PCNA co-localizing cells was determined as described in Figure X. The histogram presents the average of three independent experiments and error bars represent the standard error of the mean. Statistical analyses were performed using one-way ANOVA (p<0.0001).
Figure 36. Colocalization between Brca1 and PCNA foci in Bard1<sup>SF/SF</sup> and Bard1<sup>KAXA</sup> cells. C) Representative images from the Brca1 and PCNA co-staining experiments quantified in panels A and B.
Bard1\textsuperscript{KA/KA} cells. Indeed, if the Bard1 and Brca1 co-staining experiments are taken together, the data indicates that Bard1 BRCT phospho-recognition is required for recruitment of the Brca1/Bard1 heterodimer to HU-stalled replication forks.

4. Tumorigenesis in Bard1 BRCT mutants

Although Brca1-null and Bard1-null animals undergo embryonic lethality, mice homozygous for certain hypomorphic Brca1 mutations (e.g., Brca1\textsuperscript{SF/SF} or Brca1\textsuperscript{inf/inf}) are viable. As these mice age, they develop a broad spectrum of tumor types at increased rates relative to their wildtype and heterozygous littermates (Ludwig et al., 2001; Shakya et al., 2011). Since Brca1\textsuperscript{SF/SF} cells are defective for both HDR and SFP, it is not possible to conclude whether the tumor susceptibility of these Brca1-mutant mice reflects the loss of HDR, SFP, or both. In contrast, Bard1\textsuperscript{SF} and Bard1\textsuperscript{KA} are separation-of-function alleles that abrogate SFP without affecting HDR. Therefore, we generated cohorts of Bard1\textsuperscript{SF/SF} and Bard1\textsuperscript{KA/KA} mice and monitored them for tumor formation (Figure 37A). Although some of these animals developed tumors at an advanced age, the kinetics of tumor formation in Bard1\textsuperscript{SF/SF} and Bard1\textsuperscript{KA/KA} mice were statistically indistinguishable from that of their wild type littermate controls (Bard1\textsuperscript{SF/SF} vs. Bard1\textsuperscript{+/+} p = 0.9854; Bard1\textsuperscript{KA/KA} vs. Bard1\textsuperscript{+/+} p = 0.8387). When the Kaplan-Meier curve of tumor-free survival for Brca1\textsuperscript{SF/SF} mice (Shakya et al., 2011) is superimposed on those of the Bard1 cohorts, it is clear that the kinetics of tumor formation occur with a markedly increased frequency and decreased latency in Brca1\textsuperscript{SF/SF} mice relative to Bard1\textsuperscript{SF/SF} and Bard1\textsuperscript{KA/KA} mice (Figure 37B). Indeed, the kinetics of tumor formation in the Brca1\textsuperscript{SF/SF} mice was significantly greater when compared to either the Bard1\textsuperscript{SF/SF}, Bard1\textsuperscript{KA/KA}, or Bard1\textsuperscript{+/+} cohorts (p < 0.0001 for all comparisons). Therefore, the Bard1\textsuperscript{SF/SF} and Bard1\textsuperscript{KA/KA} mice are not tumor prone.

In the Bard1\textsuperscript{SF/SF} cohort, only 7 of 39 mice developed neoplasms. Of the seven mice that developed neoplasms, the average age at the time of tumor detection was 724 days, which was well past the T_{50} of Brca1\textsuperscript{SF/SF} mice (575 days) (Shakya et al., 2011). Given the advanced age of Bard1\textsuperscript{SF/SF} mice at the time of tumor formation, these likely represent spontaneous malignancies. Furthermore, none of the
Figure 37. Tumor formation in Bard1^{SF/SF} and Bard1^{KA/KA} mice. A) Kaplan-Meier tumor-free survival curves of the Bard1^{+/+} (n = 28), Bard1^{SF/SF} (n = 39), and Bard1^{KA/KA} (n=34) cohorts are presented. Using the log-rank (Mantel-Cox) test, no statistical significance (defined as p<0.05) was observed between the Bard1^{SF/SF} and Bard1^{+/+} curves (p = 0.9854) or the Bard1^{KA/KA} and Bard1^{+/+} curves (p = 0.8387). B) The Kaplan-Meier tumor-free survival curve of Brca1^{SF/SF} mice (n=72) from Shakya et al. (2011) has been added for comparison.
seven neoplasms were mammary tumors. Instead, most were hematological malignancies, specifically monocytoid leukemias and lymphomas. A squamous cell carcinoma was the only solid tumor detected in the *Bard1*<sup>SF/SF</sup> cohort. Similarly, only a small fraction of the *Bard1*<sup>KA/KA</sup> cohort developed tumors (4 of 34 mice). As was the case with the *Bard1*<sup>SF/SF</sup> tumors, the average appearance of malignancies occurred at an advanced age (657 days). Three of the four tumors observed in the *Bard1*<sup>KA/KA</sup> mice were leukemias or lymphomas and the lone solid tumor was a hepatic adenoma. In the *Bard1*<sup>+/+</sup> cohort, 3 of 28 mice developed tumors at an average age of 786 days. The three tumors consisted of a hepatocellular carcinoma, a renal cell carcinoma, and a monocytoid lymphoma. Thus, many of the malignancies observed in the *Bard1*<sup>KA/KA</sup> and *Bard1*<sup>SF/SF</sup> mice were also observed in the *Bard1*<sup>+/+</sup> control cohort. This supports the conclusion that the tumors observed in the Bard1 BRCT mutant mice were likely the result of spontaneous tumor formation and not due to the Bard1-BRCT mutation. These results indicate that simultaneous loss of Brca1/Bard1-mediated HDR and SFP, but not loss of Brca1/Bard1-mediated SFP alone, renders mice susceptible to spontaneous tumor formation.

C. Discussion

The ability of BRCA1 to promote genome stability is thought to be a central aspect of its tumor suppression activity. BRCA1 and BRCA2 both promote genome stability through at least two distinct pathways: homology directed repair (HDR) of DSBs and stalled fork protection (SFP). Early studies established that BRCA1 and BRCA2 are both required for HDR and that cells deficient for either protein readily accumulate chromosomal abnormalities (Moynahan et al., 1999; Moynahan, Pierce, et al., 2001). More recently, both proteins have also been implicated in the protection of stalled forks from nuclease degradation (Kolinjivadi, Sannino, de Antoni, Techer, et al., 2017; Schlacher et al., 2011; Schlacher et al., 2012; Ying et al., 2012). Since fork progression can be stalled in a transient manner by a variety of obstacles, cells employ specific mechanisms to stabilize stalled forks and restore their replication competence. For example, upon stress-induced stalling of DNA replication, the conventional three-way replication fork can be converted via branch migration into a four-way structure in which the two nascent DNA strands anneal to form a fourth arm (Figure 2) (Neelsen & Lopes, 2015). Through further
remodeling of this “reversed fork”, DNA synthesis can proceed past the obstacle by using the complementary nascent DNA strand as a template, allowing scheduled DNA synthesis to resume.

In 2003, Lomonosov et al. reported that the HU-induced stalled forks of wildtype cells, which can be detected as Y-shaped DNA junctions on 2D gel electrophoretograms, are specifically absent from BRCA2-mutant cells, suggesting that BRCA2 is required for the formation and/or stability of stalled forks (Lomonosov et al., 2003). In support of this notion, DNA fiber analyses revealed that the HU-induced stalled forks of BRCA2-mutant cells are degraded by the nuclease activity of MRE11 (Schlacher et al., 2011; Ying et al., 2012). Moreover, Schlacher et al. identified a BRCA2 mutation that specifically ablates SFP without affecting HDR, and further showed that cells bearing this mutation undergo chromosomal instability upon HU treatment (Schlacher et al., 2011). These findings support two critical notions: first, that SFP and HDR are separable aspects of BRCA2 function and, second, that SFP is itself an important contributor to genome integrity, especially in cells subjected to replication stress. In a subsequent study, Schlacher et al. reported that SFP is also dependent on BRCA1, FANCD2, and RAD51, suggesting the existence of a “stalled fork protection” pathway that preserves genome integrity by preventing nucleolytic degradation of stalled forks (Schlacher et al., 2012). Indeed, multiple components (PTIP, MLL3/4, CHD4, and PARP1) of an opposing pathway that promotes fork degradation by recruiting the MRE11 nuclease to stalled forks have also been identified (Ding et al., 2016; Ray Chaudhuri et al., 2016), and recent studies have established that the four-way reversed fork is the likely substrate for nucleolytic degradation in BRCA1/2-deficient cells (Kolinjivadi, Sannino, De Antoni, Zadorozhny, et al., 2017; Vujanovic et al., 2017).

