Functional analysis of *BRCA1* variants of uncertain significance (VUS)

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Abbreviations

ACMG	American College of Medical Genetics
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3-related
BACH1	BRCA1 associated C-terminal helicase
BARD1	BRCA1-associated RING domain protein 1
BRCA1	Breast cancer susceptibility gene 1
BRCT	BRCA1 C-terminal
DBD	DNA binding domain
DNA	Deoxyribose nucleic acid
DSB	Double stranded break
ENIGMA	Evidence-Based Network for the Interpretation of Germline Mutant Alleles variants of uncertain significant
EV	Empty vector
GAL4	Galactose-responsive transcription factor
gnomAD	Genome Aggregation Database
H	Hours
HBOC	Hereditary breast and ovarian cancer
HR	Homologous recombination
HUH	Haukeland University Hospital
L	Linker
MAF	Minor Allele Frequency
Min	Minutes
mq	MilliQ
MRN	MRE11-RAD50-NBS1
NES	Nuclear export sequence
NHEJ	Non-homologous end joining
NLS	Nuclear Localisation sequence
ON	Over night
OUH	Oslo University Hospital
PARP	Poly ADP Ribose Polymerase
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PLB	Passive Lysis Buffer
RING	Really Interesting New Gene
RT	Room temperature
SCD	Serine Cluster domain
Sec	Seconds
SSA	Single strand annealing
TA	Transactivation
TUH	Trondheim University Hospital
U	Upstream BRCT
UNN	University Hospital of Northern Norway
V	Voltage
VUS	Variant of uncertain significance
WT	Wild type

Abstract

Breast cancer is the most common form of cancer among women, and 5-10% of breast cancer cases are thought to be caused by hereditary predisposition. Hereditary breast and ovarian cancer syndrome (HBOC) is an inherited disorder, associated with increased risk of early onset breast, ovarian, pancreatic and prostate cancer. One of the main causative genes of HBOC is the tumour suppressor gene *Breast cancer susceptibility gene 1 (BRCA1*).

In genetic diagnostics, genetic alterations, also termed variants, including variants of *BRCA1*, are classified according to the AGMC guidelines, in a five-tier system that ranges from benign (class 1) to pathogenic (class 5). Unfortunately, many variants are classified as variants of uncertain significance (VUS, class 3) due to scarce or conflicting evidence. This is challenging for both patients and clinicians as they cannot be used in clinical decision making, and can lead to stress and anxiety for the carrier. Missense variants are specifically difficult to classify, as a substitution can be tolerable or detrimental, however, the consequence is difficult to predict. The use of genetic testing is increasing, and many new variants are discovered as a result, including missense variants of *BRCA1*. Functional assays are a robust tool to investigate the pathogenicity of a variant. The C-terminal BRCT domain of the BRCA1 protein is involved in transcriptional regulation *in vivo*. And thus, a transactivation assay can be used to examine the effect of missense variants located in the BRCT domain of BRCA1.

In this work, missense variants located in or close to the BRCT domain of BRCA1 were characterised based on a transactivation assay, using a dual-luciferase reporter system. Eleven *BRCA1* VUSs were examined, in addition to one class 4 (likely pathogenic) variant, which was included in order to confirm its pathogenicity. Five of the VUSs served as inter-laboratory controls between medical genetic laboratories in Norway. In summary, our findings expand the knowledge of several *BRCA1* VUS, but further examinations would be required to support the final change of the variant classifications.

1. Introduction

1.1 Cancer

Cancer is one of the leading causes of death worldwide. Although it is a common disease, it is hard to describe due to its complexity. It can however, be described by a set of features that separate cancer cells from healthy cells, commonly known as "hallmarks of cancer" [1]. In 2000, the first six hallmarks of cancer cells were defined, which were self-sufficiency in growth signals, insensitivity to anti-growth signals, ability for tissue invasion and metastasis, limitless replicative potential, sustained angiogenesis and evading apoptosis. Later, additional mechanisms like deregulating cellular metabolism, avoiding immune destruction, nonmutational epigenetic reprogramming, polymorphic microbiomes, genome instability and mutation, tumour-promoting inflammation, unlocking phenotypic plasticity and senescent cells were suggested as hallmarks (or enabling characteristics) of cancer [2, 3]. The hallmarks and enabling characteristics which together facilitate tumorigenesis are summarised in Figure 1.1.

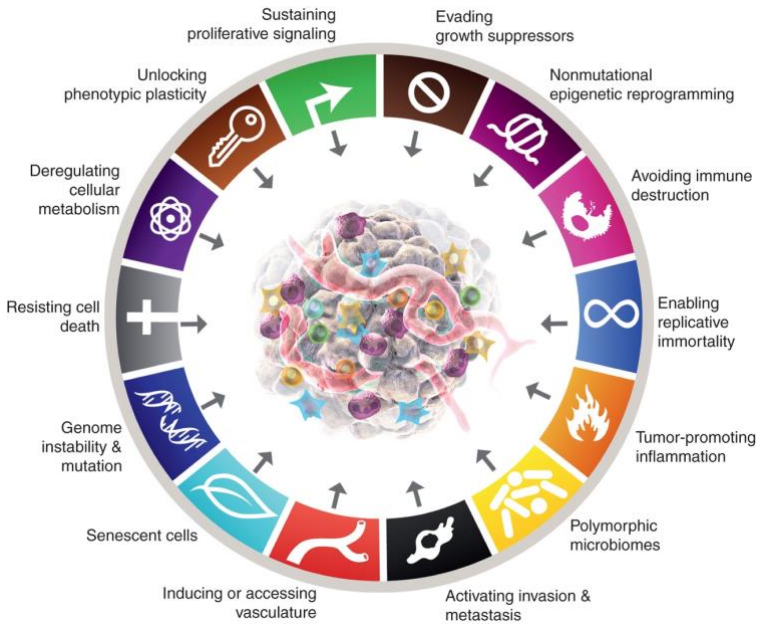

Figure 1.1 The hallmarks of cancer. The figure summaries the 14 mechanisms suggested as hallmarks of cancer (including emerging characteristics) [1-3]. The illustration is adapted from Hanahan [3].

1.2 Breast and ovarian cancer

Female breast cancer is the most commonly diagnosed cancer worldwide, with an estimated 2.3 million new cases in 2020, according to the Global Cancer Statistics 2020 [4]. Among women, breast cancer accounts for 24.5 % of all new cancer cases [4]. In Norway, breast cancer has

been the most common cancer type in women since the establishment of the Cancer Registry of Norway in 1951 [5]. According to the Cancer Registry Report from 2020, one in ten Norwegian women will be diagnosed with breast cancer by the age of 80 [5]. In 2020, there were 3 424 new cases of (female) breast cancer in Norway, and breast cancer accounted for 11.7% of cancer related deaths in women the same year. Although rare, breast cancer also occurs in males, and in 2020, 31 cases of male breast cancer were reported in Norway.

