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DNA Repair (Amst). Author manuscript; available in PMC 2024 October 01.

Published in final edited form as:

DNA Repair (Amst). 2023 October ; 130: 103563. doi:10.1016/j.dnarep.2023.103563.

Finding Significance: New Perspectives in Variant Classification of the RAD51 Regulators, BRCA2 and Beyond

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Abstract

For many individuals harboring a variant of uncertain functional significance (VUS) in a homologous recombination (HR) gene, their risk of developing breast and ovarian cancer is unknown. Integral to the process of HR are *BRCA1* and regulators of the central HR protein, RAD51, including *BRCA2, PALB2, RAD51C* and *RAD51D*. Due to advancements in sequencing technology and the continued expansion of cancer screening panels, the number of VUS identified in these genes has risen significantly. Standard practices for variant classification utilize different types of predictive, population, phenotypic, allelic and functional evidence. While variant analysis is improving, there remains a struggle to keep up with demand. Understanding the effects of an HR variant can aid in preventative care and is critical for developing an effective cancer treatment plan. In this review, we discuss current perspectives in the classification of variants in the breast and ovarian cancer genes *BRCA1, BRCA2, PALB2, RAD51C* and *RAD51D*.

Keywords

Homologous recombination deficient tumors; breast cancer; ovarian cancer; variant of unknown/uncertain significance; homologous recombination; BRCA1; BRCA2; PALB2; RAD51C; RAD51D; RAD51

1. Introduction

Since the discovery of *BRCA1* and *BRCA2* in the mid-90s, there has been an established connection between homologous recombination deficiency (HRD) and increased breast and ovarian cancer risk [1-6]. It is estimated that ~30% of all ovarian tumors and ~13% of all

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Author Statement

All authors have read and approve of the submitted manuscript.

Declaration of Interests Statement

The authors declare that they have no known conflicts of interest that would influence the contents of this manuscript.

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breast tumors are HRD [7]. HR genes can be affected by either genetic mutation including insertions, deletions, frameshifts or point mutations (Fig. 1A) or through disruption in expression such as promoter methylation, copy number alterations, or alternative splicing. Missense mutations are commonly identified genetic alterations, with 2% of people having a missense mutation in any given gene (Fig. 1B) [8]. These variants can be both somatic or germline and have presented in many cancer types including metastatic prostate, pancreatic cancer, melanoma and hereditary breast and ovarian cancer syndromes (HBOC). Although *BRCA1* and *BRCA2* are the most well studied, disruptions of several other HR genes including *PALB2, RAD51C* and *RAD51D* also contribute to HRD and cancer risk [5, 9-19]. Like the *BRCA* genes, *PALB2, RAD51C* and *RAD51D* are also included on breast and ovarian cancer multi-gene panel tests. Current guidelines classify these variants into three general categories: pathogenic, benign, or variant of uncertain significance (VUS), with most variants falling into the latter category (Fig. 1C; [20]). Attempts to classify VUS have been bolstered in recent years with expanding access to cancer mutation databases, increasingly accurate predictive tools, and improved functional assays. This review will discuss recent advances in VUS classification and the direct impact of variant classification on breast and ovarian cancer patients harboring these variants.

2. Homologous recombination pathway overview and the function of the RAD51 regulators

HR is a DNA repair mechanism required for both tolerance of replication-induced DNA damage and high-fidelity repair of DNA double-strand breaks (DSBs) [21]. Although endogenous DSBs are rare (~50 per cell, per cell cycle), a single unrepaired DSB is lethal [22]. Furthermore, inaccurate repair of DSBs contributes to genetic instability, a hallmark of cancer [23]. Thus, proper regulation and activity of HR and its key players is integral to cancer prevention and promoting overall cellular health.

HR takes place primarily during S phase and begins with the resection of the DSB ends resulting in 3' single-stranded DNA overhangs (Fig. 2A.1). BRCA1 plays a critical role in resection of DSBs along with a myriad of other factors including PALB2, which interacts directly with both BRCA1 and BRCA2 (Fig. 2A). These 3' overhangs are then coated by the single-stranded DNA binding complex, replication protein A (RPA), which is subsequently displaced by the ATPase RAD51. Formation of RAD51 nucleoprotein filaments around the 3' DSB ends is an essential HR step. RAD51 presynaptic filament formation is highly regulated with the assistance of the RAD51 mediator proteins, including BRCA2, PALB2, RAD51C, and RAD51D (Fig. 2A.2). BRCA2 is recruited to DSBs through its interaction with BRCA1, bridged through PALB2, and functions by nucleating the RAD51 filament [24]. A complex consisting of BRCA2, PALB2, RAD51C and RAD51 has also been identified [25]. Subsequently, RAD51C and RAD51D function to promote RAD51 filament assembly [26]. (Fig. 2A.3). *RAD51C* and *RAD51D* are RAD51 paralogs, ancient gene duplications of *RAD51* itself, dating back to the archaeal homolog RecA [27, 28]. Although the RAD51 paralogs share homology in the ATPase core of RAD51, they have weak ATPase activity in comparison to RAD51 [29, 30]. RAD51C and RAD51D are part of a larger complex of RAD51 paralogs, called the BCDX2 complex (containing RAD51B, RAD51C,

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RAD51D, and XRCC2). RAD51C also forms a separate, two-member complex with XRCC3, called CX3 [29]. The precise mechanism by which the RAD51 paralogs promote RAD51 filament formation is still being elucidated. While all of these RAD51 paralogs are required for HR, pathogenic germline variants (PGVs) in *RAD51C* and *RAD51D* have the strongest association with breast and ovarian cancer predisposition (*RAD51C* ovarian and breast cancer odds ratio of 8.3 and 1.93, respectively and *RAD51D* ovarian and breast cancer odds ratio of 6.94 and 1.8, respectively) [31, 32]. After RAD51 filament formation and strand invasion (Fig. 2A.4), the second end of the DSB is captured and the homologous template is used to synthesize a new DNA stand. Finally, the hemicatenated structures are resolved into gene-conversion, crossover or non-crossover products with the help of other proteins including helicases and topoisomerases (Fig. 2A.5). Although the disruption of many DNA repair factors are associated with cancer, here we will focus on the key regulators of RAD51; including *BRCA2, PALB2, RAD51C*, and *RAD51D*.

Aside from their canonical roles in HR, the RAD51 regulators also play an important function in replication fork protection, reversal and restart (Fig. 2B). When replicative DNA polymerase encounters a fork blocking lesion, the enzyme is stalled, and the fork becomes vulnerable to nucleolytic attack. RAD51 forms a nucleoprotein filament around the exposed ssDNA. The filament is stabilized with the assistance of BRCA1, BRCA2, and the BCDX2 complex, further protecting the vulnerable fork. RAD51 then enables fork reversal into a chicken foot structure and replication can restart with the aid of the CX3 complex [21, 33, 34]. In contrast to the function of the RAD51 regulators in HR, their role in replication fork protection and restart in the context of cancer predisposition and therapeutic response is less defined. Further studies will be required to elucidate the connection between efficient replication and HBOC.