The experiments discussed above demonstrated that BRCA1 is required for SFP, however, they made use of null BRCA1 alleles and were thus largely uninformative as to what functional domains of BRCA1 are necessary for SFP. Therefore, to determine whether the phospho-recognition function of the BRCA1 BRCT domain is required for SFP, we performed the DNA fiber assay on Brca1<sup>SF/SF</sup> cells. The Brca1 phospho-recognition mutant was clearly defective in SFP, showing Mre11-dependent degradation of stalled forks (Figure 29B). Furthermore, we observed extensive DNA damage upon treatment of Brca1<sup>SF/SF</sup> cells with replication stress-inducing agents like hydroxyurea and camptothecin (Figures 30 and 31). Previous studies demonstrated that the Brca1<sup>SF</sup> mutation renders mice prone tumor formation,
implying that the SFP function may contribute to Brca1-mediated tumor suppression. However, as the Brca1SF mutation is also defective in HDR, it is still unclear which of these BRCA1 functions (HDR and/or SFP) are actually required for tumor suppression.

While a role for BRCA1 in SFP had been firmly established by previous work, it was unknown whether BARD1 is also required. Previous studies had shown that BARD1 localizes, together with BRCA1, to PCNA-staining replication structures upon hydroxyurea treatment (Scully, Chen, Ochs, et al., 1997), and a subsequent biochemical assay demonstrated that BARD1 is highly enriched at HU-stalled replication forks (Dungrawala et al., 2015). However, given that BRCA1 is reliant upon heterodimerization with BARD1 for its stability and nuclear localization, and that deletion of BARD1 would result in deficiency of BRCA1, it was unclear whether BARD1 contributes unique functions to BRCA1/BARD1-mediated SFP or instead supports SFP indirectly through stabilization of BRCA1. Nonetheless, DNA fiber analyses revealed that Bard1SF/SF and Bard1KA/KA cells display Mre11-mediated degradation of stalled forks, indicating that Bard1 BRCT phospho-recognition is required for SFP (Figures 27 and 28). Significantly, as shown in Figures 13 and 14, Bard1SF/SF and Bard1KA/KA cells stably express Brca1 polypeptides, readily form Brca1/Bard1 heterodimers, and localize to the nucleus. Thus, the role of BARD1 in BRCA1/BARD1-mediated SFP cannot simply be attributed to its ability to stabilize BRCA1.

In Chapter 3, we showed that Bard1 BRCT phospho-recognition is dispensable for HDR. Specifically, Bard1SF/SF and Bard1KA/KA cells showed no defect in the ability to form Rad51 foci after IR treatment (Figures 23 and 24) and efficiently repaired induced DSBs within the DR-GFP reporter by HDR (Figure 26). Therefore, Bard1SF and Bard1KA are separation-of-function mutants that are competent for HDR but defective for SFP (i.e., they confer a “SFP-HDR” phenotype”). In contrast to Brca1-null and Brca1SF mutants, which are defective in both HDR and SFP (conferring a “SFP-HDR” phenotype”), the Bard1SF and Bard1KA mutants can be used to determine which functions of the BRCA1/BARD1 heterodimer are mediated by SFP specifically, including, as discussed further below, tumor suppression.

While the DNA fiber assay demonstrated that the Bard1SF/SF and Bard1KA/KA cells are prone to Mre11-mediated stalled fork degradation, it was unclear whether this degradation event actually led to functional consequences such as the accumulation of DNA damage. To test this hypothesis, we employed the alkaline comet assay, which can detect the presence of single-strand and double-strand
DNA breaks. The assay revealed significant accumulation of DNA damage in $\text{Bard1}^{\text{SF/SF}}$ and $\text{Bard1}^{\text{KA/KA}}$ cells, as well as $\text{Brca1}^{\text{SF/SF}}$ cells in response to replication stress. Given that the Bard1 BRCT mutants are competent for HDR, this observation implies that SFP defects are sufficient to generate DNA damage. Significantly, $\text{Bard1}^{\text{SF/SF}}$ and $\text{Bard1}^{\text{KA/KA}}$ cells accumulated DNA damage in response to at least two distinct types of replication stress: hydroxyurea (Figure 30), which stalls forks by depleting nucleotide pools, as well as camptothecin (Figure 31), which stalls forks by generating protein-DNA adducts. Interestingly, although an accumulation of DNA damage was seen in the $\text{Bard1}^{\text{SF/SF}}$ and $\text{Bard1}^{\text{KA/KA}}$ cells in response to camptothecin treatment, the same cells were not overtly sensitive to camptothecin (Figure 18). Since $\text{Brca1}^{\text{SF/SF}}$ cells, which are defective for both SFP and HDR, are hypersensitive to camptothecin, it is possible that the cytotoxic effects of camptothecin depend on combined deficiencies in both pathways. Alternatively, since a shorter camptothecin exposure was used in the sensitivity assay (1 hour vs. 5 hours in the comet assay), it is possible that the shorter treatment is sufficient to cause cytotoxicity in the SFP$^{-}$HDR$^{-}$ cells, but not SFP$^{-}$HDR$^{+}$ cells.

Regardless, the DNA fiber assays combined with the comet assays demonstrated that the Bard1 BRCT domain is required for SFP-mediated resistance to genome instability. Indeed, this conclusion is supported by experiments involving a BRCA2 separation-of-function mutant (Brca2$^{S3291A}$) reported by Schlacher et al. (2011), which also displays an SFP$^{-}$HDR$^{+}$ phenotype. Significantly, cells harboring the Brca2$^{S3291A}$ mutation displayed chromosomal instability upon induction of replication stress with hydroxyurea. Additionally, Schlacher et al. (2011) showed that the chromosomal stability of V-C8 cells, which express a grossly truncated Brca2 polypeptide defective for both HDR and SFP, is fully rescued by exogenous expression of wildtype Brca2, but only partly rescued by the Brca2$^{S3291A}$ polypeptide (Schlacher et al., 2011). These observations suggest that both HDR and SFP contribute to chromosome integrity and that a defect in SFP alone is sufficient to induce some degree of chromosomal instability, albeit not as the genome instability observed in SFP$^{-}$HDR$^{-}$ cells. Consistent with this interpretation, although both the Bard1 and Brca1 BRCT mutant cells displayed an increase in chromosomal aberrations after treatment with the DNA interstrand crosslinking reagent MMC, $\text{Brca1}^{\text{SF/SF}}$ cells displayed significantly more aberrations in response to the same treatment compared to the $\text{Bard1}^{\text{SF/SF}}$ and $\text{Bard1}^{\text{KA/KA}}$ cell lines (Figures 20, 21, and 22). Furthermore, $\text{Brca1}^{\text{SF/SF}}$ cells displayed spontaneous chromosomal
rearrangements which were not observed in Bard1^{SF/SF} and Bard1^{KA/KA} cells, suggesting that HDR, but not SFP alone, is required for suppression of spontaneous genome instability (Figures 20, 21, and 22). One explanation for these results is that endogenous fork stalling may not occur often enough under unstressed conditions to generate significant accumulation of DNA damage in SFP^-HDR^+ cells, as the intact HDR pathway can act to repair any stalled forks that collapse into DSBs. However, under conditions of significant replication stress (e.g., HU or MMC treatment), the SFP-deficient Bard1^{SF/SF} and Bard1^{KA/KA} cells may experience a greater load of fork collapse, overwhelming the capacity of the HDR pathway to act as an error-free backup mechanism and thereby leading to the accumulation of DNA damage. On the other hand, in untreated SFP^-HDR^- cells, the moderate levels of endogenous fork stalling and collapse may be sufficient to generated DNA damage, as any fork collapse that does occur is instead shuttled to error-prone mechanisms of repair (e.g., MMEJ or NHEJ) in the absence of a functional HDR pathway. Thus, both HDR and SFP likely work together to prevent genome stability, and loss of both pathways leads to a more severe phenotype than loss of SFP alone.