Globally, ovarian cancer accounted for 3.4% of female cancer cases in 2020 [4]. According to the Cancer Registry of Norway, 487 new cases of ovarian cancer were reported in 2020. The incident rate of ovarian cancer is stable in Norway, accounting for 3.0 % of female cancer cases. In Norway, ovarian cancer was the cause of 5.4 % of female cancer related deaths in 2020 [5].

1.3 Oncogenes and tumour suppressor genes

There are two major types of genes that contribute to cancer formation: oncogenes and tumour suppressor genes. A proto-oncogene is the precursor of an oncogene, which can acquire the properties of an oncogene if it is altered. An oncogene is typically altered in a sense that results in an increased amount of the encoded protein, or in increased activity of the protein. A tumour suppressor gene, as the name implies, prevent carcinogenesis through different mechanisms, such as growth inhibition, cell cycle regulation, DNA repair and more [6]. Loss of function in a tumour suppressor gene can thus lead to genome instability and mutation, one of the hallmarks of cancer, and subsequent development of cancer [7]. In most cases, the loss of, or reduced function of, a tumour suppressor gene occurs sporadically, and cause somatic cancer. In contrast, germline alterations affecting tumour suppressor genes can predispose an individual to hereditary cancer.

1.4 Hereditary breast and ovarian cancer

Approximately 5-10% of cancer cases are thought to be hereditary, caused by germline alterations [8]. In most cases, the inheritance pattern is autosomal dominant, which exhibits a 50% chance of passing on the germline variant to the offspring. Hereditary breast and ovarian cancer syndrome (HBOC) is an inherited disorder primarily associated with increased risk of early onset breast and ovarian cancer, and in some cases pancreatic and prostate cancers [8-10]. The main causative genes for autosomal dominant hereditary breast and ovarian cancer are the tumour suppressor genes *Breast cancer susceptibility gene 1* and *2* (*BRCA1* and *BRCA2*) [10].

Both *BRCA1* and *BRCA2* are involved in (among others) mediating homologous recombination (HR) after double stranded breaks (DSBs). Carriers of a disease-causing germline variant of in *BRCA1* have a lifetime risk of 56–75% for developing breast cancer and 36–51% for developing ovarian cancer [11].

1.5 *Breast cancer susceptibility gene 1 (BRCA1)*

1.5.1. The *BRCA1* gene and its protein

In 1990, a linkage study was performed in several families with typical characteristics of early onset breast cancer, which led to the discovery of the later termed *Breast cancer susceptibility gene 1* [12, 13]. *BRCA1* is located on chromosome 17q21.3, and the canonical transcript (NM_007249.3) has 23 exons. The gene encodes a 220 kDa and an 1863 amino acid long nuclear protein product with several functional domains.

The BRCA1 protein is a phosphoprotein primarily located in the nucleus. The investigation of the BRCA1 protein structure is challenging due to its large molecular size and the lack of similarity to any other proteins [14]. A schematic presentation of the BRCA1 protein is shown in Figure 1.2. The Really Interesting New Gene (RING) domain is located in the N-terminus, which contains a RING finger and two flanking helices. The RING finger consists of a central helix and three antiparallel strands forming a β-sheet, which are stabilised by two Zn^{2+} atoms [15]. The RING domain of BRCA1 binds BRCA1 Associated RING Domain protein 1 (BARD1), and the BRCA1-BARD1 complex acts as an E3 ubiquitin ligase, which is necessary for the repair of DSBs [16]. The C-terminus consists of a serine cluster domain (SCD) followed by a BRCA1 C-terminal (BRCT) domain. The SCD contains several phosphorylation sites that are phosphorylated by ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR) kinases. The BRCT domain consists of two tandem BRCT repeats, tightly connected by a linker region [17, 18]. The BRCT domain is further described in section 1.5.2. In addition to the mentioned RING, SCD and BRCT domains, BRCA1 has two nuclear localization sequences (NLS) and a nuclear export sequence (NES) [15].

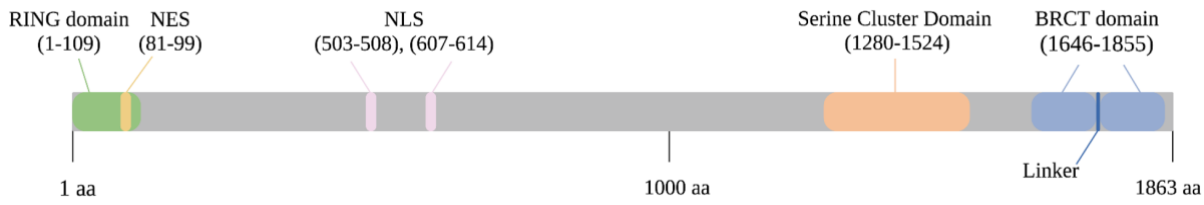

Figure 1.2 Schematic representation of the BRCA1 peptide sequence. The RING, SCD and BRCT domains are shown in green, orange and blue, along with the nuclear export sequence (NES) and nuclear localization sequences (NLS) in yellow and pink, respectively. The numbers in parentheses indicate the amino acid (aa) residue positions. The figure was made in BioRender.com, modified form Clark et al. [15].

BRCA1 plays a pivotal role in DNA repair, and therefore the NLS are critical for its function. The NES is localised within the RING domain, but the binding of BARD1 to the RING domain conceals the NES, and keeps the proteins restricted to the nucleus [19]. The middle region of the BRCA1 protein is thought to be largely unstructured and disordered [20], and although not structured, it is found to bind DNA unspecifically [21, 22].

1.5.2 The structure of the BRCT domain

This work focuses on variants in *BRCA1* located in or close to the BRCT domain. While the full structure of human BRCA1 is not yet resolved, the crystal structure of the BRCT domain is available, and the structure is shown in Figure 1.3 [23]. The BRCT domain of BRCA1 consists of two tandem BRCT repeats, BRCT1 (N-terminal repeat) and BRCT2 (C-terminal repeat). BRCT1 consists of amino acid 1646 to 1736, followed by a linker region at amino acids 1737 to 1759. BRCT2 starts at amino acid 1760, and stretches almost to the end of the protein, at position 1855 [24]. Each BRCT repeat consists of three α-helices that are packed around a β-sheet consisting of four strands.