3. Genetic Sequencing Practices and HBOC Risk

Although disruptions in many HR genes are associated with cancer, genes that regulate RAD51 are unique in that they are predominantly associated with HBOC [7, 35, 36]. Overall lifetime risk of breast and ovarian cancer for the general population is 13% and 1.2%, respectively [37]. Individuals harboring *BRCA1* and *BRCA2* PGVs pose the highest lifetime risk for breast (*BRCA1* up to 72% risk; *BRCA2* up to 69%) and ovarian cancer (*BRCA1* up to 44%; *BRCA2* up to 17%) with increased risk correlating with age [38]. Although not as high risk as *BRCA1* and *BRCA2* deficiency, disruptions in *PALB2, RAD51C* and *RAD51D* also pose significant cancer risk (*PALB2*, BC 30-60% and OC 5%; *RAD51C*, BC 21% and OC 11%, *RAD51D*, BC 20% and OC 13%; [11, 14, 39]) and should be taken into consideration accordingly. It is possible that other HR genes, like RAD51B for example, may also affect breast and ovarian cancer risk, but lack of relevant data leaves variants in these genes poorly understood.

With the popularization of precision medicine, personalized sequencing is becoming increasingly attractive to help understand cancer risk. Sequencing often occurs in advanced cancer patients as a strategic means of targeting mutational defects in the tumor, but it is also common in families with a history of HBOC [40]. This is because individuals are much more likely to develop breast or ovarian cancer if a close relative has had the

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disease. Risk increases most dramatically when this relative is an immediate family member, such as a parent or sibling, or if multiple family members have had the disease [11]. Age of onset of these family members is also an important factor to consider because PGVs are often associated with early onset of HBOC (ovarian <40, breast <55 years of age) [41-43]. For a large portion of breast and ovarian patients with PGVs, the variant is inherited as a heterozygous mutation that becomes homozygous in the tumor due to a loss of heterozygosity (LOH) event on the opposite allele [44-46]. Furthermore, in high grade serous ovarian cancer, the HRD tumors almost universally harbor p53 mutations [47]. In breast cancer, HRD tumors are largely triple negative but not exclusively [48, 49]. This finding solidified the connection between the RAD51 regulators, particularly RAD51C and RAD51D, and increased breast cancer risk. HR deficiency, irrespective of the specific HR gene disruption or variant identified, can also be detected by examining the mutation signature of the tumor itself where HR loss leads to the development of mutation signature 3 [50]. Machine learning algorithms HRDetect and CHORD examine whole genome sequencing data to determine how well it correlates with this mutational signature to identify HRD tumors [7, 51]. Clinical HRD testing is currently available through Caris (Molecular Intelligence Comprehensive Tumor Profiling) Foundation Medicine (FoundationOne CDx), Myriad (myChoice CDx) and Tempus (Tempus xT HRD test) using different combinations of BRCA sequencing, LOH determination, and other large-scale genomic aberrations. Variants in the RAD51 regulators are often observed on their own, but on rare occasion, multiple deleterious variants in the different regulator genes (i.e. *BRCA1* and *BRCA2*) can be observed in one patient or tumor [52-54]. While specific studies have seen the effects of multiple variants causing similar effects to a single variant [54], it is still not well understood how harboring multiple RAD51 mediator variants would contribute to disease risk.

Invitae, Ambry Genetics, Myriad, GeneDX and Color Diagnostics are widely used genetic testing companies. These companies developed panels of genes that are associated with different disease groups like pan cancer, Lynch syndrome, and HBOC. Many of these panels can be custom tailored by clinicians based on family phenotype. Using next-generation or sanger sequencing, the exons as well as the flanking proximal intronic regions of the genes-of-interest are analyzed. In addition to simple insertions, deletions, and point mutations, genes are also screened for large scale deletion and duplication events. Some companies like Ambry Genetics and Invitae, also offer concurrent DNA-RNA sequencing in order to determine the effects on gene expression (splicing, fusions, etc.). Unfortunately, sequencing services do not broadly include epigenetic analysis. Epigenetic alterations are known to significantly influence gene expression and its exclusion from the genetic analysis can overlook factors such as an increased methylation of gene promoters which can result in gene expression silencing. Additionally, deep intronic sequencing is also uncommon in commercial sequencing. Genetic alteration in these regions, as well as the intron-exon boundary, have the potential to activate alternative splicing and significantly disrupt protein function [55-57].

Screenings result in three kinds of outcomes: positive, inconclusive or VUS. Positive outcomes refer to the identification of a pathogenic or likely pathogenic variant in one of the examined genes that increases the risk of disease development. Individuals with this sequencing outcome are able to take advantage of preventative care and targeted treatment

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options, like PARP inhibitors. When no PGVs are found in any of the selected genes, or the only variants identified are considered benign or likely benign, the outcome is considered inconclusive and the physician may decide to expand the screening to include less commonly tested genes. Invitae, Ambry Genetics, Color Diagnostics and GeneDX sequencing services include exome sequencing with GeneDX also offering whole genome sequencing when specific gene panels fail to result in variant identification. Finally, the outcome of VUS refers to the identification of a variant in one of the tested genes whose cumulative evidence is not strong enough, or provide conflicting interpretations of phenotypic effect, preventing a classification of either benign or pathogenic.

A large portion of all identified variants are classified as VUS (*BRCA1*, 32%; *BRCA2*, 40%; *PALB2*, 48%; *RAD51C*, 52%; *RAD51D*, 48% in ClinVar as of February 2023) and even more variants present in these genes have conflicting interpretations [20]. Furthermore, VUS in *BRCA1* and *BRCA2*, are more easily characterized due to extensive structural and functional analysis, while variants in less well characterized HR genes, such as *RAD51C* and *RAD51D*, are typically less understood [58-62]. Although genetic screening can be very informative, unfortunate individuals harboring a variant with a VUS status remain uninformed about their cancer risk and/or treatment options, making VUS reclassification paramount in the age of precision medicine.

4. Evidence for Pathogenicity Classification

4.1 American College of Medical Genetics Guidelines and Variant Classification

In 2015, the American College of Medical Genetics (ACMG) together with clinicians and pathologists from the Association for Molecular Pathology and the College of American Pathologists released their general guidelines for variant classification [63]. These guidelines suggested five variant interpretation categories: benign, likely benign, variant of uncertain significance, likely pathogenic, and pathogenic, with the “likely” categories used in cases of >90% certainty. Population data, predictive algorithms, functional data, clinical and allelic information can be variably weighted, depending on validation and quality, and ultimately combined to assign variants into one of these five classifications. Importantly, the baseline for various evidence types are not uniformly weighted (Fig. 3). For example, null variants, which result in no protein or in loss of large parts of known clinically relevant domains, provide the strongest evidence towards pathogenicity under the PVS1 code [Pathogenic Very Strong (PVS)]. Besides the first expression-based analysis of these variants examining altered splicing, large deletions or early stop codon variants, few follow-up studies are required for classification of these variants as pathogenic. In contrast, more readily available but less reliable forms of evidence, like predictive analysis [baseline Pathogenic Supporting (PP) or Benign Supporting (BP)], require several additional lines of evidence to achieve a classification outside of VUS (Fig. 3). In this case, the bar for additional pathogenicity evidence is higher, and because of this, many remain variants of uncertain significance. However, each line of evidence can be modified from its baseline weight depending on validation, quality and specific gene. The ClinGen sequence variant interpretation (SVI) working group has been working to expand on these evidence classifications in order to improve classification accuracy [64, 65]. For example, it has been suggested that variants

