Functional characterization of BRCA2 variants to improve cancer risk assessment

Mesman R.L.S.

1

Introduction and outline of the thesis

Introduction

Breast cancer

Breast cancer is the most commonly diagnosed cancer type in women and continues to be a major cause of cancer-related deaths. The average risk of a woman developing breast cancer sometime in her life is approximately 13%, this means that 1 in 8 women will be diagnosed with breast cancer at some point during their lifetime (https://www.cancer.org/, accessed at 12-2019). Breast cancer is a heterogeneous disease in terms of molecular variations, cellular background, sensitivity to different treatments and clinical prognosis. Four main molecular subtypes have been identified based on the presence or absence of hormone estrogen (ER) or progesterone (PR) receptors and excess levels of human epidermal growth factor receptor 2 (HER2, a growth-promoting protein) and/or extra copies of the *HER2* gene. According to these features, breast cancer can be classified as luminal A (ER/PR+, HER2-), luminal B (ER/PR+, HER2+), HER2 type (ER/PR-, HER2+), or triple negative (ER/PR-, HER2-)[1-3]. Luminal A tumours are associated with the most favourable prognosis as they are considered as slow-growing and less aggressive than other subtypes. In addition, this subtype shows more responsiveness to anti-hormone therapy. HER2-enriched cancers tend to grow and spread more aggressively than other subtypes and are associated with poorer short-term prognosis compared to hormone receptor positive breast cancers. The majority (about 75%) of triple negative breast cancers (TNBC) fall in to the basal-like subtype defined by gene expression profiling, which are usually associated with poor short-term prognosis. Currently, there are no targeted therapies for triple negative tumours as they fail to respond to hormonal therapy (such as tamoxifen or aromatase inhibitors) or therapies that target HER2 receptors, such as Herceptin.

Breast cancer risk factors

Age is the major risk factor for breast cancer, but also lifestyle factors and genetic factors are involved in the aetiology of breast cancer. A majority of risk factors can be understood in light of their potential effects on a woman's lifetime exposure to the ovarian hormones estradiol and progesterone [4]. Endocrine-related factors include age at menarche and menopause, pregnancy and lactation history, whereby early pregnancy and breastfeeding have proven to decrease the risk of breast cancer.

Behavioural factors that are modifiable include obesity, physical inactivity, use of combined estrogen and progestin menopausal hormones and alcohol consumption [5,6].

Genetic factors

Breast cancer risk is shown to increase in women with a positive family history. If first-degree relatives are affected, the breast cancer risk increases almost two folds [7]. Although most cases of breast cancer are sporadic, it is estimated that in about 15% of all breast cancers heritable factors are involved [8]. These incidences are termed "familial breast cancer" and include all cancer cases that are likely caused by a combination of genetic and environmental risk factors. If pathogenic germline variants are identified in known breast cancer susceptibility genes the associated cancer risk

can be passed down in the family from parent to child. If this is the case, the term "hereditary breast cancer" is used. Hereditary breast cancer accounts for a small but significant proportion (up to 10%) of breast cancer cases [9]. Affected families are generally characterized by multiple breast cancer and/or ovarian cancer cases in first-, second- and third- degree relatives at young age of onset (age <50years). Over the years multiple risk alleles have been identified and together they explain about 50% of the hereditary risk. These risk alleles can be divided into three groups, i.e., high-, moderate- and low- penetrance genes.

High penetrance genes

BRCA1 and *BRCA2* are the most commonly mutated genes in families with enhanced breast and ovarian cancer risk and they explain about 15-20% of all hereditary breast cancers (www.cancer.gov, accessed at 12-2019). Autosomal dominant inheritance of pathogenic variants in *BRCA1* and *BRCA2* confers an average cumulative risk by age 70 years between 55-60% to develop breast cancer along with increased ovarian cancer risk up to 59% [10,11]. Besides predisposition to breast and ovarian cancer, pathogenic variants in *BRCA2* can also increase the risk to develop other cancer types including pancreatic cancer and prostate cancer[12,13]. Recently, *PALB2* has also been reported as a high penetrance gene. In approximately 2-5% of the familial breast cancer cases a female carries a pathogenic variant in *PALB2* which confers a similar risk for breast cancer as pathogenic variants in *BRCA2* [14].

Mutations in the high penetrance genes *TP53*, *PTEN*, STK11 and CHD1, which cause specific cancer syndromes, are exceedingly rare, and probably account for less than 0.1% of breast cancers [15,16].

Moderate penetrance genes

Mutational screening of genes functionally related to BRCA1 and/or BRCA2 has identified several other genes conferring an intermediate risk of breast cancer including; *CHEK2, ATM, BRIP1, NBS1* [17].

Low penetrance susceptibility alleles

Large genome-wide association studies have discovered multiple common breast cancer susceptibility alleles. So far, these studies have identified about 313 single-nucleotide polymorphisms (SNPs) explaining 20% of breast cancer polygenic variance. Although the individual risk of these low-penetrance breast cancer susceptibility loci is small their combined effect, summarized in polygenic risk scores (PRSs), can be substantial [18-20]. For this reason, ongoing efforts are focussed on the validation of PRSs as reliable (sub-type specific) predictors of breast cancer risk and the potential of combining PRSs and other known risk factors for risk stratification and breast cancer prevention programs [21-26].

Functional characterization of BRCA1 and BRCA2

Since the identification of *BRCA1* in 1994 and *BRCA2* in 1995 [27,28], many studies have been published that improved our knowledge about the diverse functional roles

attributed to the BRCA1/2 proteins. Insight in the mechanistic features is crucial for understanding how variants might affect the tumour suppressive activity. Both proteins contain a transcriptional-activation domain, a large exon 11 and they both show binding to RAD51, however the two proteins comprise limited overall sequence homology to each other. BRCA1 and BRCA2 have been implicated in a plethora of protein interactions and biological functions and are considered as custodians of the structural and numerical integrity of chromosomes. The proteins work in concert to repair DNA double strand breaks (DSBs) by a high-fidelity repair pathway called homologous recombination (HR) and DNA crosslinks by the Fanconi Anemia pathway. Additionally, the proteins are suggested to be involved in cell cycle checkpoint activation, R-loop metabolism, chromatin remodelling, protection and stabilization of stalled replication forks and centrosome regulation.

BRCA1

Human *BRCA1* encodes a protein of 220kDA (1863aa) with a multidomain structure. The N-terminal situated RING domain, encoded by exons 2-7 , interacts with BARD1 to form a heterodimer which supports their mutual stability and nuclear retention [29-31]. Although the BRCA1-BARD1 complex has proven to be important for homology directed repair (HDR), the physiological role of its ubiquitin E3 ligase activity need to be further defined. Intriguingly, the enzymatic activity of BRCA1 does not seem to be required for cell viability, HDR, chromosomal stability, senescence induction, centrosome duplication, spindle formation, or resistance to genotoxic stress in mouse embryonic stem (mES) cells [32] and tumour suppression in a mouse model [33]. Nevertheless, the ubiquitin ligase activity of BRCA1-BARD1 counteracts chromatin barriers to enable DNA resection and facilitates repositioning of 53BP1 on damaged chromatin and is therefore considered as a critical regulatory step in DNA repair [34,35].