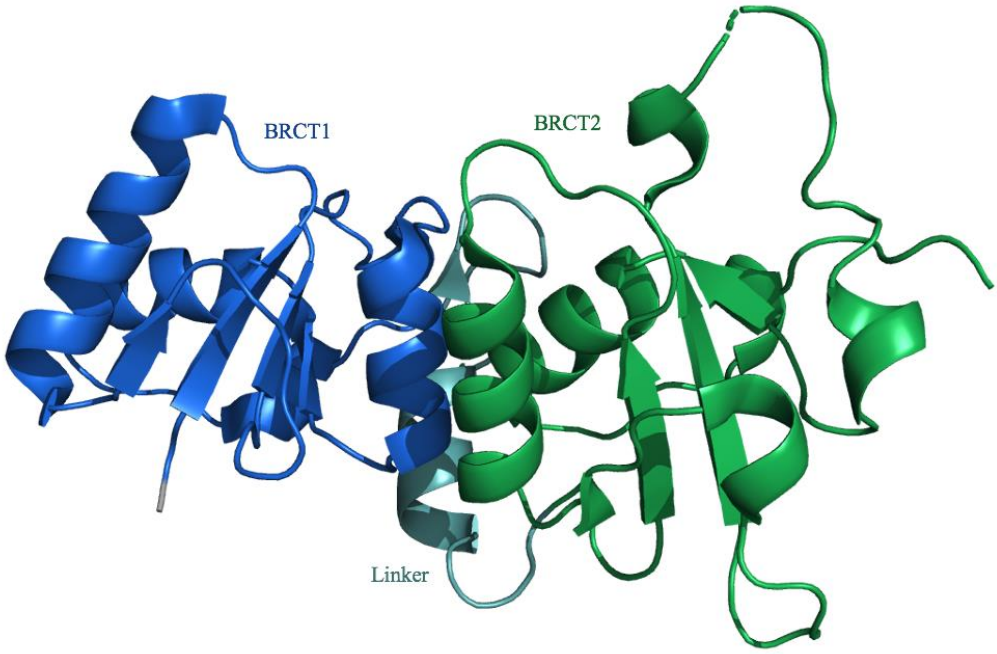

Figure 1.3 The crystal structure of the BRCT domain of BRCA1 resolved by X-ray diffraction. The structure is obtained with the PDB accession number 1T29, which includes the amino acids 1646 to 1859. BRCT1, linker and BRCT2 are show in blue, turquoise and green, respectively. The figure was made in Pymol.

One of the most important functions of the BRCT domain is the ability to bind targets through their pSer-X-X-Phe motif (p indicates phosphorylated serine) [25]. The BRCA1 BRCT domain recognizes this motif in its binding partners like BACH1, CtIP, and CCDC98 and these distinct BRCA1 macrocomplexes are selectively formed through the BRCA1 BRCT domain [26, 27].

BRCT domains are found in several other human proteins and are evolutionary conserved amongst both prokaryotes and eukaryotes [28, 29]. Several of the amino acids within the BRCT domain are highly conserved and especially prone to alterations of deleterious nature, reflecting the importance of the domain. Specifically, alterations of hydrophobic residues within the hydrophobic core of the BRCT domain inhibits the ability of BRCA1 to recognize its targets [30]. The functional role of BRCT as a transactivation domain is described in section 1.5.5.

1.5.3 The various functions of BRCA1

BRCA1 is a multifunctional protein that contributes to the homeostasis of a cell in many ways as illustrated in Figure 1.4.

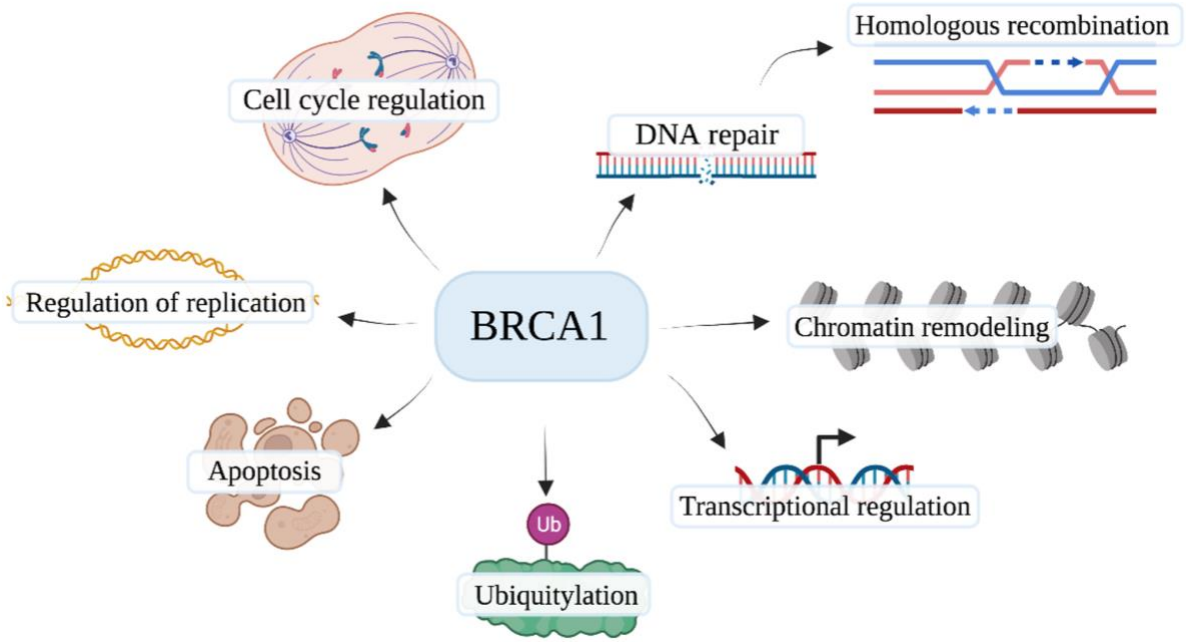

Figure 1.4 Overview of various functions of BRCA1. BRCA1 is a multifunctional protein involved in several important processes in the cell, like DNA repair, chromatin remodelling, transcriptional regulation, ubiquitylation, apoptosis, replication and cell cycle regulation. The figure was made in BioRender.com.

BRCA1 is involved in several processes, like chromatin remodelling and regulation of replication [22, 31]. Additionally, BRCA1 is specifically important in the S-phase checkpoint and the transition from G2 to M-phase [32]. *BRCA1* variants defective of ATM-mediated phosphorylation are associated with a defect in the arrest of the G2/M phase [33, 34]. Furthermore, BRCA1 is involved in ubiquitylation, through forming a complex with BARD1 [35, 36], as well as regulation of apoptosis in a p53-dependent manner [37]. Among the various functions of BRCA1, the perhaps most pivotal roles of BRCA1 is in HR and transcriptional regulation.

1.5.4 The role of BRCA1 in homologous recombination (HR)

DSBs pose a great threat to the genomic stability. Several mechanistically different pathways have evolved to repair DSBs, with the two most studied being HR and non-homologous end joining (NHEJ) [38]. NHEJ and other methods like single strand annealing (SSA) often entail deletions or insertions of several nucleotides, which can lead to chromosome translocations [39, 40]. In contrast, HR can fully restore the DSB accurately without incorporating any alterations [41]. The HR machinery prefers the usage of the sister chromatid rather than the homologous chromosome as template for DSB repair, and therefore HR is most active in S and G2 phase of

the cell cycle, when the sister chromatid is available [31]. An overview of the DSB repair mechanism by HR (and simplified mechanism of NHEJ and SSA) is shown in Figure 1.5.