The fact that Bard1^{SF/SF} and Bard1^{KA/KA} cells are deficient for SFP while proficient for HDR sheds light on the mechanism of PARPi sensitivity and resistance in BRCA1-mutant tumors. Hypersensitivity to PARP inhibitors is thought to arise as a result of the HDR and SFP defects of BRCA1, although how much each individual defect contributes to overall sensitivity is unclear. Since the PARP1 and PARP2 enzymes are required for repair of single-strand DNA breaks (SSBs), PARPi should increase the load of SSBs that can be converted, upon DNA replication, to DSBs; thus, it was proposed that although these toxic DSBs can be repaired by HDR in normal cells, in BRCA1-mutant cells the combined defects in SSB repair and HDR result in synthetic lethality. Subsequent studies revealed that the sensitivity of BRCA1-mutant cells to PARPi often correlates with the ability of the inhibitor to stabilize toxic PARP1-DNA or PARP2-DNA complexes that would generate stalled forks during DNA replication (Pommier et al., 2016). Two further observations suggest that loss of SFP may constitute a significant factor in the targeted cytotoxicity of PARPi in BRCA1/2-mutant cells. First, PARP enzymatic activity is itself required for protection of stalled forks from Mre11-dependent degradation (Ying et al., 2012). Second, in recent studies, a partial resistance to PARPi was observed in BRCA1/2-mutant cells when SFP, but not HDR, was restored by diverse mechanisms, including depletion of PTIP, SMARCAL1, EZH2, or RADX (Ray
The availability of Bard1 separation-of-function mutants has now allowed us to ask directly whether Brca1/Bard1-mediated SFP contributes to PARPi resistance. Importantly, \textit{Bard1}^{SF/SF} and \textit{Bard1}^{KA/KA} cells, which display the HDR\textsuperscript{+}/SFP\textsuperscript{−} phenotype, are clearly hypersensitive to olaparib, but to a qualitatively lesser degree than \textit{Brca1}^{SF/SF} cells with the HDR\textsuperscript{−}/SFP\textsuperscript{−} phenotype (Figure 16). These results indicate that loss of Brca1/Bard1-mediated HDR and Brca1/Bard1-mediated SFP can both contribute to the PARPi-induced cytotoxicity of Brca1-mutant cells.

We also propose a mechanistic explanation for the SFP defect seen in \textit{Bard1}^{SF/SF} and \textit{Bard1}^{KA/KA} cells. Using iPOND (isolation of proteins on nascent DNA) technology, Dungrawala \textit{et al.} demonstrated that BRCA1 and BARD1 are present at HU-stalled, but not unstressed, DNA replication forks (Dungrawala \textit{et al.}, 2015). Therefore, the ability of the BARD1 BRCT domain to bind poly(ADP-ribose) (PAR) in a phospho-dependent manner suggested a possible mechanism for the SFP defect in \textit{Bard1}^{SF/SF} and \textit{Bard1}^{KA/KA} cells (M. Li & Yu, 2013). Previous studies have shown that PAR chains are assembled at sites of stalled DNA replication by PAR polymerase 1 (PARP1) and that SFP is dependent on PARP1 activity (Ying \textit{et al.}, 2012). Therefore, to ascertain whether Bard1 BRCT phospho-recognition is required for the recruitment of Brca1/Bard1 heterodimer to stalled forks, we examined the ability of Brca1/Bard1 to localize within PCNA-staining replication factories of HU-treated cells in a series of immunostaining experiments (Scully, Chen, Ochs, \textit{et al.}, 1997). Notably, the co-localization of Brca1/Bard1 and PCNA in late S phase cells was markedly reduced in HU-treated \textit{Bard1}^{SF/SF} and \textit{Bard1}^{KA/KA} cells relative to wildtype cells. Indeed, preliminary experiments in our laboratory using the iPOND method biochemically confirm that the Brca1/Bard1 heterodimers of \textit{Bard1}^{SF/SF} and \textit{Bard1}^{KA/KA} cells fail to associate with HU-stalled replication forks (Michiko Horiguchi, personal communication). Together, these observations indicate that Bard1 BRCT phospho-recognition promotes SFP, at least in part, by mediating the recruitment of Brca1/Bard1 heterodimers to stalled replication forks.

Using the dynamics of Brca1/Bard1 and PCNA foci in untreated and HU treated cells, a rough picture of the events leading to BRCA1/BARD1 recruitment to stalled replication forks emerges. In untreated cells, Brca1/Bard1 form foci that rarely colocalize with PCNA-staining replication structures. Although the function of these S phase Brca1/Bard1 foci are not known, it has been hypothesized that
they may represent localization of the heterodimer to spontaneously-formed DSBs or stalled replication forks. However, given that the S phase Brca1/Bard1 foci of untreated cells do not colocalize with PCNA, it seems unlikely that these foci represent fork stalling events. It is also possible that the foci of unperturbed cells represent a normal process of Brca1/Bard1 sequestration during S phase, particularly if normal DNA replication is compromised by inappropriate activation of the SFP or HDR pathways. In any case, upon induction of replication fork stalling by hydroxyurea, these Brca1/Bard1 foci dissipate, leading to a transient decrease in Brca1/Bard1-staining cells. Since this phenomenon occurs in both wild type and Bard1-BRCT mutant cells, Bard1 BRCT phosho-recognition is likely to be dispensable for HU-induced dissipation of S-phase Brca1/Bard1 foci (Figures 32 and 35). Indeed, this process is accompanied by HU-induced hyper-phosphorylation of BRCA1 (Cortez et al., 1999; Scully, Chen, Ochs, et al., 1997). As such, the HU-induced dissipation of S phase BRCA1/BARD1 foci may entail the activation of DNA damage sensors, like ATM and ATR.

In HU-treated wild type cells, the Brca1/Bard1 heterodimer is recruited to stalled replication forks, as represented by the localization of Brca1 and Bard1 polypeptides to the nodular PCNA-staining replication structures during late S phase. However, the assembly of Brca1/Bard1 to these PCNA-staining structures is largely abrogated in HU-treated Bard1KA/KA and Bard1SF/SF cells, suggesting that the recruitment of Brca1/Bard1 to stalled replication forks is dependent on Bard1 BRCT phosho-recognition (Figures 34 and 36). The failure of Brca1/Bard1 to localize to PCNA-staining replication structures is manifested cytologically in either of two patterns: 1.) Brca1/Bard1 foci are present but do not overlap with PCNA foci, and 2.) Brca1/Bard1 foci are absent in cells with PCNA foci. In the first case (Brca1/Bard1+ PCNA+ non-colocalizing cells), this population might represent Bard1KA/KA and Bard1SF/SF cells in which HU treatment was insufficient to induce significant replication fork stalling and thus activation of the SFP pathway. However, this seems unlikely given that fork stalling appears to occur in the majority of late S phase Brca1+/+ cells (as reflected by the high percentage of wild type cells that display HU-induced colocalization between Bard1/Brca1 and PCNA). Another possibility is that the Brca1/Bard1 foci of HU-treated Bard1KA/KA and Bard1SF/SF cells represents sites of DSBs that arise from collapsed replication forks that no longer associate with PCNA, rather than the stalled forks of PCNA-staining replication factories. Li and Yu demonstrated that phosho-recognition by the Bard1 BRCT domain is dispensable for late
recruitment to DSBs (M. Li & Yu, 2013), thus even in the presence of the Bard1<sup>SF/SF</sup> and Bard1<sup>KA/KA</sup> mutations, the Brca1/Bard1 heterodimer should be able to eventually localize to DSBs. Accordingly, the altered staining patterns of Bard1<sup>SF/SF</sup> and Bard1<sup>KA/KA</sup> cells may reflect both the cause of the SFP defect (i.e., unprotected forks, as represented by PCNA foci that do not colocalize with Brca1/Bard1), as well as the consequences of SFP defects (i.e., collapsed forks, as represented by Brca1/Bard1 foci that do not colocalize with PCNA).

At present, it is unknown how phospho-recognition by the Brca1 BRCT domain contributes, if at all to this recruitment process. It is possible that the Brca1 BRCT domain mediates an aspect of recruitment that is distinct from the event facilitated by the Bard1 BRCT domain, similar to what was observed in the context of a DSB by Li and Yu (M. Li & Yu, 2013). Alternatively, the Brca1 BRCT domain may facilitate a non-recruitment aspect of SFP through binding to one of its phospholigands. However, the identity of the phospholigand, or the mechanism of fork protection are currently unknown. Further studies are necessary to fully explore these possibilities.