The BRCA1 RING domain is essential for tumour suppression and several germline variants within the RING finger domain of BRCA1 have been linked to development of breast and ovarian cancers. However, mutant BRCA1 proteins with a dysfunctional RING domain retain hypomorphic activity which may render tumours resistant to PARP inhibitors and platinum drugs [35-37].

The central region (aa 225-1453, exons 11-13), contains a DNA binding domain (aa 421-701) that preferably binds to splayed-arm shaped DNA under DNA damaging conditions [38].This central DNA binding domain has a critical role for genomic integrity and is suggested to be involved in the stabilization of replication forks and activation of the intra-S-phase checkpoint by modulating CHK1 phosphorylation. In addition, deletion of exon 11 of the *BRCA1* gene leads to a defect in G2–M checkpoint activation and centrosome amplification [39]. A recent paper revealed a novel attribute of the BRCA1–BARD1 complex in the repair of DNA DSBs via HDR [40]. In this particular DNA repair pathway BRCA1–BARD1 facilitates the nucleolytic resection of DNA ends by antagonizing 53BP1 to generate a single stranded template for the recruitment of another tumour suppressor complex namely PALB2-BRCA2. The data in this paper showed another mechanistic role of BRCA1-BARD1 in HDR, downstream of BRCA2

mediated RAD51 loading at resected DNA ends, whereby it promotes assembly of the synaptic RAD51 complex and subsequently support the DNA strand invasion step during D-loop formation. Exon 11 of *BRCA1* encodes the RAD51 interaction domain (aa 758-1064) involved in synaptic RAD51 complex-mediated DNA pairing.

The C-terminal region of BRCA1 is acidic and encompasses a highly conserved coiled-coil (cc) motif, a serine cluster domain (SCD) that contains ATM phosphorylation sites and two tandem BRCT repeats. The cc motif directly binds to a similar region in PALB2 which enables complex formation with BRCA2 in the HDR pathway [41,42].

The BRCT repeats interact with at least three proteins, Abraxas, a scaffold protein [43], BRIP1, a DNA helicase [44], and CtIP, an endonuclease involved in resection [45]. Mutation of the BRCT domain causes a major defect for nuclear accumulation of BRCA1, supporting a key role of the BRCT domain for BRCA1 nuclear entry [46].

Former studies showed that functional domains involved in HDR are confined to the RING domain, the cc motif and the BRCT repeats.

BRCA2

BRCA2 is a multi-faceted protein of about 384kDa encoded by 26 exons (Figure 1). Human BRCA2 is generally divided into three regions: the N-terminus; the BRC repeat region containing also one nuclear export signal (NES); and the C-terminal region containing the nuclear localization signals (NLS), another NES, the DNA binding domain (DBD) and an additional RAD51 binding domain. The N-terminus of BRCA2 (aa residues 10–40) interacts with PALB2 [47], which is required to form a complex with BRCA1 during the DNA damage response. The formation of the BRCA1-BRCA2-PALB2 repair complex is tightly regulated and suppressed during the G1 cell cycle phase by ubiquitylation of the BRCA1 interaction site of PALB2 [48]. PALB2 co-localizes with BRCA2 in nuclear foci, promotes its localization and stability and facilitates its role in HDR, replication fork protection and checkpoint functions [49]. The interaction between BRCA2 and PALB2 is relevant for tumour suppression and cancer-associated variants affecting two amino acids, namely Gly25 (changed to Arg, Gly25Arg) and Trp31 (changed to Cys or Arg, Trp31Cys and Trp31Arg, respectively) were found to disrupt the interaction with PALB2 [47].

Besides PALB2, the N-terminal region of BRCA2 can also bind to Plk1 in a cell cycle dependent manner. CDK1 and CDK2 phosphorylate the BRCA2 N-terminal region at T77 during G2/M which in turn phosphorylates RAD51 within the BRCA2 complex [50]. *In vivo* experiments revealed the importance of this molecular mechanism for the association of RAD51 with stalled replication forks and maintenance of genome stability. More recently, a research group identified Thr207 as a *bona fide* docking site for PLK1 and essential for proper alignment of the chromosomes at the metaphase plate[51] (BioRXiv Ehlén et al.,2019). Via protein secondary structure prediction tools researchers identified a zinc finger PARP like domain in the N terminus (aa 250–500) of BRCA2 that exhibits DNA binding activity and stimulates RAD51-mediated HDR. This N-terminal situated DBD is

suggested to take over the role of the DBD in the C-terminal region of BRCA2 when this part of the protein is missing [52].

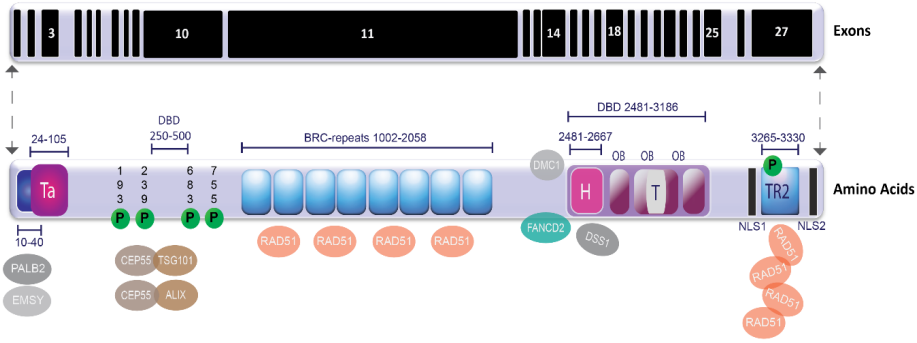

Figure 1. Schematic representation of BRCA2 domains.
Ta, transcriptional activation domain; P, phosphorylation site; H, helical domain; DBD, DNA binding domain; OB, oligonucleotide binding fold; T, Tower domain; TR2, C-terminal RAD51 binding site; NLS, nuclear localization signal.

The central region of the protein is integral to its role in RAD51 delivery on resected DNA substrates, the stimulation of RAD51 filament formation and subsequent replacement of RPA on the ssDNA lattice thereby catalysing the invasion and search for homology within a donor DNA template. Exon 11 encodes for eight BRC repeats of ~35 amino acids each whereby BRC motifs 1-4 primarily bind free RAD51 and prevent RAD51 from binding dsDNA. In contrast, BRC repeats 5-8 exhibit none of these activities but were found to bind and stabilize the RAD51-ssDNA nucleoprotein filament [53]. Although several residues within each BRC motif are highly conserved, the linear amino acid distances between the repeats vary considerably and are only moderately conserved between mammalian species.