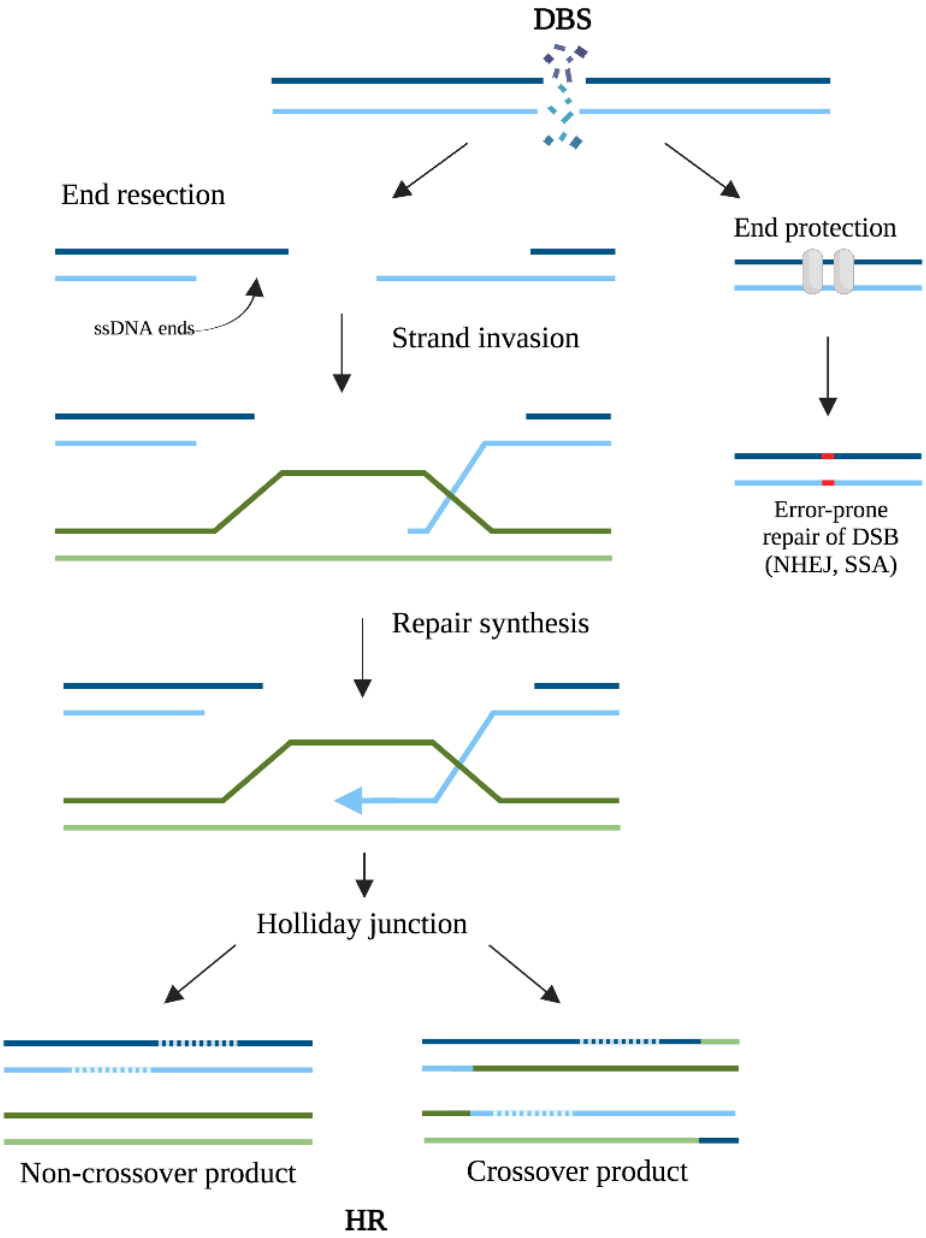

Figure 1.5. Schematic representation of DSB repair by HR. The two blue and two green DNA strands each represent a sister chromatid. Dotted lines represent newly synthesised DNA, free of alterations from HR, while red marks represent erroneously repaired DNA from NHEJ or SSA (simplified to the right). The figure was made in BioRender.com.

Deficiency in HR was initially described in cancers with *BRCA1* alterations [42]. Through the formation of several interactions with other proteins into large protein complexes, BRCA1 contributes to (at least) two distinct processes of the HR repair pathway; end resection and RAD51 loading.

BRCA1 recognises the DSB and initiates end resection, which results in single stranded DNA (ssDNA) at the site of DSB [43]. BRCA1 is thought to mediate end resection by interaction with phosphorylated CtIP and the MRE11-RAD50-NBS1 (MRN) complex through its BRCT domain [44]. In addition to specifically promoting resection, BRCA1 also appears to act as an inhibitor of the resection suppressor 53BP1 [45].

Apart from end resection, BRCA1 mediates replacement of the replication protein A (RPA) with the DNA repair protein RAD51, by loading RAD51 onto the resected ssDNA ends at the site of the DSB. RAD51 loading requires other mediation proteins and BRCA1 induces the recruitment of such a protein, PALB2, to the DSB [27, 46]. This promotes invasion of the sister chromatid and Holliday junction formation, allowing DNA polymerases to repair the DSB [47]. The importance of BRCA1 in HR is reflected in the fact that disease-causing variants of BRCA1 diminishes PALB2 and RAD51 activity, which leads to HR defects and can contribute to tumorigenesis [48]. An emerging category of drugs are being developed to cause synthetic lethality in HR deficient cells.

One such emergent therapeutic agent called Poly (ADP-ribose) polymerase (PARP) inhibitors have shown promising results. Poly ADP-ribose polymerases (PARPs) are proteins involved in base-excision repair of ssDNA. Treatment with PARP inhibitors leads to accumulation of ssDNA breaks, turning into toxic levels of DSBs eventually killing tumours cells defective in the *BRCA1/2* genes. This follows the concept of synthetic lethality based on multiple non-lethal deficiencies becoming lethal when combined [49]. The PARP inhibitor Olaparib (Lynparza) is approved as a 1st line maintenance treatment in Norway for metastasised ovarian cancer in patients with causative *BRCA1/2* variants after chemotherapy [50].

1.5.5 Transcriptional activation by BRCA1

Unlike many classical transcription factors, BRCA1 has not been found to regulate transcription through direct binding of specific DNA sequences *in vivo* [51]. However, BRCA1 binds and regulates the activity of a variety of different transcription factors, like p53, oestrogen receptor (ER), STAT1, c-Myc, NF-κB and octamer-binding transcription factor 1 (OCT1) [47, 52].
An overview of the transcription factors that BRCA1 regulates, and their respective downstream effects, are shown in Figure 1.6.