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affecting protein expression (PVS1) can range in strength from very strong (PVS1; full gene deletion) to supporting (PVS1 supporting; start codon variant in gene with alternative start site) depending on the type of variant [66]. The SVI's Bayesian classification framework, developed in 2018, gives a mathematical foundation for describing clinical and laboratory data as pathogenicity evidence [67]. In addition to the SVI, several variant curation expert panels (VCEP) have been established by ClinGen to set standard criteria regarding variant evidence requirements as well as review evidence and provide official classification of gene variants. To date, the hereditary breast ovarian and pancreatic cancer VCEP has created evidence criteria specification for both ATM and PALB2 and has utilized this criterion in the official classification of 71 ATM and PALB2 variants. However, criteria and classifications have yet to be released for RAD51C and RAD51D. The reclassified PALB2 variants can be found on the VCEP website. Separate from the hereditary breast, ovarian and pancreatic cancer VCEP, ClinGen has also included the Evidence-based Network for Interpretation of Germline Mutant Alleles (ENIGMA) consortium VCEP to focus on variants in BRCA1 and BRCA2. The ENIGMA consortium is an international group of experts focused on the classification of variants in genes connected to hereditary breast and/or ovarian cancer genes. This group has been working independently since 2009 and is responsible for the majority of the early variant classification of BRCA1/2 variants [68]. With their integration into the ClinGen VCEP system, they have recently produced their updated guidelines for BRCA1/2 variant classification approved in April 2023 and releasing in the near future. Although unofficial, classifications for BRCA1, BRCA2, PALB2, RAD51C and RAD51D variants have been submitted through ClinVar and link to important case studies as well as relevant biochemical, functional, and population analysis (ClinVar variant database with interpretations; BRCA1, BRCA2, PALB2, RAD51C and RAD51D). These studies can be easily accessed through LitVar[2], a database of variant analysis publications. In this section, we will review the types of evidence that ACMG considers for variant classification and how this evidence may be evaluated by the hereditary breast, ovarian and pancreatic cancer and ENIGMA consortium VCEPs to produce official variant classifications.

4.2. Inheritance, Co-segregation and Population Genetics

Data from sequencing analysis is considered some of the most useful evidence for determining variant pathogenicity. Variant information for *BRCA1, BRCA2, PALB2, RAD51C* and *RAD51D* can be found across somatic and/or germline databases like Catalogue of Somatic Mutations in Cancer (COSMIC; somatic only) [69]. The Cancer Genome Atlas (TCGA; somatic and germline), ClinVar (somatic and germline) [20] or gnomAD (germline only) [70]. Information from these databases are helpful in identifying common benign variants in a population. In general, frequency above 5% (BA1), or above the expected frequency from the resulting disorder (BS1), likely points to a benign variant, as PGVs are expected to have low occurrence in the population (Fig. 3). Unfortunately, these databases are disproportionately based off of Caucasian populations and therefore most likely lack evidence for variants based in non-Caucasian populations [71]. The hereditary breast ovarian and pancreatic cancer VCEP provides cutoffs for *PALB2* variant frequencies above 0.1 (BA1) and 0.01 (BS1). As of now, the SVI subgroup has not released cutoffs for *BRCA1, BRCA2, RAD51C* or *RAD51D.*

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As previously stated, family history of disease can dramatically increase the risk of developing HBOC [11, 72]. Co-segregation of these familial cancers with a VUS can help establish pathogenicity. This co-segregation has been seen in each of the RAD51 regulators and is best established in *BRCA1* and *BRCA2* variant patients with high penetrance alleles and founder variants [73-79]. With lower penetrance HBOC genes that were more recently added to the multigene panel tests, co-segregation of the variant with the disease has been harder to establish within families, even with a history of breast/ovarian cancer and requires statistical methods to detect [80]. These caveats have made classifying potentially deleterious variants challenging. This type of evidence can be considered anywhere between supporting and strong depending on the amount of data available (PP1, BS4 Fig. 3). Conversely, *de novo* variants in affected patients with no parental history of disease are also ascribed a pathogenic line of evidence (PS2, PM6).

Although uncommon, inheritance of multiple variants and their relationship to one another (cis and trans) can be a helpful indicator of pathogenicity. Bi-allelic germline variants in many of the *RAD51* regulators have been observed in patients with Fanconi Anemia (FA), a typically recessive genetic disorder associated with dysmorphism, cognitive abnormalities and increased risk of childhood cancers. Variants within the *RAD51* regulators generally promote disease in an autosomal dominant manner, requiring variants in both alleles to be deleterious. Therefore, one can assume both variants found in FA patients to be pathogenic [Pathogenic Moderate 3 (PM3)]. However, a dominant variant in *RAD51* itself and in *RAD51C* FA patients have been reported [81-83]. As a result, the effect of monoallelic germline inheritance of these non-null variants on disease risk is unknown and may have a reduced effect compared to a fully penetrant null variant. It is also hypothesized that FA variants are hypomorphic in that they produce an intermediate functional abnormality. This is because the disease phenotype presents in a wide range of severities. Take BRCA2 associated FA for example. FA patients who inherited a truncating variant in BRCA2 were born with severe intrauterine growth restrictions and congenital malformations [84]. Conversely, one missense BRCA2 FA patient exhibited such a mild phenotype, they were only diagnosed at the age of 33, when receiving treatment for breast cancer [85]. Again, the independent inheritance of two RAD51 mediator variants is rare and more often patients inherit one deleterious variant and then endure a LOH event [44]. However, continuing to analyze biallelic variants may aid in understanding how more mild disruptions in HR can impact individual disease risk.

4.3 Pathogenicity Prediction Tools

Due to its accessibility, *in silico* prediction of pathogenicity (PP3, BP4) is one of the most common types of evidence available for variant classification (Fig. 3; [86]). An estimated 15% of all variants currently rely on this data for classification status and would be downgraded in its absence [87]. Although predictive algorithms rely on a different type of data, like impact on structure, conservation of the mutated residue, or splice site information, ACMG recommends that all *in silico* and predictive results be combined and considered a single piece of evidence as to prevent redundancies. Similar to this problem, it is possible that *in silico* tools could capture mutational hotspot and functional domain information (PM1). Therefore, when combining scores (PM1, PP3), it is recommended that they do not

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surpass the distinction of strong evidence in order to prevent over counting of evidence. Currently, the hereditary breast ovarian and pancreatic cancer VCEP does not allow *in silico* prediction evidence to be considered in classification of PALB2 variants.

In the past, these predictive tools relied solely on one single characteristic. Due to this limitation, models such as Protein Variation Effect Analyzer (PROVEAN), Polymorphism Phenotyping (PolyPhen), and Sorting Intolerant from Tolerant (SIFT) were poor predictors of pathogenicity, specifically in proteins of unknown structure. Use of stand-alone PolyPhen-2 or SIFT predictive score with developer thresholding is currently not recommended for variant classification [65]. As predictive models evolve, there has been a noticeable shift from individual predictive tools towards increasingly accurate ensemble methods [88]. For example, Rare Exome Variant Ensemble Learner (REVEL) is an ensemble tool that uses a combination of 13 separate predictive algorithms to create a collective prediction score of pathogenicity ranging from 0 to 1.0 [89]. Supporting this notion, in a study analyzing the *in silico* meta predictors, REVEL was used to predict the classification of a truth set of 4,094 non-VUS missense variants from ClinVar. Of the five meta-predictors examined, REVEL had one of the best overall prediction scores (0.907 and 0.899 out of 1.0). When compared to the combined SIFT/PolyPhen2 prediction score, REVEL produced far less false positive/negative predictions (4.6% REVEL compared to 27.5% SIFT/PolyPhen2) and achieved 4.9% more correct predictions [90]. More recently, when 55 *RAD51C* variants of uncertain significance underwent functional analysis, the corresponding REVEL scores were a much better predictor of HR deficiency in comparison to SIFT, PolyPhen, or PROVEAN [91].