The BRCA2 DBD at the C-terminal end of the protein contains a helical domain (H), three oligonucleotide binding (OB) folds and a tower domain (T), which may facilitate BRCA2 binding to both single-stranded DNA and double-stranded DNA. The small peptide DSS1 associates with OB1 and an adjoining helical region in BRCA2 thereby masking a NES. For this reason, DSS1 binding is essential to prevent mis-localization of BRCA2 to the cytoplasm. The pathogenic missense variant Asp2723His, located in OB1, underlines the importance of masked nuclear export signals to control the nuclear retention of both BRCA2 and RAD51 molecules [54]. In addition, the BRCA2-DSS1 complex enables physical interaction with RPA wherein DSS1 acts as a DNA mimic to attenuate the affinity of RPA for ssDNA and subsequently promote the assembly of the RAD51 presynaptic filament [55]. The DBD of BRCA2 can also bind to PAR chains to mediate the rapid recruitment of BRCA2 to DNA lesions, suggesting a BRCA1-and RAD51-independent function of BRCA2 in HDR [56]. The C-terminal RAD51 binding site of BRCA2 (aa 3265-3330) shares no homology

with the BRC repeats and RAD51 binding by this domain is regulated in a cell-cycle dependent manner. Phosphorylation at Ser3291 by CDKs takes place in G2/M transition of the cell cycle and abrogates the interaction with RAD51 filaments, which in turn blocks HDR. Mutations in the Ser3291 phosphorylation site do not compromise HDR in the context of the full length protein. However, RAD51 binding by the C-terminus (TR2 domain) has been implicated in the protection of nascent DNA strands at stalled replication forks. Exon 27 is essential to prevent nascent DNA of stalled replication forks from MRE11-mediated degradation and enables fork restart by positioning MRE11 for stabilization of the replisome (which is independent of its nuclease activity). Deletion of exon 27, which includes phosphorylation site Ser3291, causes defects in response to both stalled and collapsed replication forks[57]. These findings suggest that the BRCA2 C-terminal interaction with RAD51 is essential for protection of stalled replication forks but is dispensable for the repair of DSB by HDR. The last 156 amino acids of BRCA2 (1707-1863) contain two nuclear localization signals (NLS) that govern its localization to the nucleus.

Important BRCA-related tumour suppressor functions

Homology Directed Repair

The most prominent tumour suppressor function of the BRCA proteins is their essential role in HDR, an evolutionarily conserved pathway that coordinates high-fidelity repair of DSBs. Double-strand breaks are the most deleterious form of DNA damage since they can lead to cell death if left unrepaired or chromosomal aberrations and subsequently tumour development when repaired by error prone pathways. To counteract the deleterious effects of DSBs, cells have evolved multiple DSB repair pathways including classical nonhomologous end-joining (c-NHEJ), alternative or microhomology-mediated end-joining (MMEJ) and HDR [58,59]. HDR operates with slower kinetics compared to NHEJ and is executed primarily during the late S and G2 phases of the cell cycle to exploit the intact sister chromatid as a template for error-free repair, while NHEJ results in more mutagenic repair outcomes throughout interphase. MMEJ is also intrinsically mutagenic and can introduce templated insertions and small deletions when joining chromosome break ends[60,61]. Whereas NHEJ and MMEJ involve limited processing of DNA ends for ligation, HDR requires the resection of the DSB to generate a 3′-ssDNA overhang that can be used by the recombination machinery to search for homology [62]. These DNA tails become coated with RPA, which is subsequently displaced by RAD51 to form a nucleoprotein complex termed the presynaptic filament. The presynaptic filament searches for, engages, and then invades a homologous duplex target to form a displacement loop (D-loop) to prime DNA synthesis and complete repair [63]. The mechanism prohibiting HDR in G1 minimally consists of the suppression of DNA-end resection coupled with a multistep block of the recruitment of BRCA2 to DNA damage sites that involves the inhibition of BRCA1–PALB2–BRCA2 complex assembly [48].

Protection of stalled replication forks and the repair of replication-associated breaks

The most common DSBs in cells are thought to be replication-associated breaks as a consequence of collapsed replication forks [64]. During DNA replication, progression of replication forks can be hampered by certain sequence and structural properties including DNA lesions (base damages, single strand DNA breaks, interstrand cross-links), secondary structures (G-quadruplex and R-loops), repetitive elements and nucleotide pool depletion [65]. Depending on the nature and the persistence of the replication stress, stabilized forks can either restart or collapse. To promote restarting of the fork, nascent strands need to undergo limited resection. This process is strictly regulated because both inhibition and over-activation of nuclease activity is detrimental for fork restart. Proteins as PTIP and PARP1 are suggested to be involved in the recruitment of the two key nucleases MRE11 and DNA2 on different types of stalled forks depending on their structures and the moment at which they appear [66]. Nascent DNA strands at stalled forks need to be stabilized and protected from excessive resection and the current model envisions a crucial role for BRCA2 in this process. BRCA2 interacts with monomeric RAD51 through its BRC repeats and stabilizes RAD51 filaments via its TR2 domain [67]. In addition, BRCA2 facilitates RAD51 loading at stalled forks by PLK1 phosphorylation. Interestingly, stalled fork protection does not depend on the ability of BRCA2 to interact with DNA, suggesting that the main role of BRCA2 at stalled replication forks is to load and stabilize polymerized RAD51[67]. The role for BRCA1-BARD1 in fork protection is an active area of investigation and is thought to involve its known function in BRCA2 recruitment to DNA. However, a recent study describes an additional process to promote fork protection independently of the canonical BRCA1–PALB2–BRCA2–RAD51 pathway [68,69]. In this newly described pathway, PIN1 induces a conformational change in BRCA1 which in turn leads to increased recruitment of RAD51 to stalled replication forks and protection of the nascent DNA strand. Restart of a replication fork implies a high degree of coordination between recombination and replication functions. It has become clear that recombination is important not only for repairing DNA lesions but also for re-initiating the replication process. HDR at stalled forks is regulated differently from HDR at DSBs arising independently of a fork. Stalled replication forks may be processed by sister chromatid recombination (SCR), generating error-free or error-prone homologous recombination outcomes [70]. Both the HDR-dependent and HDR-independent functions of BRCA1 and BRCA2 seem to be important to ensure genome integrity.

BRCA-mutated tumours

Tumours harbouring loss-of-function variants in *BRCA1* or *BRCA2* have a deficiency in the repair of DNA DSBs by HDR. Consequently, these tumours are characterized by specific genomic scars (mutational signature). The mutational signature in these tumours is known in cancer genomics as 'Signature 3' [71]. There is emerging evidence that also sporadic tumours can have a BRCAness phenotype due to a defective HDR pathway. The mutational landscape of these tumours may be of clinical relevance as they expose specific therapeutic vulnerabilities.

The therapeutic strategy of PARP-inhibition is a relatively novel approach to exploit the absence of efficient HDR in tumours. Poly (ADP-ribose) polymerases (PARPs) are a family of enzymes that play an important role in various cellular processes, such as replication, recombination, chromatin remodelling, and DNA repair. Due to inactivation of complementary DNA repair pathways by the inhibition of PARylation or trapping of PARP1 on damaged DNA, there is selective killing of HDR-deficient cells [72-75].