Since the HDR and SFP functions of BRCA1/BARD1 both contribute to chromosomal stability, it is conceivable that BRCA1/BARD1-mediated tumor suppression activity is dependent on HDR, SFP, or both. To discriminate among these possibilities, it would be helpful to evaluate tumorigenesis in mice harboring Brca1 separation-of-function mutations that yield either the HDR<sup>−</sup>SFP<sup>+</sup> or HDR<sup>+</sup>SFP<sup>−</sup> phenotype. A number of mouse strains exist that harbor Brca1 alleles modeled after human pathogenic BRCA1 mutations associated with hereditary breast and ovarian cancer. A subset of these, including the Brca1<sup>SF</sup> allele, encode polypeptides that retain at least some Brca1 function. Thus, unlike Brca1-null animals, which invariably undergo embryonic lethality, mice that are homozygous for these hypomorphic mutations (Brca1<sup>SF/SF</sup>) can survive as adults, but develop a wide spectrum of tumor types at increased rates relative to their wild type and heterozygous littermates. However, since Brca1<sup>SF/SF</sup> cells are deficient for both the HDR and SFP pathways, this observation does not illuminate the relative contributions of HDR or SFP to BRCA1-mediated tumor suppression. In contrast, Bard1<sup>SF/SF</sup> and Bard1<sup>KA/KA</sup> mice, which exhibit the HDR<sup>+</sup>SFP<sup>−</sup> phenotype, do not display increased tumor formation relative to their littermates (Figure 37), suggesting that abrogation of BRCA1/BARD-mediated SFP alone is not sufficient to elicit tumor susceptibility. While some of the Bard1<sup>SF/SF</sup> and Bard1<sup>KA/KA</sup> mice did develop tumors at a highly
advanced age, none of these were mammary tumors, and most also arose with the same kinetics in the control \textit{Bard1}^{+/+} cohort. Therefore, tumor formation in the \textit{Bard1}^{SF/SF} and \textit{Bard1}^{KA/KA} cohorts likely represents the appearance of spontaneous malignancies associated with advanced age. Together, the tumor cohort data suggests that the tumor suppression activity of BRCA1/BARD1 is dependent on its HDR function alone, or on a combination of its HDR and SFP functions, but not on SFP alone.
CHAPTER V

THE BARD1 BRCT AND BRCA1 BRCT PHOSPHO-RECOGNITION MUTANTS ARE HAPLOINSUFFICIENT FOR STALLED FORK PROTECTION
A. Introduction

In most families afflicted with BRCA1-linked hereditary breast and ovarian cancer, the tumor-prone women are heterozygous mutation carriers with one mutant and one normal BRCA1 allele (Miki et al., 1994; Wooster & Weber, 2003). Almost invariably, the normal BRCA1 allele is lost or inactivated in the tumors that arise in these women, rendering them functionally null for BRCA1 and indicating that BRCA1 acts as a tumor suppressor gene. However, as discussed in the previous chapters, BRCA1-null cells and organisms are typically inviable. This raises the question as to how these BRCA1-null tumor cells remain viable in the face of the loss of both BRCA1 alleles. One hypothesis is that prior to the loss of the second BRCA1 allele, heterozygous BRCA1 cells accumulate other genetic lesions, such as loss of p53 or p16, that permit the viability of BRCA1-null cells. Therefore, this hypothesis implies that heterozygous BRCA1 cells must possess some defect or defects that lead to genome stability, eventually leading to accumulation of genetic alterations that allow for the viability of BRCA1-null cells.

Apart from their enhanced tumor susceptibility, BRCA1-mutation carriers are healthy and fertile, as are heterozygous Brca1/Bard1-mutant mice. Indeed, studies conducted over the past 20 years have established that most biological functions attributed to BRCA1, including HDR, are unaffected in cells that are heterozygous for pathogenic BRCA1 mutations (Jiang & Greenberg, 2015; Moynahan & Jasin, 2010; Nagaraju & Scully, 2007; Venkitaraman, 2014). Nonetheless, subtle defects in heterozygous BRCA1-mutant mammary epithelial cells have been reported, including premature senescence, modest chromosomal instability, and aberrant development of mammary epithelial cell populations, suggesting that BRCA1 may be haploinsufficient for at least some of its functions (Konishi et al., 2011; Lim et al., 2009; Nolan et al., 2016). For example, Pathania et al. (2014) systematically compared the phenotypes of wild type (BRCA1+/+) and heterozygous-mutant (BRCA1mut+/) human mammary epithelial cells (Pathania et al., 2014). As expected, BRCA1mut+/ cells were fully competent for a number of BRCA1-dependent processes, such as HDR, checkpoint signaling, centrosome duplication, and spindle formation. In contrast, however, these cells displayed signs of replication stress, including a failure to stabilize HU-stalled DNA replication forks and an increased level of UV-induced DNA damage (Pathania et al., 2014).

In this chapter, we examine the effect of heterozygous Bard1 (Bard1SF+/ or Bard1KA+/) or Brca1 (Brca1SF+/) BRCT mutations on BRCA1/BARD1-mediated stalled fork protection. Additionally, we also determine whether these heterozygous mutant cells accumulate DNA damage after replication stress.
B. Results

1. Stalled fork protection is also defective in heterozygous cells harboring BRCT mutations in either Brca1 or Bard1

i. Stalled fork protection is defective in Brca1SF/+ cells

Although cells that are heterozygous for pathogenic BRCA1 mutations retain most BRCA1-mediated functions, including HDR, Pathania et al. observed a defect in stalled fork protection in heterozygous-mutant (BRCA1mut/+ human mammary epithelial cells (Pathania et al., 2014). To determine whether heterozygosity for the Brca1 BRCT mutation also impairs SFP, we conducted DNA fiber analysis of immortalized MEFs according to the conditions described in Chapter 4. Briefly, immortalized MEFs were subjected to two sequential 20-minute pulses with the nucleoside analogs 5′-iodo-2-deoxyuridine (IdU) and 5′-chloro-2-deoxyuridine (CldU) (Figure 38A). The track lengths of single DNA fibers were then measured immediately following CldU treatment (–HU condition) or after a 90-minute treatment with hydroxyurea (HU, +HU condition). SFP was once again assessed by calculating the ratio of the lengths of adjacent CldU and IdU replication tracts, with a decrease in the CldU/IdU ratio indicating stalled fork degradation and thus an SFP defect.

Two independent Brca1SF/+ MEF clones (O and V), along with an isogenic Bard1+/+ (Q) and an isogenic Brca1SF/SF clone (P) were treated as described above. As expected, in all untreated (–HU) clones the median ratio of CldU/IdU was close to 1 (Figure 38B). Additionally, when Brca1+/+ cells were exposed to HU, the median CldU/IdU ratio was statistically indistinguishable from that of untreated Brca1+/+ cells (0.87 in the –HU condition vs. 0.89 in the +HU condition), indicating that stalled replication forks are stable in wild type cells. Consistent with the results shown in Chapter 4 (Figure 29), HU-treated Brca1SF/SF cells showed a significant reduction in the CldU/IdU ratio compared to untreated Brca1SF/SF cells (0.89 –HU vs. 0.47 +HU). Notably, in both heterozygous-mutant Brca1SF/+ clones, HU treatment also induced a marked decrease in the CldU/IdU ratio (clone O: 0.88 –HU vs 0.49 +HU; clone V: 0.90 –HU vs. 0.49 +HU), indicating a profound defect in SFP. Interestingly, the SFP defect observed in the heterozygous Brca1SF/+ cell lines appears to be qualitatively similar to that of homozygous Brca1SF/SF cells. Furthermore, exposure to the Mre11 nuclease inhibitor mirin rescued the CldU/IdU ratio in the HU-treated cells in the two heterozygous Brca1SF/+ clones (O: 0.49 +HU vs. 0.88 +HU +mirin; V: 0.47 +HU vs. 0.91
Figure 38. DNA fiber analysis reveals an SFP defect in heterozygous Brca1SF/± cells. A) A schematic of the DNA fiber assay. Cycling immortalized MEFs were exposed to sequential 20-minute pulses of the nucleoside analogs 5'-iodo-2-deoxyuridine (IdU) and 5'-chloro-2-deoxyuridine (CldU). Cells were then either harvested (–HU control) or treated with 2 mM hydroxyurea (HU) for 1.5 hours and then harvested. To inhibit Mre11 nuclease activity, cells were exposed to 50 μM mirin throughout both nucleoside analogue pulses as well as the subsequent HU treatment. As Mre11-dependent fork degradation in BRCA1/2-mutant cells occurs in a directional manner such that the most recently synthesized DNA of the nascent strands is degraded first, the CldU and IdU tract lengths were measured and the CldU/IdU ratio calculated to ascertain fork degradation. B) DNA fiber analysis of two independent Brca1SF/± MEF clones (O and V), an isogenic Brca1+/+ clone (Q), and an isogenic Brca1SF/± clone (P). For each condition, the CldU/IdU ratios of at least 150 individual DNA fibers are presented as a dot plot, and the median CldU/IdU ratio is denoted by a horizontal red line. Statistical analyses were conducted using the Mann-Whitney rank sum test (**** p<0.0001). The experiment presented here is representative of three independent experiments, each yielding the same statistically significant biological results.
+HU +mirin). Therefore, the degradation of HU-stalled forks observed in Brca1\textsuperscript{SF/+} cells is also mediated by the Mre11 nuclease. Taken together, these results show that the Brca1 BRCT phospho-recognition mutation is haploinsufficient for the SFP pathway.