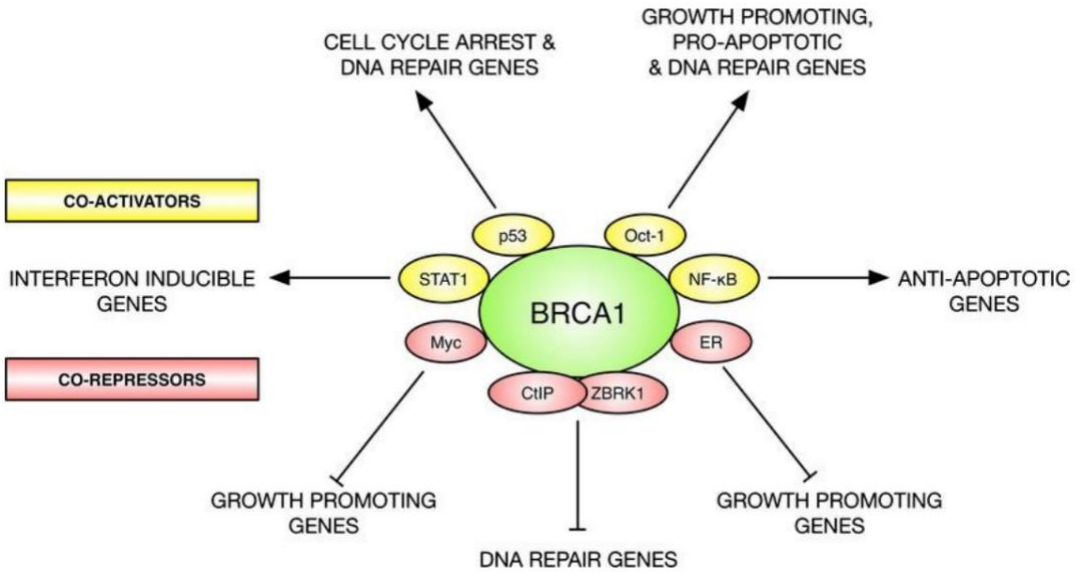

Figure 1.6 The regulation of transcription by BRCA1. BRCA1 regulates transcription by binding several different transcription factors that either activate (yellow) or repress (red) transcription. The figure is adapted from Savage and Harkin [47].

BRCA1 interacts with the tumour suppressor protein TP53 trough the C-terminus of BRCA1 [53-55]. This interaction both stabilises and stimulates the transcriptional activity of TP53, inducing downstream target genes involved in DNA repair and cell cycle arrest, like p21 [56, 57]. This activity is dependent on BRCA1 phosphorylation in response to DNA damage by ATM and ATR, at serines–1423 and –1524 in the SCD [58].

Another important aspect of the role of BRCA1 in regulation of transcription in response to DNA damage, is through binding and activating the basal transcriptional machinery [32]. The BRCA1 protein forms a complex with the RNA polymerase II holoenzyme. The link between BRCA1 transcriptional regulation and tumour suppression has been demonstrated, as cancer associated BRCA1 alterations were found to disrupt the interaction of BRCA1 and the RNA polymerase II holoenzyme [59, 60].

Early experiments in the yeast strain *Saccharomyces cerevisiae* transformed with human *BRCA1* fused to the activation domain of the yeast transcription factor Galactose-responsive transcription factor (GAL4), showed that the BRCT domain induced transcription of a reporter gene [61]. Similar experiments, all performed by fusing the C-terminal region of BRCA1 (aa 1560– 1863) to the GAL4 DNA-binding domain (DBD) (producing a GAL4 DBD BRCA1 BRCT fusion protein), showed that the BRCT domain of BRCA1 functioned as a

transactivation domain, activating gene transcription both in mammalian and yeast cells [62, 63]. Simultaneously, it was shown that some variants in the BRCT domain of BRCA1 exhibited a reduction in transactivation activity [62]. The fusion construct GAL4 DBD BRCA1 BRCT was used in this study to measure the ability of different fusion protein variants to transcriptionally activate its target.

1.6 Genetic testing of patients with suspected HBOC

In Norway, patients with suspected HBOC are referred to medical genetic laboratories and offered genetic counselling, and sequencing of a panel of genes involved in breast and ovarian cancer, including *BRCA1*. Suspicion of HBOC is often based on early age of breast cancer or ovarian cancer, multiple related primary cancer sites (e.g., breast and ovarian) as well as several relatives with related cancer forms. The genetic tests are performed by Sanger sequencing and/or Next Generation sequencing. If a variant is discovered in the test, it has to be interpreted to evaluate its clinical significance, which is important for the cancer risk assessment, genetic counselling, and clinical follow up of the patients and potentially, their relatives [64].

1.7 Variant interpretation

In general, when interpreting genetic variants in the *BRCA1* gene, most medical genetic laboratories use the classification criteria recommended by the American College of Medical Genetics (ACMG) and the Evidence based Network for the Interpretation of Germline Mutant Alleles (ENIGMA) Consortium [65, 66] . These criteria consist of a five-tier system that classifies variants as benign (class 1), likely benign (class 2), variant of uncertain significance, also termed VUS (class 3), likely pathogenic (class 4) or pathogenic (class 5). During variant interpretation, benign and/or pathogenic evidence of different strength are gathered and summarised to reach a final classification. If available, information regarding the type of variant (frameshift, splice site, missense, etc), frequency of the variant in the general population, functional analysis of the variant described in literature, evolutionary conservation, biochemical properties of the altered amino acid, previous classification performed by other laboratories in public databases (such as ClinVar) and clinical data of the patient and the patient's family history are of interest.

Genetic variants are categorised into different types, depending on how they affect the DNA sequence. Insertions or deletions can result in a change of the reading frame, which is likely to

alter the proteins' structure and function. A substitution can produce a missense or a nonsense variant, where a missense variant results in a change of one amino acid to another, and a nonsense variant causes a premature stop codon. There are more than 4.6 million missense variants in different genes gathered in the Genome Aggregation Database (gnomAD), of which 99% are rare missense variants and only 2% have a clinical impact in the ClinVar database [67]. The number of novel *BRCA1* variants increases, and today there are more than 11 000 *BRCA1* variants registered in ClinVar [68]. Missense variants are especially hard to interpret, as they are often rare, and only limited information of each of the variants is available. Missense variants in *BRCA1* are the focus of this work.

The allele frequency of a variant in the general population can reflect its pathogenicity. The international online reference database of human genetic variation, gnomAD, contains variant allele frequency data (minor allele frequency, MAF) from 140 000 unrelated individuals of different ethnicities, which includes 270 million genome variants [69]. This information is useful during variant interpretation, as most diseases of hereditary origin, including HBOC, are rare in the general population, and cannot be caused by variants that are common in a normal, healthy population.