Currently, machine learning is used to teach artificial intelligence models to predict pathogenicity. Evolutionary model of Variant Effect (EVE) was trained using multiple sequence alignments from 250 million protein sequences across varying species [92]. Variants are cross-referenced with EVE's 'evolutionary index' to determine pathogenicity. When analyzing *BRCA1* variants, EVE predicted variant pathogenicity to the same accuracy as functional experiments (equal area under the curve of 0.97; [92]). Retrospective analysis using the functional analyses previously reported for *PALB2* and *RAD51C* [91, 93], we found that EVE was not an accurate predictor of HRD (Supplemental Fig. 1). More studies may be required to produce the most accurate methods and thresholding to use with EVE predictions, as we have seen with REVEL. However, these results are consistent with VCEP specification criteria that excludes PALB2 *in silico* analysis. Although these predictive tools are currently considered to be a type of supportive evidence at their baseline weight, a recent study from the Clinical Genome Resource (ClinGen) SVI Working Group have determined that predictive evidence can be considered up to pathogenic strong or benign very strong depending on the tool and the score assigned. For example, REVEL scores above 0.932 can be considered pathogenic very strong, whereas intermediate scores can range between benign strong and pathogenic moderate (BS, BM, BP, PPS, PM) and scores below 0.003 are benign very strong [65]. The inconsistency in which these different *in silico* predictive tools correlate with HRD in multiple large variant analysis assays suggests that the machine learning approaches may have limited utility as a preliminarily informing clinicians and scientists. Ultimately, functional analysis of individual variants is key for determining pathogenicity.

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4.4. Functional assays for variant screening

The specific functions and properties of *BRCA1, BRCA2, PALB2, RAD51C* and *RAD51D* have been exploited to examine and determine the pathogenicity of potential disease-causing variants. VUS that alter or disrupt native protein activity can be identified through functional testing (Fig. 4). Functional evidence is ascribed a strong baseline weight (PS3, BS3) by ACMG guidelines (Fig. 3) and requires stringent validation to meet this evidence metric, including 11 known pathogenic and benign variant controls. Traditionally, wild-type genes and knockout models have been utilized as the positive and negative controls for function, but with this new evidence metric and the increasing number of classified variants, more groups are turning to standardized variant controls [94-96]. In the absence of these controls, functional evidence is downgraded to moderate or supportive evidence [64]. The continuous addition of functional evidence, contributing to the identification of functional domains, can also be considered moderate or supportive functional evidence for future variant classification (PP2; Fig. 3).

DNA repair foci and localization: *BRCA2, PALB2, RAD51C* and *RAD51D* are required for RAD51 filament formation, which can be indirectly assayed through fluorescence microscopy by the accumulation of RAD51 into distinct puncta, or foci, following DNA damage (Fig. 4A. Taking advantage of this phenomenon, a new method called Repair CAPacity, or RECAP, was developed to determine HRD directly in patient tissue samples by analyzing RAD51 foci formation following tumor irradiation [97]. Similarly, RAD51 foci experiments have also been performed in *BRCA1, BRCA2* and *RAD51C* organoid models of patient-derived tumor samples, which maintain the mutational signatures of the original tumor [98]. Recruitment of these proteins to RAD51 foci and other DNA damage sites, visualized by γH2AX foci, or into the nucleus, where DNA repair occurs, can also be examined to assess protein function (Fig. 4A).

HR reporter assays and HR gene knockout systems: HR efficacy can be directly assessed through fluorescence-based reporter assays in human cells (Fig. 4B). For example, in the direct repeat recombination (DR-GFP) assay an endonuclease-induced DSB is introduced in an inactive *GFP* sequence that is repaired to an active *GFP* sequence using a homologous donor template on the same chromosome (pictured in Fig. 4B) [99]. Analogously, sister chromatid recombination (SCR) assays use a homologous template on the opposite sister chromatid as the repair template instead of a homologous donor on the same sister chromatid [100]. Lastly, in the CRISPR Clover-LMNA/Ruby-LMNA HDR assays, a donor plasmid containing fluorescently-tagged LMNA is used as the template to repair a CRISPR/Cas9 created DSB in the genomic sequence of *LMNA* [101]. In each of these methods, fluorescence is used as a readout for HR proficiency which can be easily assessed by fluorescence activated cell sorting (FACS) or microscopy-based approaches (Fig. 4B). In the largest screen of *PALB2* variants to date, 84 VUS and 7 truncation *PALB2* variants were screened for homologous recombination efficacy utilizing the DR-GFP reporter assay in B400 *Palb2*$^{-/-}$ *Trp53*–/– mouse mammary tumor cell line [93]. The effect of the *PALB2* variants on HRD in human U2OS cells was confirmed using a CRISPR Clover-LMNA reporter assay, where the endogenous *PALB2* was knocked down by siRNA and the variant *PALB2* was transiently expressed. This screen resulted in the identification

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of four HRD *PALB2* variants [93]. While this study and others utilized mouse knockout cell lines for their functional screening, Chinese hamster ovary cells (CHO) and DT40 chicken cell lines have been used extensively for variant analysis since many of the HR factors are non-essential in these models, including *RAD51C* and *RAD51D* [33, 102-106]. In addition, human cell line knockout models for *BRCA2, RAD51C* and *RAD51D* are also available in both inducible and non-inducible cell lines [91, 96, 107-109]. While these HR assays can be extremely useful when a variant is either clearly proficient or deficient for HR, more intermediate results of partial HR function are difficult to interpret. Therefore, analysis of other protein characteristics like protein-protein interaction capabilities, DNA damage sensitivity or cell cycle effects may be required to determine whether intermediate HR variants function normally or abnormally.

HR can also be directly assessed through yeast recombination experiments, which express the mammalian proteins of interest (Fig. 4B). Using this approach, VUS in *BRCA1* that were predicted to be pathogenic using *in silico* approaches were analyzed for gene reversion events using a budding yeast reporter assay [110]. Using this method, variants can usually be screened much faster than in mammalian cell-based assays. Furthermore, due to the high level of conservation between the yeast system and the mammalian HR pathway, the genetic dependencies on specific HR factors, such as *MRE11* or *XRS2*, can be assessed [111]. On the other hand, while the yeast system can be used to preliminarily screen many variants, yeast do not have *BRCA1, BRCA2, RAD51C*, or *RAD51D* homologs and so the applicability of the findings in yeast to humans requires additional experimentation.

Replication fork protection and dynamics.—As unrepaired DNA can halt cell growth, accurate BRCA1, BRCA2, PALB2, and RAD51C protein function are required for unhindered cell cycle progression. Non-functional protein variants can produce a cell cycle block, causing an accumulation of cells at the G2/M checkpoint [33, 96, 112, 113]. In addition, these HR proteins have a secondary function during replication, which can be assessed through DNA fiber spreading experiments which enables the observation of replication fork stalling, restart, regression, protection and collapse (Fig 4.C) [33]. In these assays, cells are treated with a DNA damaging agent and then pulsed with thymidine analogs CldU and IdU to create the fluorescent DNA tracks that can be visualized by fluorescence microscopy (example in Fig. 4C). The lengths of these DNA tracks elude to the fate of the replication fork. For example, when examining *BRCA2* VUS, dysfunctional variants were less efficient in fork protection, exhibited by an 11-24% reduction in the IdU/CldU length ratio when compared to that of the wild-type *BRCA2* [59]. In some cases, a disease-causing variant can uncouple HR from its role in replication protection. For example, a FA patient with a *RAD51* variant was previously identified as replication fork deficient while HR proficient [81, 114], suggesting that other functions should be taken into consideration when examining the potential pathogenicity of a particular variant. Furthermore, recent studies have suggested that the replication-associated functions are critical for PARP inhibitor sensitivity, as it has been argued that the ssDNA gaps that accumulate upon PARP inhibition, and are the predominant mechanism of cell death for these HRD tumors [109].