ii. Stalled fork protection is also defective in Bard1\textsuperscript{SF/+} and Bard1\textsuperscript{KA/+} cells

To determine whether the Bard1 BRCT mutations are also haploinsufficient for SFP, we performed the DNA fiber assay with two independent heterozygous Bard1\textsuperscript{SF/+} clones (B and D), along with an isogenic Bard1\textsuperscript{+/+} clone (E) and an isogenic Bard1\textsuperscript{SF/SF} clone (A). As expected, in all untreated (–HU) clones, the CldU/IdU approached unity (Figure 39). Additionally, when Bard1\textsuperscript{+/+} cells were exposed to HU, the median CldU/IdU ratio was statistically indistinguishable from that of untreated Bard1\textsuperscript{+/+} cells (1.04 –HU vs. 1.00 +HU), confirming that stalled replication forks are stable in wild type cells. Consistent with the results of Chapter 4 (Figure 27), Bard1\textsuperscript{SF/SF} cells showed a significant reduction of the CldU/IdU ratio upon HU treatment (1.04 –HU vs. 0.81 +HU), indicative of an SFP defect. Notably, in both heterozygous-mutant Bard1\textsuperscript{SF/+} clones, HU treatment also induced a marked decrease in the CldU/IdU ratio (B: 1.04 –HU vs. 0.82 +HU; D: 1.02 –HU vs. 0.79 +HU), indicating a profound defect in SFP. Again, the SFP defect observed in the heterozygous Bard1\textsuperscript{SF/+} clones appears to be qualitatively similar to that of homozygous Bard1\textsuperscript{SF/SF} cells, and treatment with the Mre11 nuclease inhibitor mirin restored the CldU/IdU ratio to levels similar to untreated Bard1\textsuperscript{SF/+} cells (B: 0.82 +HU vs. 1.02 +HU +mirin; D: 0.79 +HU vs. 1.00 +HU +mirin).

Similarly, we performed the DNA fiber assay on a heterozygous Bard1\textsuperscript{KA/+} MEF clone (K), along with an isogenic Bard1\textsuperscript{+/+} clone (I) and an isogenic Bard1\textsuperscript{KA/KA} clone (J). As expected, HU treatment induced a significant decrease in the CldU/IdU ratio in the Bard1\textsuperscript{KA/KA} cells (0.89 –HU vs. 0.49 +HU) but not Bard1\textsuperscript{+/+} cells (0.88 –HU vs. 0.88 +HU) (Figure 40). Consistent with the Bard1\textsuperscript{SF/+} and Brca1\textsuperscript{SF/+} heterozygotes, heterozygous Bard1\textsuperscript{KA/+} cells also displayed a significant decrease in the CldU/IdU ratio in response to HU treatment (0.91 –HU vs. 0.51 +HU) that was qualitatively similar to that of homozygous Bard1\textsuperscript{KA/KA} cells. Furthermore, Mre11 inhibition restored the CldU/IdU to close to unity in both HU-treated Bard1\textsuperscript{KA/+} and Bard1\textsuperscript{KA/KA} cells. Together, these results indicate that the Bard1 BRCT phospho-recognition mutations are haploinsufficient for SFP.
Figure 39. DNA fiber analysis reveals an SFP defect in heterozygous \( \text{Bard}^{1SF+} \) cells. A) Schematic of the DNA fiber assay. B) DNA fiber analysis of two independent \( \text{Bard}^{1SF+} \) clones (B and D), an isogenic \( \text{Bard}^{1+/+} \) clone (E), and an isogenic \( \text{Bard}^{1SF/SF} \) clone (A). Red bar is median CldU/IdU ratio. For each condition, the CldU/IdU ratios of at least 150 individual DNA fibers are presented as a dot plot, and the median CldU/IdU ratio is denoted by a horizontal red line. Statistical analyses were conducted using the Mann-Whitney rank sum test (**** \( p <0.0001 \)). The experiment presented here is representative of three independent experiments, each yielding the same statistically-significant biological results.
Figure 40. DNA fiber analysis reveals an SFP defect in heterozygous Bard1<sup>KA/+</sup> cells. A) A schematic of the DNA fiber assay, as described in Figure 38. B) DNA fiber analysis of isogenic Bard1<sup>KA/+</sup> (K), Bard1<sup>+/+</sup> (I), and Bard1<sup>KA/KA</sup> (J) clones. For each condition, the CldU/IdU ratios of at least 150 individual DNA fibers are presented as a dot plot, and the median CldU/IdU ratio is denoted by a horizontal red line. Statistical analyses were conducted using the Mann-Whitney rank sum test (**** p<0.0001). The experiment presented here is representative of three independent experiments, each yielding the same statistically significant biological results.
2. Replication stress induces DNA damage in heterozygous Bard1 and Brca1 BRCT-mutant cells

While the DNA fiber assay demonstrated that stalled replication forks are susceptible to Mre11-mediated degradation in \( \text{Bard1}^{\text{SF}+/} \), \( \text{Bard1}^{\text{KA}+/} \), and \( \text{Brca1}^{\text{SF}+/} \) cells, the assay does not reveal whether this degradation translates into an increased burden of DNA damage and genome instability. Therefore, we used the alkaline comet assay to determine whether replication stress induces increased levels of DNA damage in \( \text{Bard1}^{\text{SF}/} \) and \( \text{Bard1}^{\text{KA}/} \) cells. As described in Chapter 4, the alkaline comet assay detects single-strand and double-strand DNA breaks. Briefly, cells are treated with a DNA damaging agent, embedded in agarose, and incubated in an alkaline denaturing buffer before being subjected to gel electrophoresis. Single-strand and double-strand DNA breaks will cause the damaged DNA to migrate though the gel faster than undamaged DNA. Upon staining with a fluorescent nucleic acid dye (GelRed); the damaged DNA can be visualized as a "comet tail" trailing the undamaged DNA (i.e., the "comet head"). The amount of DNA damage can then be quantified from the tail moment, a measurement of the length and amount of DNA present in the comet tail.

Thus, isogenic clones of \( \text{Brca1}^{+/} \) (Q), \( \text{Brca1}^{\text{SF}/} \) (O), and \( \text{Brca1}^{\text{SF}/} \) (P) immortalized MEFs were exposed to 2mM HU for 5 hours (Figure 41). After HU treatment, the average amount of DNA damage per cell was quantified through measurement of the tail moment. Consistent with the data shown in Figure 30, the \( \text{Brca1}^{\text{SF}/} \) clone showed a significant increase in the average tail moment (10.45), and thus an increase in the DNA damage burden, when compared to its isogenic \( \text{Brca1}^{+/} \) control (4.11). Interestingly, the \( \text{Brca1}^{\text{SF}/} \) clone also displayed a significant increase in the average tail moment (7.09) when compared to the wild type clone. Of note, the level of DNA damage observed in the heterozygous clone (7.09) was modestly, but significantly lower, than what was observed in the corresponding homozygous-mutant clone (10.45).

Similarly, isogenic clones of \( \text{Bard1}^{+/} \) (E), \( \text{Bard1}^{\text{SF}/} \) (B), and \( \text{Bard1}^{\text{SF}/} \) (A) immortalized MEFs were treated and analyzed as described above. As shown in Figure 41, consistent with the data shown in Figure 30A, the \( \text{Bard1}^{\text{SF}/} \) clone displayed a high level DNA damage after HU exposure as reflected by a significant increase in the average tail moment (12.19) when compared to its isogenic \( \text{Bard1}^{+/} \) control (3.31). As was observed in heterozygous \( \text{Brca1}^{\text{SF}/} \) cells, heterozygous \( \text{Bard1}^{\text{SF}/} \) cells also displayed an
Figure 41. *Brd1*<sup>SF</sup>, *Brd1*<sup>KA</sup>, and *Brc1*<sup>SF</sup> cells accumulate DNA damage during replication stress. Alkaline comet assay using isogenic sets of wild type, heterozygous, and homozygous clones of *Brc1*<sup>SF</sup>, *Brd1*<sup>SF</sup>, and *Brd1*<sup>KA</sup> mutants. For each condition, the comet tails of at least 75 cells were analyzed using CometScore Software Version 1.5, and the individual tail moments are presented as dot plots. The mean tail moment is denoted by a horizontal red line, and the error bars represent the standard error of the mean. Statistical analyses were conducted using one-way ANOVA (* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001).
average tail moment (7.66) that was significantly increased compared to the \textit{Bard1}^{+/+} clone, but significantly less than the \textit{Bard1}^{SF/SF} clone.