Functional analysis described in the literature available in databases like PubMed are useful when interpreting variants. Functional characterisation can be a robust tool to clinically annotate variants [70]. Importantly, it is recommended to critically evaluate factors such as whether the assay models the disease mechanism of the gene, and whether an appropriate number of control variants are included for comparison [71].

The evolutionary conservation of a domain or segment in the amino acid sequence often reflects its importance in the protein. Amino acid alterations in proteins are random, and alterations that are beneficial or neutral will persist during the course of evolution. Especially for missense variants, the conservation of the substituted amino acid provides knowledge on its necessity and also the potential consequence of the alteration, as highly conserved amino acids are likely imperative for the protein structure and function. It is thus informational to assess the degree of conservation between species to gain insight into the importance of a particular amino acid [72].

The biochemical consequence of an amino acid substitution is an important factor when assessing the pathogenicity of a missense variant. A conservative change, in which both the

original and the new amino acid have the similar physicochemical properties and size, will not necessarily introduce major structural and functional consequences. On the other hand, a non-conservative change, like replacing a hydrophobic and small residue with a charged and larger amino acid, could potentially introduce major structural and functional damage.

Previous classification shared by other laboratories and public databases can also be useful during variant interpretation. ClinVar is a public website archive where human genetic variants and interpretations are gathered. Submitters are clinical and research laboratories, independently submitting their suggested classification of a variant, and ClinVar is thus a useful source to guide variant interpretation.

Clinical data including patient and family history is essential for variant interpretation. Several family members with cancer, especially early onset cancer and several related tumour forms are all indication of a pathogenic variant. In addition, segregation data is also useful in variant interpretation. During segregation analysis, the genotypes and phenotypes of multiple generations in a family can be used to establish if a variant segregates with disease within a family. The variant should be found in disease-affected family members, but not in unaffected family members [73]. Apart from clinical data, the factors presented under variant interpretation) were used in this study if available and applicable in order to interpret several *BRCA1* variants of uncertain significance.

1.8 *BRCA1* variants of uncertain significance

Accurate interpretation of the pathogenicity of *BRCA1* variants is vital for the patient and its family, as it affects the risk assessment, genetic counselling and treatment. Carriers of benign and likely benign *BRCA1* variants do not harbour increased cancer risk compared to the general population, and can be released from the physiological burden of possibly carrying a pathogenic variant. If there is strong suspicion of familial cancer, further testing for additional cancer related genes (gene panels) can be offered. Carriers of variants classified as likely pathogenic or pathogenic have an increased risk of cancer, and are offered screening, surveillance and prophylactic surgery [64]. Examples of such variants are the carriers of among others the four Norwegian *BRCA1* founder variants (c.1016dup, c.1556del, c.3328_3229del, c.697_698del), found through haplotyping studies in in 2001 [74, 75].

However, variants of uncertain significance are challenging, as they cannot be used in risk assessment and clinical decision-making. Furthermore, VUSs may represent a major burden for the patient, leading to stress and anxiety. One study found that patients harbouring *BRCA1* VUS showed higher levels of anxiety and distress compared to patients with clear benign or pathogenic variants [76]. Over the years, reduced cost and technical development in the sequencing field has led to a rapid growth of genetic testing of patients with suspected HBOC. Consequently, an increasing number of novel *BRCA1* variants are being discovered. A large number of the new *BRCA1* variants detected are classified as VUS, as the knowledge of these variants are either very limited or conflicting.

Although *BRCA1* is a well characterised gene, the interpretation of missense *BRCA1* variants is still a challenge for clinical laboratories. In a study by Hovland et al., all *BRCA1* variant in Norway were collected and the classification was compared, aiming to reveal potential discrepancies in variant classification between the hospitals [68]. There are, as of 2021, in total 463 unique *BRCA1* variants detected in Norway, of which 25% are classified as VUS. In addition, 6% of the variants are classified as both VUS and likely benign in different Norwegian hospitals. Given this background, it is of great importance to functionally study *BRCA1* VUSs, particularly the challenging missense variants for which there is often limited or conflicting evidence, aiming to clarify their pathogenicity.

1.9 Aims

The *BRCA1* variants studied in this work are missense substitutions located close to or within the BRCT domain of *BRCA1*. A total of 11 variants classified as VUS by one or several of the medical genetics' laboratories in Norway or reported as VUSs in ClinVar were examined [68]. In addition, one likely pathogenic and six (likely) benign or (likely) pathogenic controls variants were included. The main aim of this thesis was to generate new knowledge to support variant interpretation of these *BRCA1* VUSs primarily based on TA assay.

More specific sub-aims are:

- Analyse *BRCA1* variants classified as VUS by transactivation assay through comparison to benign and pathogenic control variants.
- Compare the TA activity of inter-laboratory control variants observed in this study with similar experiments previously performed at University Hospital of North Norway and Oslo University Hospital.
- Assess the relative protein expression of the BRCA1 protein variants.
- Examine potential correlation between the localisation of the variants in the BRCT domain and the functionality of the corresponding protein.
- Compare two methods for normalisation of western blot data (actin and total protein), as normalisation against total protein is an emerging method of normalisation.

2. Materials

2.1 Variant nomenclature and *BRCA1* reference sequence

The data in this study was based on the Genome Reference Consortium Human Build 37 (GRCh37). The *BRCA1* GenBank reference sequence NM_007294 was used, and the exons are numbered systematically from 1 to 23. The *BRCA1* variants were named according to recommendations by the Human Genome Variation Society (HGVS). Variants written with the prefix c. denotes a change in the coding DNA sequence, and the format is "prefix""position_substituted""reference_nucleotide"">"new_nucleotide"**.** As an example, a variant with a T to C substitution at the nucleotide position 4315 is written as c.4315T>C. Similarly, the prefix p. is used to denote a change in the protein sequence, and the format is "prefix""amino_acid""position""new_amino_acid". For instance, the variant c.4315T>C describes a change (T to C) in the nucleotide position 4315 which corresponds to a predicted change in the amino acid at position p.(Leu1439Phe). The parentheses indicate that the change at protein level is predicted and has not been verified.

2.2 *BRCA1* wild type and variant plasmids

The pcDNA3 GAL4 DBD:BRCA1 BRCT (aa 1396– 1863) wild type plasmid and the corresponding empty vector pcDNA3 were kindly gifted from J. Langerud and N. Iversen at the Department of Medical Genetics at Oslo University Hospital. A schematic illustration of the WT plasmid is shown in Figure 2.1. For simplicity, the pcDNA3 GAL4 DBD:BRCA1 BRCT wild type plasmid will hereafter be referred to as wild type (WT), the empty vector pcDNA3 will be referred to as empty vector (EV) and the plasmids with alterations in the *BRCA1* sequence are called *BRCA1* variant plasmids. The sequence of the WT protein is listed din supplementary data (section 7.1).