The identification of BRCAness tumours can therefore have significant implications for treatment decisions.

Genetic counseling

Genetic testing to identify pathogenic variants in the high-penetrance breast cancer susceptibility genes *BRCA1, BRCA2* and *PALB2* and moderate-penetrance genes *CHEK2* and *ATM* is currently offered for patients with clinically presumed hereditary breast and/or ovarian cancer.

In about 7% of the genetic tests a pathogenic variant in *BRCA1* or *BRCA2* is found and carriers are offered intensive screening programs and/or prophylactic surgeries (www.oncoline.nl accessed December 2019). Moreover, patients with *BRCA1*- and *BRCA2*-related cancer (both germline and somatic) and, expectedly *PALB2*-related cancer, may benefit from dedicated therapy regimes, such as PARP inhibitors as mentioned above. However, up to 20% of tests will report a variant of uncertain clinical significance (VUS) [76-78]. Due to the lack of sufficient clinical or functional evidence, carriers of VUS are counselled in a similar way as carriers of benign variants or tested individuals in which no variant was found. Worldwide, in over 10.000 families, carriers of VUS are identified which cannot take advantage of the risk assessment, prevention, and therapeutic measures that are available to carriers of known deleterious variants. Women in which a VUS is identified experience considerable psychological distress, not only due to the possibility that they may have a cancer risk as high as that for known pathogenic variants, but also due to the uncertainty of this cancer risk [79,80].

Variants of uncertain significance in *BRCA1* and *BRCA2*

Most pathogenic variants are sequence alterations for which the deleterious impact on protein function can be readily deduced from the genetic test result. Frame-shifting insertions/deletions and nonsense substitutions are protein truncating variants and are therefore classified as pathogenic. Variants located in the canonical splice donor/acceptor sites, conserved 5' or 3' intron sequences at intron-exon boundaries, are also considered as pathogenic/likely pathogenic due to their high probability to interfere with mRNA splicing.

Besides typical pathogenic variants that truncate the protein, genetic testing identifies an increasing number of variants for which the impact on protein function is unknown. These so called "variants of uncertain clinical significance" (VUS) are mostly missense or silent variants, intronic variants or small in-frame insertion and deletions.

Variant classification

In order to make informed clinical decisions regarding surveillance and risk reducing surgery a 5-tier classification system has been proposed in which each class is associated with specific recommendations for clinical management [81]. In the current multifactorial likelihood model (MLM), a prior probability of pathogenicity is combined with likelihood ratios (LRs) estimated from clinical data resulting in a final posterior probability that assigns the variant to one of the five classes [78,81]. Class 1/2 variants have very low posterior probabilities (<0.05) for pathogenicity and are probably benign, whereas class 4/5 variants have very high posterior probabilities (>0.95) for being associated with cancer risk equivalent to classical pathogenic variants that encode a truncated protein. By definition, VUS are classified as class 3.

The prior probability is an *in silico* prediction of the functional impact based on evolutionary conservation across species and bioinformatic predictions of variant effect on protein sequence or mRNA splicing [82,83]. Many of these algorithms are not very accurate and tend to 'over predict' missense changes as pathogenic [84,85]. Moreover, due to the high prior probability assigned to nonsense (0.99) and splice site (0.97) variants the prior heavily impacts the final classification of these variants.

The LRs for pathogenicity are estimated from clinical data, such as co-segregation with disease, co-occurrence with a pathogenic variant in the same gene, reported family history, breast tumour pathology, and more recently, case-control data [86-92].

The accuracy of the MLM relies heavily on the amount of clinical data, which is scarce for the majority of variants because most of them are very rare. Consequently, there are often insufficient clinical data to make clinically meaningful inferences about their associated cancer risks and therefore it has been difficult to move a VUS into either class 1/2 or class 4/5 on the basis of clinical data. To address this problem and support classification of VUS, biological assays have been developed that can measure the impact of a variant on mRNA splicing and BRCA-related tumour suppressor functions [93-100]. Information on protein function can serve as independent classifiers of VUS.

Functional assays for the characterization of *BRCA2* VUS

A "well-established" functional assay can provide strong evidence for the classification of variants according to gene-specific variant classification guidelines by ENIGMA (https://enigmaconsortium.org/) as well as the generic guidelines published by the American College of Medical Genetics (ACMG) and Association for Molecular Pathology (AMP) [101] (http://dx.doi.org/10.1101/709428 Brnich et al.,2019). A well-established assay is an assay that have been validated using previously classified pathogenic and benign variants and shown to be reproducible and robust. Ideally, the functional analysis is performed in a stable cell line that can be modified genetically in the lab and cultured for several weeks. The cell line must allow stable and efficient expression of a human variant of interest.

For this reason, most functional assays to study *BRCA2* variants are performed in Brca2 deficient V-C8 hamster cells or mouse embryonic stem cells (mESC) with a conditional knockout allele of *mBrca2* using either cDNA constructs [102,103] or bacterial artificial chromosomes (BACs) carrying the complete gene to express variants [104,105]. In the latter approach, sequence alterations are introduced in the full-length human gene which allows the assessment of all types of variants including those that affect mRNA splicing. Many intronic *BRCA2* variants have been identified that affect mRNA splicing by either disrupting canonical splice sites or activating/creating a cryptic splice site. In addition, 20-25% of all reported exonic variants are predicted to affect mRNA splicing because they modify splicing regulatory elements, such as exonic splicing enhancers or silencers (ESE and ESS)[106-109]. Although spliceogenic variants can successfully be identified by *in silico* tools and RNA analysis in minigene assays or lymphocytes from variants carriers, these methods fall short in their ability to determine to what extent the aberrant splicing events affect protein function and predispose to cancer development. This underscores the need for a more sophisticated assay whereby the expression of alternative transcripts, either naturally occurring or induced by a variant can be linked to protein function. The mESC-based functional assay is eminently suited for this purpose due to the presence of only a single human *BRCA2* allele. For this reason, our research project was focused on the optimization and validation of this particular model system.

Application of functional data in variant classification

Another import aspect is the applicability of functional data for clinical variant interpretation. The assay readout should model pathogenesis and therefore provide insight into the effect of a variant on the tumour suppressor activity of the protein. As HDR is thought to be the most prominent tumour suppressor function of BRCA2, the non-crossover gene conversion assay is a commonly used method to study the impact of variants on protein function [110,111]. The assay is based on homology-directed repair of an I-SceI-induced DSB in a green fluorescent protein (DR-GFP) reporter construct. The DR-GFP construct consists of two mutated GFP genes, SceGFP, which is disrupted by insertion of the 18-bp recognition site for the I-SceI endonuclease, and iGFP, which is truncated at both the 5' and 3' ends. I-SceI cleavage of SceGFP followed by HDR results in a functional GFP gene of which the constitutive expression can be measured by flow cytometry. Quantitation of the proportion of GFP positive cells serves as a measure for HDR activity. This functional readout has successfully been used, in combination with Align-GVGD prediction results, in a Bayesian hierarchical model (VarCall) to estimate the overall probability of pathogenicity for *BRCA2* VUS [84].