Finally, we also performed the alkaline comet assay using isogenic clones of \textit{Bard1}^{+/+} (I), \textit{Bard1}^{KA/+} (K), and \textit{Bard1}^{KA/KA} (J) immortalized MEFs (Figure 41). Consistent with our previous data, \textit{Bard1}^{KA/KA} cells showed a significant increase in DNA damage after HU treatment (8.94, average tail moment) when compared to its isogenic \textit{Bard1}^{+/+} control (3.42). Once again, the heterozygous \textit{Bard1}^{KA/+} cell line showed an average tail moment (5.37) that was significantly more than its isogenic \textit{Bard1}^{+/+} control but significantly less than an isogenic \textit{Bard1}^{KA/KA} clone. Together, these results indicate that both the heterozygous \textit{Brca1} BRCT phospho-recognition mutant as well as the heterozygous \textit{Bard1} BRCT phospho-recognition mutants accumulate DNA damage during replication stress.

C. Discussion

Here we show that SFP is also defective in cells heterozygous for mutations that ablate the BRCT phospho-recognition activities of either \textit{Bard1} (\textit{Bard1}^{SF/+} and \textit{Bard1}^{KA/+}) or \textit{Brca1} (\textit{Brca1}^{SF/+}) using the DNA fiber assay (Figures 38 – 40). Additionally, we also show that this fork defect in these heterozygous cells leads to an accumulation of DNA damage during replication stress. Based on the results of the DNA fiber assay, the heterozygous-mutant cells do not exhibit an intermediate phenotype with respect to SFP; instead, the degree of nascent DNA degradation observed in heterozygous-mutant cells (e.g., \textit{Bard1}^{SF/+}) is qualitatively equivalent to that observed in the corresponding homozygous-mutant cells (\textit{Bard1}^{SF/SF}). However, the heterozygous mutant cells accumulated DNA damage after HU-mediated replication stress to a lesser degree than the corresponding homozygous mutant. It is possible that this discrepancy may be due to an inherent limit of the assay to discern between degrees of fiber degradation.

Interestingly, however, although both heterozygous and homozygous \textit{Bard1}^{SF} and \textit{Bard1}^{KA} cells displayed a clear defect in SFP and an accumulation of DNA damage after replication stress, only the homozygous \textit{Bard1}^{SF/SF} and \textit{Bard1}^{KA/KA} cells were hypersensitive to fork-stalling drugs, such as the inter-strand crosslinking agent MMC and the PARP inhibitor olaparib (Figures 15 and 16). In contrast, Pathania et al. (2014) observed that \textit{Brca1}^{mut/+}
mammary epithelial cells are more sensitive than Brca1<sup>−/−</sup> cells to a different DNA inter-strand crosslinking reagent (cisplatin) and to ultraviolet light (UV) (Pathania et al., 2014).

While there may be several explanations for this discrepancy, the simplest, and probably least satisfying, is that differences in cell type and/or experimental conditions account for the differing results. For example, the genotoxic sensitivity experiments of Pathania et al. (2014) were performed in human mammary epithelial cells using a longer treatment period and a different crosslinking reagent (cisplatin). Nonetheless, it is still quite puzzling that homozygous Bard1<sup>Sf/Sf</sup> and Bard1<sup>KA/KA</sup> MEFs, but not the corresponding heterozygous MEFs, would display hypersensitivity to MMC and PARPi in our clonogenicity assays. While it is possible that the hypersensitivity to these two agents is due to some unknown, non-SFP pathway, defect caused by the Bard1 BRCT mutations, it is also possible that the DNA fiber assay is not sensitive enough to discern subtle differences in the severity of SFP defects between homozygous and heterozygous cells, as mentioned above. If so, then heterozygous Bard1<sup>Sf/+</sup> and Bard1<sup>KA/+</sup> cells may have a SFP defect that is less severe than that of the corresponding homozygous-mutant cells. As a result, heterozygous-mutant cells might not accumulate sufficient DNA damage upon MMC or PARPi treatment to impair colony formation in our clonogenicity assays. Indeed, this model is supported by the intermediate level of DNA damage displayed by heterozygous cells in the alkaline comet assay (Figure 41). To further test this model, chromosomal instability (assessed by giemsa karyotyping and T-FISH) could be evaluated in heterozygous Bard1 BRCT mutant cells in response to different DNA damaging agents (e.g., MMC, PARPi, HU, or UV treatment), with the model predicting that heterozygous cells would accumulate more DNA aberrations than wildtype cells but fewer than homozygous cells.

Interestingly, Pathania et al. invoke the concept of “conditional haploinsufficiency” (Bartek, Lukas, & Bartkova, 2007) to explain the genotoxic sensitivity of heterozygous mammary epithelial Brca1<sup>mut/+</sup> cells (Pathania et al., 2014). They demonstrate that upon induction of replication stress and treatment with a DSB-inducing agent like ionizing radiation, Brca1<sup>mut/+</sup> heterozygotes that were previously competent for HDR now displayed HDR deficiencies. Therefore, it is possible that the sensitivity of Brca1<sup>mut/+</sup> heterozygotes to genotoxins may be triggered in a similar manner, such that MMC-induced replication stress would uncover an HDR deficiency that in turn leads to a toxic accumulation of DNA damage. However, in Bard1<sup>Sf/+</sup> and Bard1<sup>KA/+</sup> cells there would be no HDR deficiency to “uncover”, as even homozygous cells are competent for HDR, explaining the lack of genotoxic
hypersensitivity in our clonogenicity assays. This model could be tested by karyotype analysis of heterozygous Bard1 (Bard1^{50/-} or Brca1^{60/-}) and Brca1 (Brca1^{50/-}) BRCT mutant cells subjected to replication stress, with the model predicting that Bard1 heterozygotes would display lower levels of chromosomal aberrations compared to Brca1 heterozygotes.

In the final chapter, we will integrate the haploinsufficiency data presented above, along with the HDR, SFP, and tumor formation data presented in previous chapters, into a model of BRCA1/BARD1-mediated breast cancer.
CHAPTER VI

CONCLUSIONS AND FUTURE DIRECTIONS
BRCA1 and its obligate binding partner, BARD1, form a heterodimer that performs a diverse set of functions in the cell. Most importantly, BRCA1 and BARD1 are both mutated in cases of hereditary breast cancer (Antoniou et al., 2003; Couch et al., 2015; De Brakelere et al., 2016; De Brakelere et al., 2010; Hall et al., 1990; Ratajska et al., 2012; Roy et al., 2011; Sabatier et al., 2010). Therefore, a primary goal of the BRCA1 field is to determine how loss of BRCA1 or BARD1 leads to tumorigenesis. However, two problems complicate matters: first, the BRCA1/BARD1 heterodimer has been implicated in a wide variety of cellular processes making it difficult to determine which of its functions are actually required for tumor suppression. Second, since BRCA1 and BARD1 are reliant upon each other for their stability and thus, many of their functions, it is unclear which activities of the BRCA1/BARD1 heterodimer are mediated by each protein individually.

While most research into the BRCA1/BARD1 heterodimer has focused on the contributions of BRCA1, the interpretation of these experiments in addressing the two problems described above is often limited by the use of null alleles. Indeed, it is possible that the primary function of BARD1 is simply to stabilize BRCA1, and that BARD1 contributes few, if any, other unique functions to the BRCA1/BARD1 heterodimer. However, the existence of cancer-associated mutations that are predicted to affect BARD1 without disrupting its interaction with BRCA1 argues against this theory. Moreover, a recent study showed that the BARD1 BRCT domain is required for $\gamma$H2AX-independent recruitment of the BRCA1/BARD1 heterodimer to damaged DNA (M. Li & Yu, 2013). We therefore hypothesized that BARD1 may contribute unique functions to the BRCA1/BARD1 heterodimer that are required for tumor suppression.