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DNA damage sensitivity and cell viability.—Assays which measure sensitivity to DNA damaging agents have been used for decades in the discovery of important DNA repair proteins. PGVs, having disrupted DNA repair capabilities, show increased sensitivity to DNA damaging agents such as ionizing radiation and cisplatin (Fig. 4D). This approach was used to analyze 74 *BRCA1* VUS for sensitivity to chemotherapeutics cisplatin and the PARP inhibitor, Olaparib [96]. Surprisingly, a subset of the *BRCA1* variants that are considered HRD are insensitive to these therapeutic agents. These results suggest that either partial HR function may not be enough to predict drug sensitivity or that other functions, outside of HR, may also be important for therapeutic response.

Embryonic lethality experiments are performed as a way of determining the essential requirement of HR genes [115-119]. This experiment can also be performed in the presence of variants, where embryonic lethality equates to non-functional variants (Fig. 4D). Saturation genome editing (SGE) is a new tool used for variant analysis that takes advantage of this essential requirement to simultaneously functionally screen thousands of possible variants [120]. SGE is a CRISPR/Cas9 based method where variants are created in HAP1 cells, which require efficient HR for viability (Fig. 4D). Cells are continuously collected over days and their gDNA and mRNA are sequenced. In this population data, non-functional variants will quickly die off, while functionally WT variants will continue to replicate. In a study screening *BRCA1* single nucleotide variants, SGE was used to simultaneously analyze 3,893 variants across 13 exons [121]. Here, 72.5% of *BRCA1* variants were labeled as functional, 21.1% were non-functional, and 6.4% were considered intermediate. In ClinVar, 169 of these *BRCA1* variants are labeled as pathogenic. Of these 162/169 were in agreeance of the non-functional label, 2/169 were oppositely labeled functional and 5/169 were labeled intermediate (likely pathogenic). Overall, this method showed a sensitivity of 96.7% and a specificity of 98.2% [121]. Using this data, a recent publication determined that, in addition to publicly available frequency and predictive data, the SGE results from this study could reclassify 49 VUS to likely benign and 5 VUS to likely pathogenic, making it a viable option for VUS screening in the future [122].

Protein-protein interactions.—Variants that result in the loss of essential protein-protein interactions can have a drastic effect on protein function (Fig. 4E). A recent study used the yeast-hybrid system to screen 56 *RAD51C* variants for their interaction capabilities with native binding partners RAD51B, RAD51D and XRCC3 as part of the BCDX2 and CX3 complexes [91]. In this system the *GAL4* transcription factor is split into the DNA binding and activating domains (BD and AD, respectively). The BD is fused to the end of one interacting partner, while the AD is fused to the other. If protein binding is unimpaired, these two proteins come together to reconstitute the *GAL4* transcription factor which stimulates transcription of a reporter gene essential for yeast growth (Example in Fig. 4E). Therefore, interaction efficiency can be measured by the extent of yeast growth. In this study, the binding interaction of the RAD51C variant with RAD51D was predictive of HR proficiency [91]. A similar experiment can also be performed in mammalian cells where GAL4 transcription factor reconstitution allows for expression of a reporter protein like luciferase. This approach has previously been used to analyze PALB2 VUS interaction with BRCA2 [93].

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Other functions.—In addition to these overarching functional assays, *BRCA1* and *BRCA2* have other functions that can be quickly assessed for variant screening. For example, BRCA1 has important E3 ubiquitin ligase functions that can be examined by observing the ubiquitylation status of its substrates (Fig. 4F)[123, 124]. On the other hand, dysfunctional BRCA1 and BRCA2 have been observed to increase centrosome production (Fig. 4F)[125, 126]. Therefore, using microscopy, centrosome number can be used as an appropriate read out for variant *BRCA2* function [127, 128].

5. The Effects of Variant Classification

Knowing disease risk and variant functionality can expand available options for both preventative care and post-diagnosis targeted treatment. Generally, patients at high risk of breast and ovarian cancer (family history, personal history, etc.) should start routine disease monitoring early in life and may choose to undergo double mastectomy or oophorectomy to significantly lower their cancer risk. These surgeries can have life-altering effects on patients, such as inducing an early menopause. Therefore, not all high-risk women choose this option and prefer extra surveillance for early cancer detection [129]. Research shows that the decision to have children or have risk-reducing surgery in BRCA positive patients is significantly associated with age ($p < 0.001$) and those at reproductive age described a more negative self-concept and higher vulnerability ratings ($p < 0.01$) [130]. Many individuals report increased stress and anxiety around genetic testing that significantly decreases with time from screening [131]. These stress levels are reportedly similar among patients of all outcomes (positive, negative uninformative or unclassified variant), but are increased when there is a significant discrepancy in their perceived risk [132]. Having clear and accurate genetic counseling available to these patients may aid in quelling these negative emotions.

In the event that cancer does develop, knowing the variant effect on the tumor is helpful when designing an effective treatment plan. Variants known to contribute to HRD in the tumor can be targeted through several precision medicine techniques. PARP inhibitors, including Olaparib, Niraparib, Rucaparib and Talazoparib, are approved by the FDA for treatment of breast and/or ovarian cancer [133]. They are thought to work by blocking other DNA repair pathways, leading to the accumulation of toxic intermediates that result in the generation of DSBs. Recently, it was suggested that the accumulation of ssDNA gaps, and not DSBs, results in PARP inhibitor sensitivity [109, 134]. *BRCA1*-deficient cells treated with the PARP inhibitor, Olaparib, produce less nascent DNA at replication forks and show an increase in ssDNA gaps due to low levels of XRCC1. These gaps are left during replication between Okazaki fragments and are usually filled by XRCC1 in conjunction with ligase 3. Depletion of *53BP1* restored the gap-filling functions in *BRCA1* KO cells and thus conferred PARPi resistance [109]. HRD tumors are also particularly sensitive to DNA polymerase theta or REV1 inhibition, which similarly block competing DNA repair pathways used to repair toxic DSBs. In particular, DNA polymerase Theta inhibitors sensitize *BRCA1, BRCA2, PALB2* and *RAD51C* KO or depleted cells to standard chemotherapy [135-139]. These small molecule inhibitors are currently in clinical trials as a monotherapy, or in combination with Talazoparib for treatment of metastatic and advanced solid tumors and in combination with Niraparib to treat breast cancer.

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While targeted treatment of HRD variants has improved outcomes in patients, relapse can occur to due reversion mutations and have been clinically observed in each of the RAD51 regulators (*BRCA1, BRCA2, PALB2, RAD51C* and *RAD51D*) [108, 140-142]. In this case, variants that create frame shifts and large deletions or early termination codons are reverted to restore expression of the full-length protein and prevent further PARPi sensitivity. It has been suggested that not all variants are equally revertible and that missense variants that occur in essential motifs are less likely to revert because a reversion to the exact, original amino acid is needed to restore protein function [143]. Overall, understanding the biological impact of a variant can have major psychological and lifestyle effects on a patient but can also help improve disease surveillance, and provide more tailored treatment options.