Besides HDR, BRCA2 has been suggested to be involved in additional genome maintenance processes like centrosome amplification, cell cycle checkpoint regulation and replication fork stabilization which might be also important for tumour suppression [112,113]. A centrosome amplification assay, DNA fiber assay, nuclear localization assay and protein–protein interaction-based assays are developed to study cellular processes other than the repair of DSBs. It remains yet to be determined if these assays are required for the functional characterization of VUS to ensure the identification of all

variants associated with increased cancer risk. So far, there is little known about the existence of high-risk separation-of-function variants that have no effect on HDR.

Aim of this thesis

This thesis is aimed at improving the classification of variants in *BRCA2* by providing information on the functional impact of a variant and by establishing a quantitative relationship between residual tumour suppressor activity and cancer risk. To this end we optimized and validated a mESC-based functional assay suited to test all types of *BRCA2* variants, including those that affect mRNA splicing.

By improving the interpretation of genetic test-results in terms of risk estimation, this project will allow women to make more informed choices with respect to their cancer risk category (low, moderate, high). This will contribute to cancer prevention among those at increased risk, as well as reduced anxiety and avoidance of unnecessary treatments among those at low risk (better quality of life). Moreover, patients with BRCA1/2-related cancer (both germline and somatic) and in the near future PALB2 may be subjected to a dedicated therapy regime that specifically targets HDR deficient tumours, such as PARP inhibitors.

Outline of the thesis

Offering genetic testing to identify pathogenic variants in individuals with clinically presumed hereditary breast and/or ovarian cancer is currently routine clinical practice. In case a pathogenic variant is identified, carriers might benefit from risk reducing measures as intensified screening programmes or prophylactic surgery. Pathogenic variants associated with high cancer risk typically disrupt protein function via the introduction of a premature stop codon due to a nonsense mutation or via frame shifting insertions/deletions. However, for many of the variants identified by genetic testing it is uncertain if the function of the variant protein is impaired to such an extent that cancer risk is enhanced. Those variants are therefore classified as variants of uncertain significance (VUS) and they represent a major challenge for genetic counselling and clinical management of the families in which they are identified. Most VUS are rare and insufficient information can be mined to compute a reliable cancer risk estimation, leaving large numbers of families in uncertainty. Driven by a clear clinical need to classify variants in relevant cancer risk categories (i.e. high, moderate and population level), biological assays are developed which allow functional characterization of VUS.

In **Chapter 2** we validated a mESC-based model system that allows efficient generation and reliable functional characterization of *BRCA2* variants. This was done by evaluating its ability to correctly discriminate between (likely) pathogenic (class 4/5) and (likely) benign (class 1/2) missense variants that have been classified on basis of genetic and clinical

data. Furthermore, we determined the functional impact of VUS identified in the clinic. The mESC assay is based on the ability of human *BRCA2* variants to complement the loss of endogenous mouse *Brca2* (*mBrca2*) and the subsequent quantification of their ability to perform crucial BRCA2 tumour suppressor functions such as HDR. Based on the data presented in chapter 2, variants with an HDR level higher than 50% can be classified as non-high risk variants (class 1/2), whereas high-risk variants (class 4/5) are either unable to rescue the lethal cell phenotype of *mBrca2* loss or display an HDR level below 30% of WT BRCA2. Most VUS (29 out of 43) showed functional complementation in the range of non-high risk variants and ten variants revealed a severe impact on protein function comparable with high-risk missense variants. Additionally, we identified variants with hypomorphic BRCA2 activity. More extensive research is required to specify the relationship between residual HDR activity and breast cancer risk. For this purpose a large case-control study was performed in which more than 42.000 breast cancer cases and controls were genotyped. In this study, included in **Chapter 3** of the thesis**,** we provide evidence that hypomorphic variants with an intermediate effect on HDR activity (30-50% of WT), confer clinically relevant, moderately increased risks of breast cancer.

In **Chapter 4** we used the mESC-based functional assay to characterize spliceogenic *BRCA2* variants in exon 12. Presumed loss of function (LoF) variants i.e. nonsense variants and variants in the canonical donor and acceptor sequences, induced increased expression levels of the naturally occurring Δ(E12) isoform and only partially affected BRCA2 functionality. The true null effect of nonsense variants was evaded by in-frame skipping of the functionally redundant exon 12. In the resulting alternative mRNA transcripts the premature termination codon is missing which prevents degradation of transcripts by the nonsense mediated decay pathway. These data provide unprecedented evidence for a cancer predisposition gene that variant-induced alternative splicing can rescue tumour suppressor activity of presumed LoF variants in *BRCA2.* To elaborate on this potential rescue mechanism, we systematically characterized a large panel of alternative transcripts for their ability to encode for (partial) functional BRCA2 protein and assessed presumed LoF variants throughout the *BRCA2* gene. The results from this study, included in **Chapter 5** of this thesis, show that complete loss of BRCA2 function may be prevented through sufficient expression of wild type transcript by the variant allele or through the expression of in-frame alternative *BRCA2* transcripts encoding for (partial) functional protein. The study reveals the functional consequences of variant-related aberrant splicing events and provides a framework for accurate clinical application of the current classification guidelines for presumed LoF variants.

Finally, the findings described in this thesis are discussed in **Chapter 6** .