To address this hypothesis, we analyzed novel Bard1 mouse lines bearing mutations that disrupt phospho-recognition by the Bard1 BRCT domain ($Bard1^{SF}$ and $Bard1^{KA}$). We selected these mutations to study because 1) they were not predicted to affect the formation of the BRCA1/BARD1 heterodimer, 2) cancer-associated mutations that disrupt the BARD1 BRCT domain have been identified, and 3) the Brca1 BRCT domain had been shown to be vital for homology-directed repair of DNA breaks and for tumor suppression (Shakya et al., 2011).

As predicted by previous structural studies, the $Bard1^{SF}$ and $Bard1^{KA}$ mutations did not disrupt the formation or stability of the Brca1/Bard1 heterodimer (Figures 13 and 14), confirming that the analysis of these mutations might potentially uncover unique functional contributions of Bard1 to the BRCA1/BARD1 heterodimer. Our key findings from this study can be summarized in three points: 1) BRCT phospho-recognition by Bard1 is
dispensable for homology-directed repair, 2) phospho-recognition by Bard1 is required for stalled fork protection and maintenance of chromosomal stability in the face of replication stress, and 3) Bard1 BRCT phospho-recognition is not essential for Brca1/Bard1-mediated tumor suppression. Thus, based on the analysis of these mice and the cell lines derived from these mice, we have identified novel contributions that Bard1 makes to BRCA1/BARD1-mediated functions in genome stability. We have also addressed one of the fundamental questions of BRCA1 research; specifically, what functions of the BRCA1/BARD1 heterodimer are or are not required for tumor suppression? The consequences of these findings are discussed below.

In Chapter 4 we demonstrated that the Bard1 BRCT mutations abrogate stalled fork protection (SFP) in homozygous mutant cells (Figures 27 and 28). Mechanistically, this defect represents a failure of the Brca1/Bard1 heterodimers to mobilize at stalled replication forks in HU-treated Bard1^{SF/SF} and Bard1^{KA/KA} cells (Figures 34 and 36). Of note, in Chapter 3 we observed that the same cells are competent for homology-directed repair (HDR). Thus, the Bard1^{KA} and Bard1^{SF} alleles represent separation-of-function mutations that confer homozygous mutant cells with a novel SFP^− HDR^− phenotype.

From our studies, it appears that phospho-recognition by the Bard1 BRCT domain or the Brca1 BRCT domain mediate distinct, but possibly overlapping, functions. Specifically, we confirmed in Chapter 3 that Brca1^{SF/SF} cells are defective for HDR of double-strand DNA breaks (Shakya et al., 2011), while in Chapter 4 we demonstrated that these same cells harbor a previously unreported defect in SFP (Figure 29). Clearly, the BRCT phospho-recognition properties of Brca1 and Bard1 are functionally distinct with respect to their impact on HDR, with only Brca1 BRCT phospho-recognition making an essential contribution to this process. In contrast, SFP is dependent on phospho-recognition by the BRCT domains of both Brca1 and Bard1. At present, we do not understand how SFP is impaired mechanistically by the Brca1^{SF} mutation. On one hand, is Brca1 BRCT phospho-recognition, like Bard1 BRCT phospho-recognition, also required for the recruitment of Brca1/Bard1 heterodimers to stalled replication forks? And if so, how is this achieved at the molecular level and what are the Brca1 BRCT phospho-ligands required for this recruitment? On the other hand, does Brca1 BRCT phospho-recognition make other essential contributions to SFP that are independent of Brca1/Bard1 recruitment to stalled replication forks?

In any case, the SFP^− HDR^− phenotype of Bard1^{SF/SF} and Bard1^{KA/KA} cells clearly contrasts with the SFP^+ HDR^− phenotype of Brca1^{SF/SF} cells. Thus, further analysis of the Bard1 separation-of-function mutations described here
should provide new insights into the relative contributions of HDR and SFP to BRCA1/BARD1-mediated genome stability and tumor suppression.

The phenotypes of \(\text{Bard}1^{\text{SF/SF}}, \text{Bard}1^{\text{KA/KA}}, \text{and Brca1}^{\text{SF/SF}}\) cells suggest that SFP and HDR work in concert to prevent genome instability. As such, we present a cellular model of how the two functions interact to prevent genome instability. It seems likely that replication stress represents a common endogenous source of DNA damage, including double-strand breaks. In the absence of an intact SFP pathway, endogenous replication stress may lead to a greater incidence of fork collapse. However, in unstressed conditions this SFP defect alone may be insufficient to generate significant chromosomal instability. Instead, in cells that have a functional HDR pathway, like \(\text{Bard}1^{\text{SF/SF}}\) and \(\text{Bard}1^{\text{KA/KA}}\) MEFs, HDR may act as a backup mechanism that effectively repairs DSBs that accumulate as a result of collapsed forks, thus preventing the accumulation of significant spontaneous chromosomal instability. Thus, it may require heightened levels of replication stress—not found in normal cycling cells—to generate enough DNA damage to overwhelm the capacity of HDR to suppress chromosomal instability. In support of this model, we observed the accumulation of DNA damage in MMC- or HU-treated \(\text{Bard}1^{\text{SF/SF}}\) and \(\text{Bard}1^{\text{KA/KA}}\) cells (Figures 20, 21, and 30), but no increase in spontaneous chromosomal rearrangements (Figures 20 and 21). Furthermore, in the \(\text{Bard}1^{\text{SF/SF}}\) and \(\text{Bard}1^{\text{KA/KA}}\) mice we observed none of the obvious developmental defects that typically arise in Brca1-mutant mice as a result of growth retardation induced by excessive DNA damage.

On the other hand, in cells that are deficient for both HDR and SFP, like the \(\text{Brca1}^{\text{SF/SF}}\) mutant, there is no HDR pathway to act as an error-free backup mechanism to process DNA damage that arises from replication stress. As a result, the DSBs formed from collapsed replication forks may instead be handled by more error-prone pathways such as MMEJ and NHEJ, resulting in significant chromosomal instability. Indeed, endogenous fork stalling and collapse appear to be sufficient to accumulate spontaneous chromosomal instability in SFP\(^{-}\)HDR\(^{-}\) cell lines, as demonstrated by T-FISH analysis of unperturbed \(\text{Brca1}^{\text{SF/SF}}\) primary MEFs (Figure 22). Furthermore, the growth and viability defects of \(\text{Brca1}^{\text{SF/SF}}\) mice suggest that spontaneous DNA damage also occurs during embryogenesis. Additionally, exposure to MMC-induced replication stress generates an even greater level of chromosomal aberrations in \(\text{Brca1}^{\text{SF/SF}}\) cells than in SFP\(^{-}\)HDR\(^{+}\) cells (Figure 22).
In Chapter 4, we used our separation-of-function $\text{Bard1}^{SF/SF}$ and $\text{Bard1}^{KA/KA}$ mice to ascertain whether loss of the SFP function of the Brca1/Bard1 heterodimer is sufficient to induce tumor formation. In contrast to $\text{Brca1}^{SF/SF}$ mice, which develop tumors at an accelerated rate (Shakya et al., 2011), the $\text{Bard1}^{SF/SF}$ and $\text{Bard1}^{KA/KA}$ mice were not prone to tumor development, as compared to their littermate wild type controls (Figure 37). Thus, we conclude that SFP defects alone are not sufficient to induce BRCA1/BARD1-mediated tumorigenesis. Instead, SFP and HDR defects in concert or HDR defects alone are likely to be required for tumor formation. Unfortunately, the latter possibility cannot be evaluated at this time since Brca1 separation-of-functions mutations that generate a SFP$^-$HDR$^-$ phenotype have not yet been identified.

In Chapter 5, we showed that both the Bard1 and Brca1 BRCT mutants are haploinsufficient for SFP (Figures 38, 39, and 40), confirming previous reports of BRCA1 haploinsufficiency for SFP in human mammary epithelial cells (Pathania et al., 2014). Additionally, this haploinsufficiency for SFP leads to an accumulation of DNA damage during replication stress (Figure 41) This finding is potentially significant because the tumor-prone women of families afflicted with BRCA1-linked hereditary breast and ovarian cancer are heterozygous mutation carriers, bearing one mutant and one normal BRCA1 allele (Miki et al., 1994) (Wooster & Weber, 2003). Therefore, the haploinsufficiency of BRCA1 for SFP may contribute to the tumor susceptibility of BRCA1-mutation carriers. Based on these data, we integrated our data regarding SFP and HDR function at the cellular level (described above) with a model of BRCA1/BARD1-mediated tumorigenesis.