In total, 18 variants were studied in this work in addition to WT and EV. Two (likely) benign [77] and four (likely) pathogenic [77-80] controls were included, as well as 5 inter-laboratory control variants [77, 78], for comparison of the results obtained with the same assay at other clinical laboratories in Norway. Of the remaining seven variants of interest, three were classified as VUS in at least one of the Norwegian medical genetics laboratories [68], three were reported as VUSs in the variant database ClinVar (marked – in Table 2.1), and one was

classified as likely pathogenic in Norway, but was recently submitted in ClinVar as VUS (Table 2.1).

Figure 2.1: Schematic representation of the pcDNA3 GAL4 DBD:BRCA1 (aa 1396– 1863) WT plasmid. Purple represents the GAL4 DNA Binding Domain, and pink represents the *BRCA1* BRCT insert. Figure adapted from Langerud et al [77].

While seven of the variant plasmids were kindly gifted by Langerud and Iversen, four variant plasmids were made by a former master student in the group (Nikara Pedersen). The remaining seven variant plasmids were made by site-directed mutagenesis as a part of this thesis using the WT plasmid as template.

In addition, the two plasmids used in the transactivation assay, pGAL4-e1b-Luc (Firefly) and phRG-TK (Renilla), were kindly gifted by J. Langerud and N. Iversen at the Department of Medical Genetics, Oslo University Hospital.

Table 2.1: *BRCA1* variants and controls investigated in this study.

Nucleotide substitution	Amino acid substitution	Protein domain	Variant	Norwegian Classification^	Obtained from
c.4315C>T	p.(Leu1439Phe)	Upstream BRCT	Variant of interest	2*, 3Δ□○	Made by N.P
c.4956G>A	p.(Met1652Ile)	BRCT 1	Benign control	2	Made by J.L/N.I
c.4964C>T	p.(Ser1655Phe)	BRCT 1	Pathogenic control	4	Made by J.L/N.I
c.5002T>C	p.(Phe1668Leu)	BRCT 1	Variant of interest	3	Made by N.P
c.5095C>T	p.(Arg1699Trp)	BRCT 1	Pathogenic control	5	Mutagenesis
c.5101C>A	p.(Leu1701Met)	BRCT 1	Variant of interest	-	Mutagenesis
c.5123C>T	p.(Ala1708Val)	BRCT 1	Inter-laboratory control	3	Made by J.L/N.I
c.5125G>A	p.(Gly1709Arg)	BRCT 1	Inter-laboratory control	3	Made by J.L/N.I
c.5131A>C	p.(Lys1711Gln)	BRCT 1	Inter-laboratory control	3	Made by J.L/N.I
c.5153G>C	p.(Trp1718Ser)	BRCT 1	Variant of interest	4	Mutagenesis
c.5245C>G	p.(Pro1749Ala)	Linker	Variant of interest	-	Mutagenesis
c.5324T>G	p.(Met1775Arg)	BRCT 2	Pathogenic control	-	Mutagenesis
c.5411T>A	p.(Val1804Asp)	BRCT 2	Benign control	2	Mutagenesis
c.5429T>C	p.(Val1810Ala)	BRCT 2	Variant of interest	3	Made by N.P
c.5477A>T	p.(Gln1826Leu)	BRCT 2	Inter-laboratory control	2*, 3□○	Made by J.L/N.I
c.5504G>A	p.(Arg1835Gln)	BRCT 2	Inter-laboratory control	3	Made by N.P
c.5506G>A	p.(Glu1836Lys)	BRCT 2	Variant of interest	-	Mutagenesis
c.5513T>G	p.(Val1838Gly)	BRCT 2	Pathogenic control	4	Made by J.L/N.I

N.P = Variant plasmids made previously by former master student N. Pedersen by similar manner as described in this thesis.
J.L/N.I = Variant plasmids kindly gifted from J. Langerud and N. Iversen, Department of Medical Genetics, Oslo University Hospital.
For *BRCA1* variants with conflicting classifications between medical genetic laboratories in Norway, the following symbols indicate the corresponding laboratories [68]:
* = Oslo University Hospital (OUH)
Δ = Haukeland University Hospital (HUH)
□ = University Hospital of North Norway (UNN)
○ =Trondheim University Hospital (TUH)
^ = Represents the classification of the variant at the time the variants were selected (fall 2021).

2.3 Experimental materials

Table 2.2 Bacterial culturing, plasmid purification and site-directed mutagenesis

Product	Manufacturer	Catalogue number
One Shot TOP10 Competent cells	Invitrogen	C404010
ImMedia Amp Liquid	Invitrogen	45-0035
ImMedia Amp Agar	Invitrogen	45-0034
S.O.C. medium	Invitrogen	15544-034
QIA filter Plasmid Maxi Kit	QIAGEN	12243
QIAPREP Spin Miniprep Kit	QIAGEN	12263
Quick-Change II XL Site-Directed mutagenesis kit	Agilent Technologies	200522-5
Quick-Change XL 10-Gold Ultracompetent Cells	Agilent Technologies	200516-4
TE buffer	Invitrogen	12090015

Table 2.3 Primers for site-directed Mutagenesis from Sigma-Aldrich. The nucleotide substitutions are shown in bold.

Variant	Direction	Sequence 5´ - 3´
BRCA1 c.5095C>T	Forward	CTAGAAAATATTTCAGTGTCC**A**TTCACACACAAACTCAGCATCTG
	Reverse	CAGATGCTGAGTTTGTGTGTGAA**T**GGACACTGAAATATTTTCTAG
BRCA1 c.5101C>A	Forward	CCTAGAAAATATTTCA**T**TGTCCGTTCACACACAAACTCAGCAT
	Reverse	ATGCTGAGTTTGTGTGTGAACGGACA**A**TGAAATATTTTCTAGG
BRCA1 c.5153G>C	Forward	GACTGGGTCACC**G**AGAAATAGCTAACTACCCATTTT
	Reverse	AAAATGGGTAGTTAGCTATTTCT**C**GGTGACCCAGTC
BRCA1 c.5245C>G	Forward	TTCTCTTGCTCGCTTTG**C**ACCTTGGTGGTTTCTTC
	Reverse	GAAGAAACCACCAAGGT**G**CAAAGCGAGCAAGAGAA
BRCA1 c.5324T>G	Forward	GATCTGTGGGC**C**TGTTGGTGAAGGGCCCATAGC
	Reverse	GCTATGGGCCCTTCACCAACA**G**GCCCACAGATC
BRCA1 c.5411T>A	Forward	CCACAATTGGGTGG**T**CACCTGTGCCAAGG
	Reverse	CCTTGGCACAGGTG**A**CCACCCAATTGTGG
BRCA1 c.5506G>A	Forward	TCCAACACCCACT**T**TCGGGTCACCACAGG
	Reverse	CCTGTGGTGACCCGA**A**AGTGGGTGTTGGA