References

1. Perou, C.M. *et al.* Molecular portraits of human breast tumours. *Nature* **406**, 747-52 (2000).
2. Sorlie, T. *et al.* Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci U S A* **98**, 10869-74 (2001).
3. Sotiriou, C. *et al.* Breast cancer classification and prognosis based on gene expression profiles from a population-based study. *Proc Natl Acad Sci U S A* **100**, 10393-8 (2003).
4. Bernstein, L. Epidemiology of endocrine-related risk factors for breast cancer. *J Mammary Gland Biol Neoplasia* **7**, 3-15 (2002).
5. Tamimi, R.M. *et al.* Population Attributable Risk of Modifiable and Nonmodifiable Breast Cancer Risk Factors in Postmenopausal Breast Cancer. *Am J Epidemiol* **184**, 884-893 (2016).
6. McPherson, K., Steel, C.M. & Dixon, J.M. ABC of breast diseases. Breast cancer-epidemiology, risk factors, and genetics. *BMJ* **321**, 624-8 (2000).
7. Colditz, G.A., Kaphingst, K.A., Hankinson, S.E. & Rosner, B. Family history and risk of breast cancer: nurses' health study. *Breast Cancer Res Treat* **133**, 1097-104 (2012).
8. Kraus, C. *et al.* Gene panel sequencing in familial breast/ovarian cancer patients identifies multiple novel mutations also in genes others than BRCA1/2. *Int J Cancer* **140**, 95-102 (2017).
9. Prakash, R., Zhang, Y., Feng, W. & Jasin, M. Homologous recombination and human health: the roles of BRCA1, BRCA2, and associated proteins. *Cold Spring Harb Perspect Biol* **7**, a016600 (2015).
10. Antoniou, A.C. *et al.* The BOADICEA model of genetic susceptibility to breast and ovarian cancers: updates and extensions. *Br J Cancer* **98**, 1457-66 (2008).
11. Mavaddat, N. *et al.* Cancer risks for BRCA1 and BRCA2 mutation carriers: results from prospective analysis of EMBRACE. *J Natl Cancer Inst* **105**, 812-22 (2013).
12. Easton, D.F. *et al.* Cancer risks in two large breast cancer families linked to BRCA2 on chromosome 13q12-13. *Am J Hum Genet* **61**, 120-8 (1997).
13. van Asperen, C.J. *et al.* Cancer risks in BRCA2 families: estimates for sites other than breast and ovary. *J Med Genet* **42**, 711-9 (2005).
14. Antoniou, A.C., Foulkes, W.D. & Tischkowitz, M. Breast-cancer risk in families with mutations in PALB2. *N Engl J Med* **371**, 1651-2 (2014).
15. Hilbers, F.S., Vreeswijk, M.P., van Asperen, C.J. & Devilee, P. The impact of next generation sequencing on the analysis of breast cancer susceptibility: a role for extremely rare genetic variation? *Clin Genet* **84**, 407-14 (2013).
16. Turnbull, C. & Rahman, N. Genetic predisposition to breast cancer: past, present, and future. *Annu Rev Genomics Hum Genet* **9**, 321-45 (2008).
17. Couch, F.J. *et al.* Associations Between Cancer Predisposition Testing Panel Genes and Breast Cancer. *JAMA Oncol* **3**, 1190-1196 (2017).
18. Mavaddat, N. *et al.* Polygenic Risk Scores for Prediction of Breast Cancer and Breast Cancer Subtypes. *Am J Hum Genet* **104**, 21-34 (2019).
19. Michailidou, K. *et al.* Association analysis identifies 65 new breast cancer risk loci.

Nature **551**, 92-94 (2017).
20. Milne, R.L. *et al.* Identification of ten variants associated with risk of estrogen-receptor-negative breast cancer. *Nat Genet* **49**, 1767-1778 (2017).
21. Li, J.J. *et al.* Polygenic Risk, Personality Dimensions, and Adolescent Alcohol Use Problems: A Longitudinal Study. *J Stud Alcohol Drugs* **78**, 442-451 (2017).
22. Li, H. *et al.* Breast cancer risk prediction using a polygenic risk score in the familial setting: a prospective study from the Breast Cancer Family Registry and kConFab. *Genet Med* **19**, 30-35 (2017).
23. Maas, P. *et al.* Breast Cancer Risk From Modifiable and Nonmodifiable Risk Factors Among White Women in the United States. *JAMA Oncol* **2**, 1295-1302 (2016).
24. Garcia-Closas, M., Gunsoy, N.B. & Chatterjee, N. Combined associations of genetic and environmental risk factors: implications for prevention of breast cancer. *J Natl Cancer Inst* **106**(2014).
25. Rudolph, A. *et al.* Joint associations of a polygenic risk score and environmental risk factors for breast cancer in the Breast Cancer Association Consortium. *Int J Epidemiol* **47**, 526-536 (2018).
26. Lakeman, I.M.M., Schmidt, M.K., van Asperen, C.J. & Devilee, P. Breast Cancer Susceptibility—Towards Individualised Risk Prediction. *Current Genetic Medicine Reports* **7**, 124-135 (2019).
27. Miki, Y. *et al.* A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. *Science* **266**, 66-71 (1994).
28. Wooster, R. *et al.* Identification of the breast cancer susceptibility gene BRCA2. *Nature* **378**, 789-92 (1995).
29. Fabbro, M., Rodriguez, J.A., Baer, R. & Henderson, B.R. BARD1 induces BRCA1 intranuclear foci formation by increasing RING-dependent BRCA1 nuclear import and inhibiting BRCA1 nuclear export. *J Biol Chem* **277**, 21315-24 (2002).
30. Hashizume, R. *et al.* The RING heterodimer BRCA1-BARD1 is a ubiquitin ligase inactivated by a breast cancer-derived mutation. *J Biol Chem* **276**, 14537-40 (2001).
31. Ruffner, H., Joazeiro, C.A., Hemmati, D., Hunter, T. & Verma, I.M. Cancer-predisposing mutations within the RING domain of BRCA1: loss of ubiquitin protein ligase activity and protection from radiation hypersensitivity. *Proc Natl Acad Sci U S A* **98**, 5134-9 (2001).
32. Reid, L.J. *et al.* E3 ligase activity of BRCA1 is not essential for mammalian cell viability or homology-directed repair of double-strand DNA breaks. *Proc Natl Acad Sci U S A* **105**, 20876-81 (2008).
33. Shakya, R. *et al.* BRCA1 tumor suppression depends on BRCT phosphoprotein binding, but not its E3 ligase activity. *Science* **334**, 525-8 (2011).
34. Densham, R.M. & Morris, J.R. The BRCA1 Ubiquitin ligase function sets a new trend for remodelling in DNA repair. *Nucleus* **8**, 116-125 (2017).
35. Drost, R. *et al.* BRCA1 RING function is essential for tumor suppression but dispensable for therapy resistance. *Cancer Cell* **20**, 797-809 (2011).
36. Drost, R. *et al.* BRCA1185delAG tumors may acquire therapy resistance through expression of RING-less BRCA1. *J Clin Invest* **126**, 2903-18 (2016).
37. Powell, S.N. BRCA1 loses the ring but lords over resistance. *J Clin Invest* **126**, 2802-