The presence of subtle phenotypic defects in heterozygous BRCA1-mutant mammary epithelial cells supports emerging, but as yet unproven, models for the development of breast tumors in BRCA1-mutations carriers (Konishi et al., 2011; Lim et al., 2009; Martins et al., 2012; Nolan et al., 2016; Pathania et al., 2014). Since heterozygous BRCA1$^{\text{mut/+}}$ cells are defective for SFP (but not HDR), BRCA1 mutation carriers should experience a “field effect” in which all mammary epithelial cells are prone to replication stress due, at least in part, to their inability to protect stalled replication forks (Figure 42). The downstream consequences of this stress, especially chromosomal instability, would then allow for the emergence of cells that have acquired genetic lesions that circumvent the premature senescence and inviability typically associated with BRCA1 mutant homozygosity (such as p53 or p16 inactivation, which occur in the vast majority of BRCA1-mutant basal-like triple-negative breast tumors). In these cells or their progeny, subsequent loss of the wildtype BRCA1 allele would yield viable cells that
Figure 42. Model for tumorigenesis in a BRCA1 mutation carrier. All mammary epithelial cells (blue) of a BRCA1 mutation carrier (Brca1\textsuperscript{mut+}) are defective for SFP but competent for HDR. As a result of hormone-mediated (estrogen or progesterone) replication stress, Brca1\textsuperscript{mut+} mammary epithelial cells experience minor genome instability. In time, a rare mutation(s) would arise that increases the viability of HDR-defective cells (such as loss of p53 or p16), allowing subsequent loss of the wild type BRCA1 allele and the emergence of rare Brca1\textsuperscript{mut–} variants (red) that are defective for both HDR and SFP. As such, these cells and their progeny would then experience more extensive genomic instability at levels sufficient to drive malignant progression.
lack HDR activity. By virtue of their inability to support HDR, these variants would then experience more extensive genomic instability at levels sufficient to drive malignant progression. In this scenario, loss of BRCA1-mediated SRF stability would play a critical role in the early stages of tumorigenesis, allowing the subsequent evolution (and viability) of rare homozygous BRCA1-mutant cells which, having also lost HDR activity, would then be susceptible to full neoplastic progression. This model implies that the SFP and HDR functions of BRCA1 mediate distinct, but complementary, aspects of tumor suppression, and that both functions must be abrogated for tumor development in BRCA1-mutation carriers. Specifically, the SFP defect is necessary to facilitate the initial low level of genome instability which leads, firstly, to genetic lesions that allow cell viability in the absence of BRCA1 (e.g., p53/p16 inactivation) and, secondly, to loss of the second BRCA1 allele, albeit after a long latency. Consistent with this, most BRCA1-mutant breast cancers do not present until the 6th or 6th decade of life (Brose et al., 2002). Curiously, a recent case report described a rare patient with a biallelic germline BRCA1 mutation that developed breast cancer at age of 23 (Sawyer et al., 2015). Furthermore, the tumor cells from this patient were deficient for Rad51 focus formation, indicative of an HDR defect. While acknowledging the limits of these descriptive case reports, the model presented in Figure 42 would predict that these tumor cells are also defective for SFP.

This model also predicts that women who carry BARD1 mutations that are functionally comparable to $Bard1^{SF}$ or $Bard1^{KA/+}$ would not display susceptibility to breast and ovarian cancer. Although $Bard1^{SF/+}$ and $Bard1^{KA/+}$ cells would be deficient for SFP and likely accumulate low levels of genome instability, loss of the wild type allele would not “uncover” an HDR defect, since homozygous cells harboring the Bard1-BRCT mutations are competent for HDR. Thus, even $Bard1^{SF/-}$ and $Bard1^{KA/-}$ mammary epithelial cells would not be able to acquire the “mutator” phenotype necessary to drive malignant transformation. The differential tumor susceptibility of the $Bard1^{KA/KA}$ and $Bard1^{SF/SF}$ mice compared to that of the $Brca1^{SF/SF}$ mice supports this hypothesis. In principle, the best test of this model would be to compare tumor formation in heterozygous $Bard1^{KA/+}$ and $Bard1^{SF/+}$ mice to that of heterozygous $Brca1^{SF/+}$ mice. Unfortunately, however, unlike women, mice heterozygous for a Brca1 null mutation ($Brca1^{+/–}$) do not develop tumors (X. Liu et al., 2007). This difference between human and mouse BRCA1-mediated mammary tumorigenesis may be due to the shorter lifespan of mice and the markedly fewer mammary epithelial cell divisions that occur in mice relative to humans. Therefore, the
lifetime risk of a loss-of-heterozygosity event at the mouse Brca1 locus is sufficiently low to be essentially non-existent. Nevertheless, heterozygous Brca1 mice have been shown to be tumor prone when treated with genotoxic stress such as ionizing radiation (Jeng et al., 2007). Thus, it is possible that this model could be tested by applying genotoxic stress to heterozygous Bard1 and Brca1-BRCT mutants and monitoring for tumor formation.

Furthermore, the model presented above might be consistent with the tissue specificity of BRCA1/2 pathway cancers. Even though a human BRCA1/2 mutation carrier harbors the mutation in every cell type, tumors almost exclusively arise in the breast and ovaries. The most obvious connection between these two tissues is that they are hormonally responsive to estrogen and progesterone. Strikingly, estrogen and its metabolites have been reported to induce DNA damage through adduct formation in mammary cells (Savage et al., 2014). Furthermore, estrogen may also generate replication stress by inducing rapid cell proliferation (Caldon, 2014; Musgrove & Sutherland, 2009) or increased R-loop formation (Stork et al., 2016). Consistent with the idea that estrogen can contribute to breast cancer formation, conditions that increase lifetime estrogen exposure, such as early menarche, late menopause, nulliparity, and treatment with exogenous estrogen, are all associated with an increased breast cancer risk (Clemons & Goss, 2001; Foulkes et al., 2010). Furthermore, oophorectomy has been shown to reduce breast cancer risk in BRCA1-mutant carriers (Rebbeck et al., 2002). Another recent paper reported that progesterone signaling through the RANKL pathway induces aberrant proliferation and accumulation of DNA damage in BRCA1^{mut/+} cells (Nolan et al., 2016). Thus, it is possible that estrogen and progesterone act as replication stress-inducing agents, and that mammary epithelial cells responsive to these hormones would experience a significant increase in replication stress compared to other cell types. In BRCA1^{mut/+} cells that harbor an SFP defect, this stress would lead to genetic instability and eventual loss of the wild type BRCA1 allele as described above, finally resulting in malignant transformation.

We now know that mice with the SFP^HDR− phenotype (Bard1^{SF/SF}) are tumor-prone (Shakya et al., 2011) while those with the SFP^HDR+ phenotype (Bard1^{SF/SF} and Bard1^{KA/KA}) are not (Figure 37). At this point, evaluation of mice with the SFP^HDR− mice would provide a critical test of the model presented in Figure 42. In principle, the SFP^HDR− mice should not be tumor prone, as the genome instability conferred by the SFP defect is required to
enable the early steps of tumorigenesis, such as p53/p16 inactivation and loss of the second Brca1 allele (Figure 42). Unfortunately, as noted above, Brca1 separation-of-function mutations that confer this phenotype have not yet been identified. Nonetheless, recent studies demonstrate that deletion of certain DNA translocases required for reversed fork formation (i.e., SMARCAL1 and ZRANB3) can rescue the SFP defects of BRCA1- and BRCA2-mutant cells and, in doing so, restore chromosomal stability (Kolinjivadi, Sannino, De Antoni, Zadorozhny, et al., 2017; Taglialetela et al., 2017). Thus, by creating double-mutant mice that harbor a SMARCAL1 or ZRANB3 mutation along with a Brca1 hypomorphic mutation known to be defective for both SFP and HDR (such as Brca1^{SF/SF}), it should be possible to generate, and monitor tumor formation in, SFP^{+}HDR^{−} mice.

Overall, these findings advance our understanding of the individual contributions of BRCA1 and BARD1 to the functions of the BRCA1/BARD1 heterodimer. We demonstrate that BARD1, and specifically its BRCT phospho-recognition ability, is required for SFP and maintenance of chromosomal stability in the face of replication stress, indicating that BARD1 makes contributions to BRCA1/BARD1 function over and above stabilization of BRCA1 polypeptides. Finally, we demonstrate that loss of the SFP ability of the BRCA1/BARD1 heterodimer is insufficient for tumor formation. Based on these findings, we have proposed a model of BRCA1/BARD1-mediated tumorigenesis that may have implications for the prevention of BRCA1-pathway hereditary breast cancer. Further testing of this model is necessary to fully reveal the individual contributions of SFP and HDR to BRCA1-mediated tumor suppression.
CHAPTER VII

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