6. Conclusions and Future Directions

Recent advances in DNA sequencing and in functional analysis of variants has greatly improved in the last five years. As more and more variants are being uncovered, the functional impact of these variants on cancer and therapeutic response needs to be thoroughly investigated. One important area to consider is the need for reliable high throughput functional studies to keep up with the speed at which new variants are being uncovered. These functional studies are agnostic to race and ethnicity, unlike many other lines of evidence which are skewed towards observed variants or more frequently tested populations leading to bias.

With the swift identification of the more obvious HRD variants by these high throughput systems, we will soon be left with the conundrum of hypomorphic variant classification. Therefore, having universally defined gene-specific thresholding in functional studies to indicate non-functional variants would be helpful in identifying variants that potentially compound disease risk. For example, if a variant exhibits a 50% reduction in HR, does this indicate an increased cancer susceptibility in an individual and if so, how is this cancer risk increased upon environmental exposures? Further exploring the possible hypomorphic nature of FA may aid us in setting an appropriate cut off for functional abnormalities like HRD. Toward this end, it would also be helpful to understand how HRD status relates to chemotherapeutic sensitivity. When HR is disrupted enough to confer sensitivity to HRD-targeted therapies? A current obstacle in the HBOC variant classification field is the conflation of pathogenicity and HRD status. To date, there is no official HRD delineation in variant classification, and as a result, HRD status is included under pathogenicity. Although pathogenic and likely pathogenic variants are often HRD, there are many HRD variants that have yet to be officially classified due to a lack of pathogenicity evidence. Knowing HRD status is critical for cancer patients seeking the most effective therapeutic interventions. This begs the question whether HRD status should be reviewed separately from pathogenicity. This way, HRD status does not necessarily affect risk assessment and preventative care decision making for patients carrying an unclassified variant and clinicians can still be assured that they are making the best therapeutic decisions for their breast and ovarian cancer patients. One could argue that the idea of BRCA-ness covers this missing identifier. Yet, this term is usually reserved for actual patient tumors, not specific variants. BRCA-ness refers to a tumor phenotype that mimics the effects of pathogenic BRCA1 or BRCA2 variants tumors. This includes the specific mutational signature, signature 3, the suppression

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of specific HR genes like ATM, ATR and the FA family of proteins and sensitivity to PARP inhibitors [144]. This means that, although an HRD RAD51C variant may possibly be found in a high BRCA-ness scoring tumor, the BRCA-ness marker cannot be assigned to the variant itself. Thus, we suggest that an official HRD scoring system be applied to HBOC related gene variants to ensure accurate and effective treatment of HRD VUS.

Unfortunately, access to state-of-the-art sequencing platforms is not universally available. In March 2023, the Pennsylvania Senate unanimously passed Bill 8 which provides no cost breast MRI, ultrasound to individuals at high-risk of developing breast cancer and genetic testing, and genetic counseling for Pennsylvanians who carry or are expected to carry *BRCA1* or *BRCA2* variants. The lowering of these financial barriers to make genetic testing more accessible will no doubt result in the discovery of more VUS for which characterization will be have to be analyzed. Similarly, All of Us, an NIH sequencing program, has sequenced over 400,000 Americans in order to identify variants in diverse populations across the United States. As these are the first of their kind, many bills and programs are expected to follow. Particularly programs in underdeveloped nations, which, as of current, are an untapped source of disease variants and would benefit from variant driven treatment. Together, this underscores the necessity for fast and effective variant analysis and classification in the present time. By combining high-throughput variant functional analysis with increased patient data using specific variant criteria will enable reclassification of VUS to provide the best clinical guidance and patient outcomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Dr. Kristie Darrah, Dr. Sarah Hengel and Dr. Marcy Richardson for their helpful comments. This work is supported by the National Institutes of Health (R01 ES031796 to K.A.B. and F31 CA264889-01A1 to H.L.R.) and the Department of Defense (BC201356 to K.A.B.).

Abbreviations

ACMG	American College of Medical Genetics
BA	benign stand alone
BP	benign supporting
BS	benign strong
COSMIC	Catalogue of Somatic Mutations in Cancer
ClinGen	Clinical Genome Resource
DR-GFP	direct repeat GFP recombination
DSB	double-strand break

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ENIGMA	Evidence-based Network for Interpretation of Germline Mutant Alleles consortium
EVE	Evolutionary model of Variant Effect
HBOC	hereditary breast and ovarian cancer syndromes
HR	homologous recombination
HRD	homologous recombination deficient
LOH	loss of heterozygosity
PGV	pathogenic germline variants
PM	pathogenic moderate
PolyPhen	Polymorphism Phenotyping
PP	pathogenic supporting
PROVEAN	Protein Variation Effect Analyzer
PS	pathogenic strong
PVS	pathogenic very strong
RECAP	Repair CAPacity
REVEL	Rare Exome Variant Ensemble Learner
RPA	replication protein A
SCR	sister chromatid recombination
SGE	Saturation genome editing
SIFT	Sorting Intolerant from Tolerant
ssDNA	single-stranded DNA
SVI	Sequence Variant Interpretation group
TCGA	The Cancer Genome Atlas
VCEP	Variant Curation Expert Panel
VUS	variant of uncertain significance

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Highlights

- Variants in RAD51 regulators, BRCA1/2, RAD51C/D, PALB2 and breast/ovarian cancer
- Guidelines for variant classification of homologous recombination genes in cancer
- Use of population, co-segregation and functional data in variant re-classification
- Re-classification of variants of unknown significance will improve patient care