4 (2016).
38. Masuda, T., Xu, X., Dimitriadis, E.K., Lahusen, T. & Deng, C.X. "DNA Binding Region" of BRCA1 Affects Genetic Stability through modulating the Intra-S-Phase Checkpoint. *Int J Biol Sci* **12**, 133-43 (2016).
39. Xu, X. *et al.* Centrosome amplification and a defective G2-M cell cycle checkpoint induce genetic instability in BRCA1 exon 11 isoform-deficient cells. *Mol Cell* **3**, 389-95 (1999).
40. Zhao, W. *et al.* BRCA1-BARD1 promotes RAD51-mediated homologous DNA pairing. *Nature* **550**, 360-365 (2017).
41. Zhang, F., Fan, Q., Ren, K. & Andreassen, P.R. PALB2 functionally connects the breast cancer susceptibility proteins BRCA1 and BRCA2. *Mol Cancer Res* **7**, 1110-8 (2009).
42. Zhang, F. *et al.* PALB2 links BRCA1 and BRCA2 in the DNA-damage response. *Curr Biol* **19**, 524-9 (2009).
43. Wang, B. *et al.* Abraxas and RAP80 form a BRCA1 protein complex required for the DNA damage response. *Science* **316**, 1194-8 (2007).
44. Cantor, S.B. *et al.* BACH1, a novel helicase-like protein, interacts directly with BRCA1 and contributes to its DNA repair function. *Cell* **105**, 149-60 (2001).
45. Sartori, A.A. *et al.* Human CtIP promotes DNA end resection. *Nature* **450**, 509-14 (2007).
46. Anantha, R.W. *et al.* Functional and mutational landscapes of BRCA1 for homology-directed repair and therapy resistance. *Elife* **6**(2017).
47. Xia, B. *et al.* Control of BRCA2 cellular and clinical functions by a nuclear partner, PALB2. *Mol Cell* **22**, 719-29 (2006).
48. Orthwein, A. *et al.* A mechanism for the suppression of homologous recombination in G1 cells. *Nature* **528**, 422-6 (2015).
49. Hartford, S.A. *et al.* Interaction with PALB2 Is Essential for Maintenance of Genomic Integrity by BRCA2. *PLoS Genet* **12**, e1006236 (2016).
50. Yata, K. *et al.* BRCA2 coordinates the activities of cell-cycle kinases to promote genome stability. *Cell Rep* **7**, 1547-1559 (2014).
51. Addis Jones, O., Tiwari, A., Olukoga, T., Herbert, A. & Chan, K.L. PLK1 facilitates chromosome biorientation by suppressing centromere disintegration driven by BLM-mediated unwinding and spindle pulling. *Nat Commun* **10**, 2861 (2019).
52. von Nicolai, C., Ehlen, A., Martin, C., Zhang, X. & Carreira, A. A second DNA binding site in human BRCA2 promotes homologous recombination. *Nat Commun* **7**, 12813 (2016).
53. Chatterjee, G., Jimenez-Sainz, J., Presti, T., Nguyen, T. & Jensen, R.B. Distinct binding of BRCA2 BRC repeats to RAD51 generates differential DNA damage sensitivity. *Nucleic Acids Res* **44**, 5256-70 (2016).
54. Jeyasekharan, A.D. *et al.* A cancer-associated BRCA2 mutation reveals masked nuclear export signals controlling localization. *Nat Struct Mol Biol* **20**, 1191-8 (2013).
55. Zhao, W. *et al.* Promotion of BRCA2-Dependent Homologous Recombination by DSS1 via RPA Targeting and DNA Mimicry. *Mol Cell* **59**, 176-87 (2015).
56. Zhang, F., Shi, J., Bian, C. & Yu, X. Poly(ADP-Ribose) Mediates the BRCA2-

Dependent Early DNA Damage Response. *Cell Rep* **13**, 678-689 (2015).
57. Kim, T.M., Son, M.Y., Dodds, S., Hu, L. & Hasty, P. Deletion of BRCA2 exon 27 causes defects in response to both stalled and collapsed replication forks. *Mutat Res* **766-767**, 66-72 (2014).
58. Mehta, A. & Haber, J.E. Sources of DNA double-strand breaks and models of recombinational DNA repair. *Cold Spring Harb Perspect Biol* **6**, a016428 (2014).
59. Hustedt, N. & Durocher, D. The control of DNA repair by the cell cycle. *Nat Cell Biol* **19**, 1-9 (2016).
60. McVey, M. & Lee, S.E. MMEJ repair of double-strand breaks (director's cut): deleted sequences and alternative endings. *Trends Genet* **24**, 529-38 (2008).
61. Schimmel, J., van Schendel, R., den Dunnen, J.T. & Tijsterman, M. Templated Insertions: A Smoking Gun for Polymerase Theta-Mediated End Joining. *Trends Genet* **35**, 632-644 (2019).
62. Helleday, T., Lo, J., van Gent, D.C. & Engelward, B.P. DNA double-strand break repair: from mechanistic understanding to cancer treatment. *DNA Repair (Amst)* **6**, 923-35 (2007).
63. Morrical, S.W. DNA-pairing and annealing processes in homologous recombination and homology-directed repair. *Cold Spring Harb Perspect Biol* **7**, a016444 (2015).
64. Syeda, A.H., Hawkins, M. & McGlynn, P. Recombination and replication. *Cold Spring Harb Perspect Biol* **6**, a016550 (2014).
65. Zeman, M.K. & Cimprich, K.A. Causes and consequences of replication stress. *Nat Cell Biol* **16**, 2-9 (2014).
66. Liao, H., Ji, F., Helleday, T. & Ying, S. Mechanisms for stalled replication fork stabilization: new targets for synthetic lethality strategies in cancer treatments. *EMBO Rep* **19**(2018).
67. Schlacher, K. *et al.* Double-strand break repair-independent role for BRCA2 in blocking stalled replication fork degradation by MRE11. *Cell* **145**, 529-42 (2011).
68. Daza-Martin, M., Densham, R.M. & Morris, J.R. BRCA1-BARD1: the importance of being in shape. *Mol Cell Oncol* **6**, e1656500 (2019).
69. Daza-Martin, M. *et al.* Isomerization of BRCA1-BARD1 promotes replication fork protection. *Nature* **571**, 521-527 (2019).
70. Kowalczykowski, S.C. Initiation of genetic recombination and recombination-dependent replication. *Trends Biochem Sci* **25**, 156-65 (2000).
71. Polak, P. *et al.* A mutational signature reveals alterations underlying deficient homologous recombination repair in breast cancer. *Nat Genet* **49**, 1476-1486 (2017).
72. Livraghi, L. & Garber, J.E. PARP inhibitors in the management of breast cancer: current data and future prospects. *BMC Med* **13**, 188 (2015).
73. Cerrato, A., Morra, F. & Celetti, A. Use of poly ADP-ribose polymerase [PARP] inhibitors in cancer cells bearing DDR defects: the rationale for their inclusion in the clinic. *J Exp Clin Cancer Res* **35**, 179 (2016).
74. Yuan, Z. *et al.* PARP inhibitors as antitumor agents: a patent update (2013-2015). *Expert Opin Ther Pat* **27**, 363-382 (2017).
75. Keung, M.Y.T., Wu, Y. & Vadgama, J.V. PARP Inhibitors as a Therapeutic Agent for Homologous Recombination Deficiency in Breast Cancers. *J Clin Med* **8**(2019).