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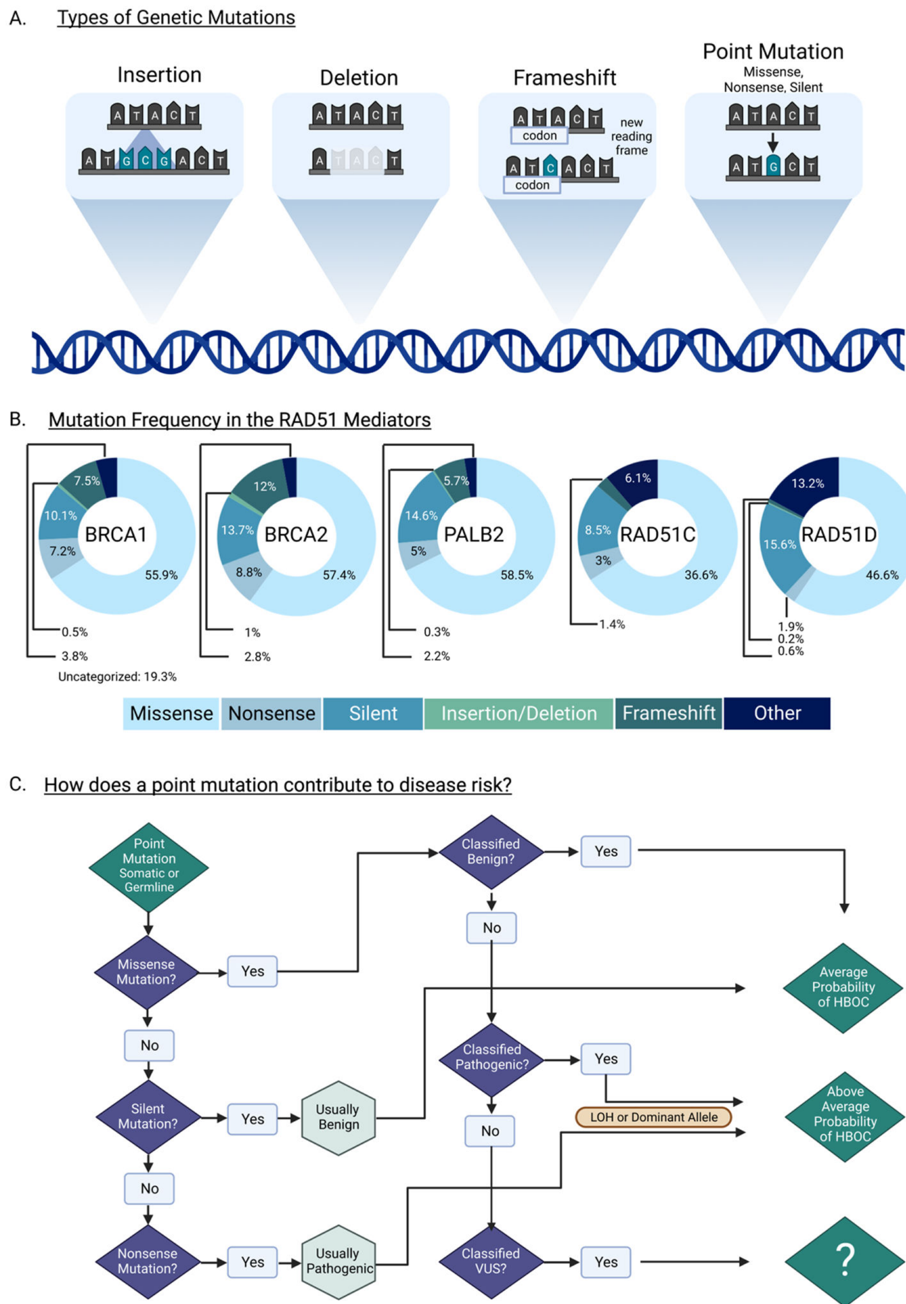

Fig. 1. Types of genetic alterations in the RAD51 regulators and a classification schematic

(A) Simplified types of genetic alterations including insertions, deletions, frameshifts and point mutations. The inserted or mutated residues are in blue and deleted residues in light grey. (B) Mutation distribution represented as a pie chart in the HR genes; *BRCA1, BRCA2, PALB2, RAD51C, RAD51D* reported in COSMIC [69]. Missense mutations (sky blue), nonsense mutations (grey/blue), silent point mutations (medium blue) along with in frame insertion/deletion mutations (light green) and frameshift mutations (dark green). Mutations within the “other” category (navy) include promoter mutations, mutations in intronic regions and combinations of mutations. Percentages indicated are the number of samples where

the mutation type is present divided by total number of samples analyzed. In some cases, the total is not 100% due to the addition of multiple uncategorized mutations and multiple mutations found in a single sample. (C) The flow chart determines disease risk contribution of five types of point mutations; silent, nonsense, missense benign, missense pathogenic and missense VUS. The prior four mutations have a known contribution to disease risk, while it is unknown how missense VUS contribute to disease risk. Created with BioRender.com

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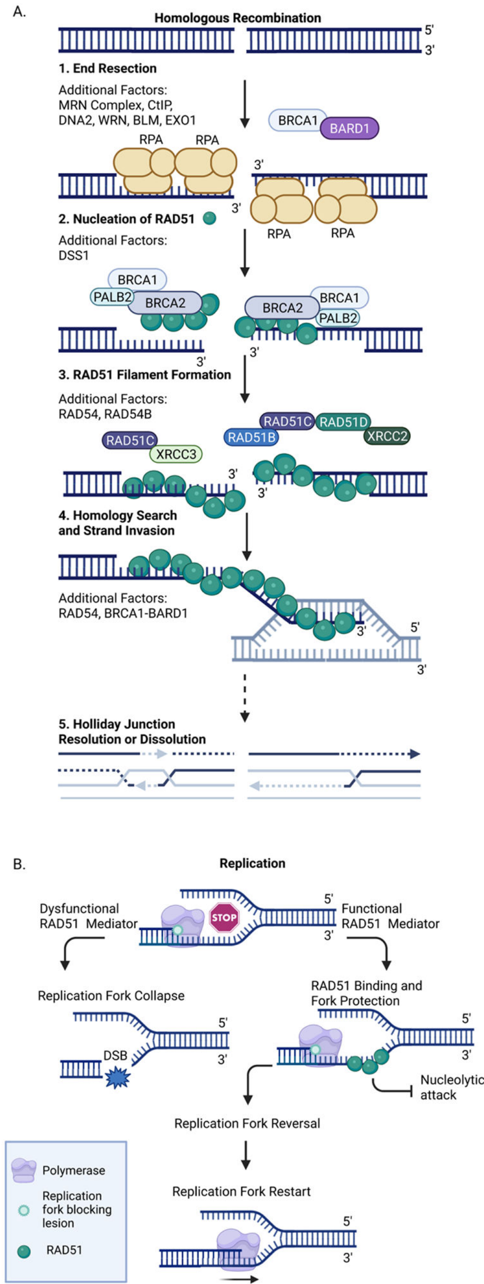

Fig. 2. The roles of the RAD51 regulators during HR and replication

(A1-5) The steps of homologous recombination are shown from the perspective of BRCA1 and the RAD51 mediator proteins BRCA2, PALB2, RAD51C and RAD51D. Additional proteins required for each step are listed on the left of the arrows.

1. After a DSB occurs, the DNA ends are resected through the activities of MRN complex, CtIP, DNA2, WRN, BLM, EXO1 with BRCA1 (blue oval) and BARD1 (purple oval). The ssDNA is coated with the ssDNA binding complex RPA, which is a heterotrimer (beige ovals)

2. BRCA2 (blue oval) with BRCA1 and PALB2 (light teal oval) bring RAD51 to ssDNA and enable RAD51 filament nucleation (green circles).

3. RAD51 presynaptic filament formation is promoted by the CX3 complex (RAD51C (navy oval) and XRCC3 (light green oval) and the BCDX2 complex (RAD51B (blue oval); RAD51C (navy oval); RAD51D (green oval); XRCC2 (dark green oval). Additional factors include RAD54, RAD54B etc.
4. RAD51 filaments perform the homology search and strand invasion steps of a homologous sequence (light blue DNA; a sister chromatid or a homologous chromosome) with the assistance of RAD54, BRCA1-BARD1, among others.
5. The catentated DNA Holliday junctions are either resolved or dissoluted by many additional factors including helicases and topoisomerases (not shown).
(B) The effects of functional and non-functional RAD51 mediator proteins during replication. When DNA polymerase (purple) is halted by a fork blocking lesion (green starburst), the RAD51 regulators are required for fork protection and replication restart (right side). RAD51 binding to the ssDNA gap protects the fork from nucleolytic attack and enables replication fork reversal that promotes replication fork restart. Dysfunction of the RAD51 mediator proteins results in nucleolytic degradation and replication fork collapse. Created with BioRender.com