76. Eccles, B.K., Copson, E., Maishman, T., Abraham, J.E. & Eccles, D.M. Understanding of BRCA VUS genetic results by breast cancer specialists. *BMC Cancer* **15**, 936 (2015).
77. Eggington, J.M. *et al.* A comprehensive laboratory-based program for classification of variants of uncertain significance in hereditary cancer genes. *Clin Genet* **86**, 229-37 (2014).
78. Lindor, N.M. *et al.* A review of a multifactorial probability-based model for classification of BRCA1 and BRCA2 variants of uncertain significance (VUS). *Hum Mutat* **33**, 8-21 (2012).
79. Vos, J. *et al.* Opening the psychological black box in genetic counseling. The psychological impact of DNA testing is predicted by the counselees' perception, the medical impact by the pathogenic or uninformative BRCA1/2-result. *Psychooncology* **21**, 29-42 (2012).
80. Vos, J. *et al.* The counsellees' view of an unclassified variant in BRCA1/2: recall, interpretation, and impact on life. *Psychooncology* **17**, 822-30 (2008).
81. Plon, S.E. *et al.* Sequence variant classification and reporting: recommendations for improving the interpretation of cancer susceptibility genetic test results. *Hum Mutat* **29**, 1282-91 (2008).
82. Tavtigian, S.V., Greenblatt, M.S., Lesueur, F., Byrnes, G.B. & Group, I.U.G.V.W. In silico analysis of missense substitutions using sequence-alignment based methods. *Hum Mutat* **29**, 1327-36 (2008).
83. Vallee, M.P. *et al.* Adding In Silico Assessment of Potential Splice Aberration to the Integrated Evaluation of BRCA Gene Unclassified Variants. *Hum Mutat* **37**, 627-39 (2016).
84. Guidugli, L. *et al.* Assessment of the Clinical Relevance of BRCA2 Missense Variants by Functional and Computational Approaches. *Am J Hum Genet* (2018).
85. Boonen, R. *et al.* Functional analysis of genetic variants in the high-risk breast cancer susceptibility gene PALB2. *Nat Commun* **10**, 5296 (2019).
86. Spurdle, A.B. *et al.* Clinical classification of BRCA1 and BRCA2 DNA sequence variants: the value of cytokeratin profiles and evolutionary analysis--a report from the kConFab Investigators. *J Clin Oncol* **26**, 1657-63 (2008).
87. Easton, D.F. *et al.* A systematic genetic assessment of 1,433 sequence variants of unknown clinical significance in the BRCA1 and BRCA2 breast cancer-predisposition genes. *Am J Hum Genet* **81**, 873-83 (2007).
88. Spurdle, A.B. *et al.* Refined histopathological predictors of BRCA1 and BRCA2 mutation status: a large-scale analysis of breast cancer characteristics from the BCAC, CIMBA, and ENIGMA consortia. *Breast Cancer Res* **16**, 3419 (2014).
89. Thompson, D., Easton, D.F. & Goldgar, D.E. A full-likelihood method for the evaluation of causality of sequence variants from family data. *Am J Hum Genet* **73**, 652-5 (2003).
90. de la Hoya, M. *et al.* Combined genetic and splicing analysis of BRCA1 c.[594-2A>C; 641A>G] highlights the relevance of naturally occurring in-frame transcripts for developing disease gene variant classification algorithms. *Hum Mol Genet* **25**, 2256-2268 (2016).
91. Goldgar, D.E. *et al.* Integrated evaluation of DNA sequence variants of unknown clinical significance: application to BRCA1 and BRCA2. *Am J Hum Genet* **75**, 535-44

(2004).
92. Tavtigian, S.V., Byrnes, G.B., Goldgar, D.E. & Thomas, A. Classification of rare missense substitutions, using risk surfaces, with genetic- and molecular-epidemiology applications. *Hum Mutat* **29**, 1342-54 (2008).
93. Guidugli, L. *et al.* Functional assays for analysis of variants of uncertain significance in BRCA2. *Hum Mutat* **35**, 151-64 (2014).
94. Toland, A.E. & Andreassen, P.R. DNA repair-related functional assays for the classification of BRCA1 and BRCA2 variants: a critical review and needs assessment. *J Med Genet* **54**, 721-731 (2017).
95. Bonnet, C. *et al.* Screening BRCA1 and BRCA2 unclassified variants for splicing mutations using reverse transcription PCR on patient RNA and an ex vivo assay based on a splicing reporter minigene. *J Med Genet* **45**, 438-46 (2008).
96. Anczukow, O. *et al.* Unclassified variants identified in BRCA1 exon 11: Consequences on splicing. *Genes Chromosomes Cancer* **47**, 418-26 (2008).
97. Thomassen, M. *et al.* Characterization of BRCA1 and BRCA2 splicing variants: a collaborative report by ENIGMA consortium members. *Breast Cancer Res Treat* **132**, 1009-23 (2012).
98. Roy, R., Chun, J. & Powell, S.N. BRCA1 and BRCA2: different roles in a common pathway of genome protection. *Nat Rev Cancer* **12**, 68-78 (2011).
99. Chen, C.C., Feng, W., Lim, P.X., Kass, E.M. & Jasin, M. Homology-Directed Repair and the Role of BRCA1, BRCA2, and Related Proteins in Genome Integrity and Cancer. *Annu Rev Cancer Biol* **2**, 313-336 (2018).
100. Takaoka, M. & Miki, Y. BRCA1 gene: function and deficiency. *Int J Clin Oncol* **23**, 36-44 (2018).
101. Richards, S. *et al.* Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med* **17**, 405-24 (2015).
102. Guidugli, L. *et al.* A classification model for BRCA2 DNA binding domain missense variants based on homology-directed repair activity. *Cancer Res* **73**, 265-75 (2013).
103. Bouwman, P. *et al.* A high-throughput functional complementation assay for classification of BRCA1 missense variants. *Cancer Discov* **3**, 1142-55 (2013).
104. Kuznetsov, S.G., Liu, P. & Sharan, S.K. Mouse embryonic stem cell-based functional assay to evaluate mutations in BRCA2. *Nat Med* **14**, 875-81 (2008).
105. Chang, S., Biswas, K., Martin, B.K., Stauffer, S. & Sharan, S.K. Expression of human BRCA1 variants in mouse ES cells allows functional analysis of BRCA1 mutations. *J Clin Invest* **119**, 3160-71 (2009).
106. Gaildrat, P. *et al.* Multiple sequence variants of BRCA2 exon 7 alter splicing regulation. *J Med Genet* **49**, 609-17 (2012).
107. Ke, S. *et al.* Quantitative evaluation of all hexamers as exonic splicing elements. *Genome Res* **21**, 1360-74 (2011).
108. Lim, K.H., Ferraris, L., Filloux, M.E., Raphael, B.J. & Fairbrother, W.G. Using positional distribution to identify splicing elements and predict pre-mRNA processing defects in human genes. *Proc Natl Acad Sci U S A* **108**, 11093-8 (2011).

109. Sterne-Weiler, T., Howard, J., Mort, M., Cooper, D.N. & Sanford, J.R. Loss of exon identity is a common mechanism of human inherited disease. *Genome Res* **21**, 1563-71 (2011).
110. Kass, E.M. *et al.* Double-strand break repair by homologous recombination in primary mouse somatic cells requires BRCA1 but not the ATM kinase. *Proc Natl Acad Sci U S A* **110**, 5564-9 (2013).
111. Moynahan, M.E., Pierce, A.J. & Jasin, M. BRCA2 is required for homology-directed repair of chromosomal breaks. *Mol Cell* **7**, 263-72 (2001).
112. Fradet-Turcotte, A., Sitz, J., Grapton, D. & Orthwein, A. BRCA2 functions: from DNA repair to replication fork stabilization. *Endocr Relat Cancer* **23**, T1-T17 (2016).
113. Venkitaraman, A.R. Cancer susceptibility and the functions of BRCA1 and BRCA2. *Cell* **108**, 171-82 (2002).