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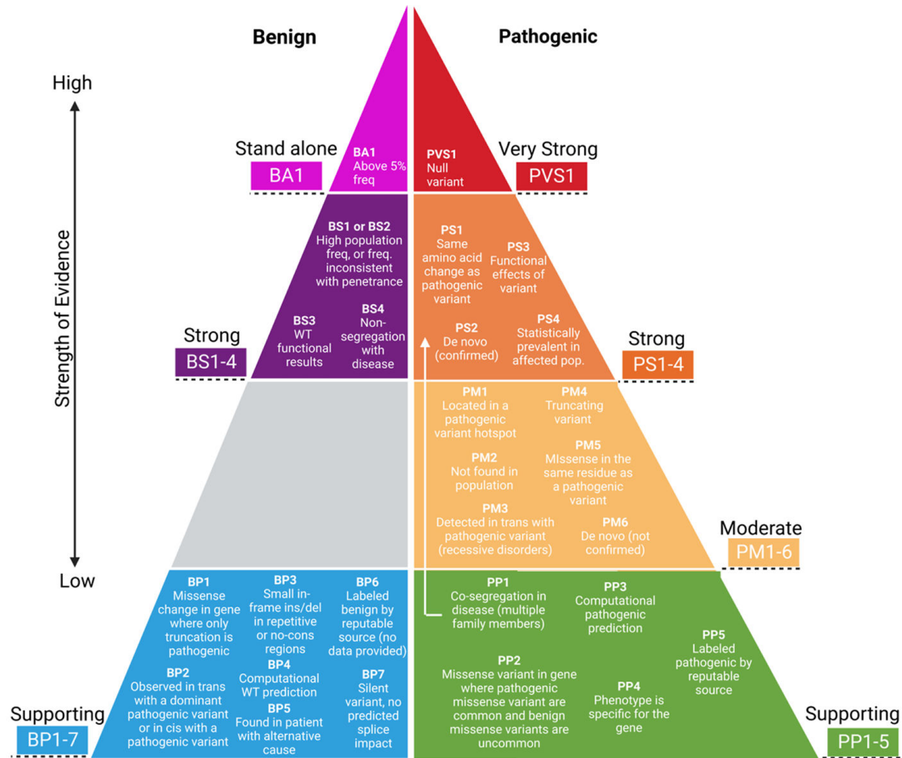
A. Strength and Type of Available Evidence



B. Evidence Combination for Classification

Likely Benign =

1. BS1-4 BP1-7 OR
2. BP1-7 BP1-7

Benign =

1. BA1 OR
2. BS1-4 BS1-4

Likely Pathogenic =

1. PVS1 PM1-6 OR
2. PS1-4 PM1-6 OR
3. PS1-4 PP1-5 OR
4. PM1-6 PM1-6 PM1-6 OR
5. PM1-6 PM1-6 PP1-5 PP1-5 OR
6. PM1-6 PP1-5 PP1-5 PP1-5 PP1-5

Pathogenic =

1. PVS1 PS1-4 OR
2. PVS1 PM1-6 PM1-6 OR
3. PVS1 PM1-6 PP1-5 OR
4. PVS1 PP1-5 PP1-5 OR
5. PS1-4 PS1-4 OR
6. PS1-4 PM1-6 PM1-6 PM1-6 OR
7. PS1-4 PM1-6 PM1-6 PP1-5 PP1-5 OR
8. PS1-4 PM1-6 PP1-5 PP1-5 PP1-5 PP1-5

Fig. 3. Variant classification pyramid for benign and pathogenicity and evidence combination for classification key

Adapted from Richards et al. [63] (A) Evidence for variant classification is organized into three benign groups (left side of triangle) and four pathogenic groups (right side of triangle), with evidence decreasing in strength down to the bottom of the pyramid. Each evidence grouping is given a specific label (i.e. BA, BS, BP, PVS, PS, PM and PP) and each type of evidence in the grouping is given a numbered label as well (1-7). PP1 evidence classification is shown as supporting but this base classification can increase to moderate or strong with increasing segregation data (white arrow). The described variant classification pyramid is based off of the general ACMG guidelines. Variant classification pyramids should be edited to fit VCEP criteria for specific genes.

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(B) Evidence combinations needed for variant classification as likely benign, benign, likely pathogenic and pathogenic classification are listed. Each colored box represents a piece of evidence from the specified category in (A). The number of times a specific box is listed represents different pieces of evidence required under the same category. For example, there are two ways for a variant to be classified as likely benign; 1) one piece of evidence from BS1-3 and one from BP1-7 (i.e. BS3 WT functional results and BP4 computational WT prediction) or two pieces of evidence from BP1-7 (i.e. BP4 computational WT prediction and BP5 found in patients with alternative cause). Created with BioRender.com

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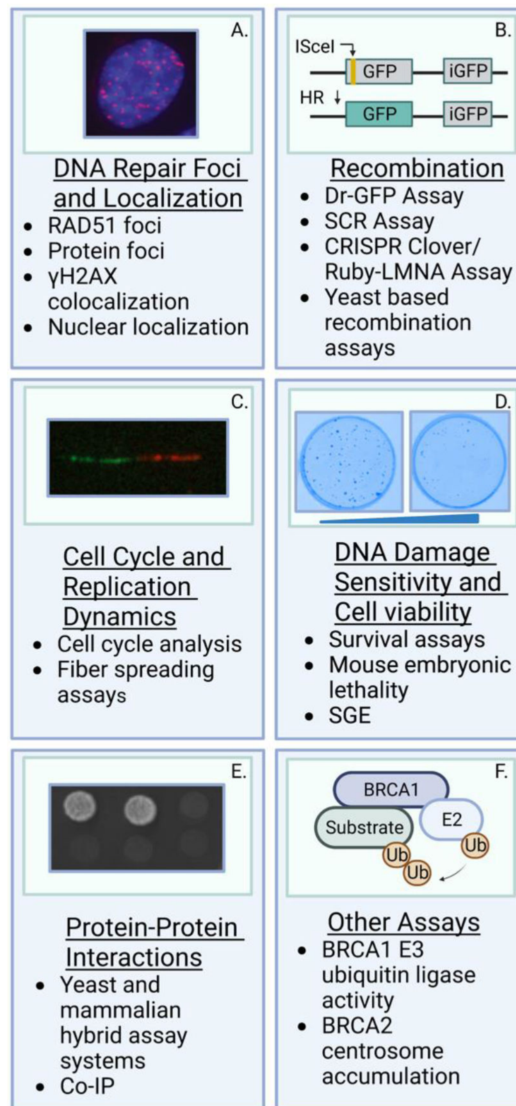

Fig. 4. Functional assays for variant classification.

Functional Assays can be utilized to examine how variants change the activities of the RAD51 mediator proteins. These activities include (A) formation of DNA repair foci (including RAD51 and the protein of interest), colocalization with gamma-H2AX (γH2AX; shown with DAPI colocalization) and nuclear localization), (B) recombination capabilities, which can be measured using the DR-GFP assay (shown), SCR assay, CRISPR Clover/Ruby-LMNA Assay, and yeast-based recombination assays, (C) perturbed cell cycle progression and replication which can be measured by cell cycle analysis and fiber spreading assays (shown), (D) contribution to cell viability and sensitivity to DNA damaging agents, measured by survival assays (plates shown), early mouse development studies, and SGE, (E) protein-protein interaction capabilities as measured by yeast and mammalian hybrid assay systems (yeast shown) and co-immunoprecipitation (co-IP), *in vitro* pull downs (F) and other non-recombination or replication-based functions like the E3 ubiquitin ligase

activity of BRCA1, examined by western blotting and centrosome accumulation in the absence of functional *BRCA2*, visualized by microscopy. Created with BioRender.com

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