Table 2.4 Sequencing reagents

Product	Manufacturer	Catalogue number
Big Dye version 3.1	Applied biosystems	4336911
Sequencing Buffer	Applied biosystems	4336699

Table 2.5 Sequencing primers

Primer name	Direction	Sequence 5´- 3´
F10	Forward	CAATGGAAGAAACCACCAAG
F9	Forward	AACCCCTTACCTGGAATCTG
F8	Forward	TGATGAAGAAAGAGGAACGG
R2	Reverse	AACCCCTTACCTGGAATCTG
T7	Forward	TAATACGACTCACTATAGGG
BGH	Reverse	TAGAAGGCACAGTCGAGG

Table 2.6 Cell culture

Product	Manufacturer	Catalogue number
Fetal Bovine Serum (FBS)	Sigma-Aldrich	F7524
Penicillin-Streptomycin (PS)	Sigma-Aldrich	P4333
Dulbecco's Modified Eagle Medium (DMEM)	Sigma-Aldrich	41966-029
Trypsin	Sigma-Aldrich	T3924
Phosphate Buffered Saline (PBS)	Sigma-Aldrich	D8537
jetPRIME	Polyplus transfection	117-01
HEK293FT cells	Invitrogen	R70007
Automated cell counter	Scepter 2.0	Merk
Cell counter sensors	60 µm	Merk

Table 2.7 Mycoplasma test, fingerprinting of cell lines and agarose electrophoresis

Product	Manufacturer	Catalogue number
Universal Mycoplasma Detection Kit	ATCC	30-1012K
QIAamp DNA Mini Kit	QIAGEN	51306
AmpFlSTR® Identifiler Plus® PCR Amplification Kit (50 rxn)	Life Technologies	A26182
GeneScanTM-500 LIZ Size Standard	Life Technologies	4322682
HiDi Formamide	Life Technologies	4311320

Table 2.8 Agarose gel electrophoresis

Product	Manufacturer	Catalogue number
Elite 2-in-1 Agarose Tablets	ProteinArk	PAL-E-2-in-1-100
Quick-Load® 100 bp DNA Ladder	New England Biolabs	N0467L/S
Gel Loading Dye, Purple (6X)	New England Biolabs	B7025S
Tris-Borate-EDTA (TBE) buffer	-	-

Table 2.9 Western blot analysis and protein quantification

Product	Manufacturer	Catalogue number
cOmplete™, EDTA-free Protease Inhibitor Cocktail	Merck	1187358001
Pierce Ripa Buffer	Thermo Scientific	89901
Pierce BCA Protein Assay Kit	Thermo Scientific	23227
4x Lammeli Sample Buffer	BIO-RAD	1610747
NuPAGE Sample Reducing Agent	Invitrogen	NP0009
Mini-Protean TGX Stain-Free Gels, 4-20%	BIO-RAD	4568095
10x Tris/Glycine/SDS Buffer	BIO-RAD	1610772
Trans-Blot Turbo RTA Transfer Kit, LV PVDF	BIO-RAD	1704274
Precision Plus Protein Unstained Standard	BIO-RAD	161-03
Every Blot Blocking Buffer	BIO-RAD	12010020
GAL4 (DBD) (RK5C1) mouse monoclonal	Santa Cruz Biotechnology	sc 510
B-actin (C4) mouse monoclonal IgG	Santa Cruz Biotechnology	sc 47778
m-IgGk BP-HRP (secondary antibody)	Santa Cruz Biotechnology	sc 516102
Precision Protein StrepTactin-HRP Conjugate	BIO-RAD	161380
PBS Tablets	Gibco	18912-014
Tween 20 (T)	Merck	8221840500
Clarity Western ECL Substrate	BIO-RAD	170-5061

Table 2.10: Transactivation assay

Products	Manufacturer	Catalogue number
Dual-Luciferase Reporter Assay System	Promega Corporation	E1960
96 Well Polystyrene Microplate, white	Greiner Bio-One	655075

Table 2.11 Laboratory instruments

Instrument	Model name	Manufacturer
Microplate Luminometer	Centro XS^3 LB 960	Berthold
Microplate reader	Synergy HT	BioTek Instruments
Microplate reader	Big Lunatic	Unchained Labs
UV-VIS Spectrophotometer	NanoDrop 1000	Thermo Scientific
Capillary electrophoresis 3730	DNA analyser	Applied Biosystems
Thermal cycler	Veriti	Applied Biosystems
Gel running system	Mini-Protean Tetra system	BIO-RAD
Transfer Machine	Trans-Blot Turbo transfer system	BIO-RAD
Gel and blot imager	ChemiDoc MP	BIO-RAD

Table 2.12 Software and databases

Application	Developer	Version
SeqScape	Applied Biosystems	2.5
NanoDrop 1000 Operating Software	Thermo Scientific	03.08.2001
Gen5 Microplate Reader	BioTek	02.06.2010
ImageLab	BIO-RAD	6.1
ICE Luminometer software	Berthold Technologies	1.0.9.8
Exel	Microsoft	16.43
Word	Microsoft	16.43
Power Point	Microsoft	16.43
BioRender.com	app.biorender.com	-
ClinVar	National Center for Biotechnology Information (NCBI)	-
Alamut Visual	Interactive Biosoftware	2.15
Genome Aggregation Database	Broad institute	2.1.1
PubMed	National Center for Biotechnology Information (NCBI)	-
PyMOL	Schrödinger	2.4.2

3. Methods

3.1 Preparation of liquid growth medium and agar plates

For preparation of agar plates, imMedia™ Growth Medium agar with ampicillin was used. The content of a pouch was mixed with 200 ml of millliQ (mq) H_2O and the solution was heated in the microwave until boiling and poured into plates. To make liquid growth medium, imMedia™ Growth Medium liquid with ampicillin was used. The content of a packet was mixed with 200 µl of mqH_2O and the mixture was heated in a microwave until boiling, and the medium was capped and cooled to room temperature (RT) before use. Hereafter, the imMedia™ Growth Medium agar will be referred to as agar plates, and the imMedia™ Growth Medium liquid will be referred to as growth medium (the concentrations of ampicillin are not specified for neither of the products by the manufacturer).

3.2 Transformation of One Shot TOP10 cells

Transformation of One Shot TOP10 Competent *E. coli* cells was performed according to the manufacturer's protocol for chemically competent cells. In short, 45 µl of competent cells and 2 µl of purified plasmid DNA were used for each variant. Reactions were incubated on ice for 30 min before cells were heat-shocked for 30 seconds (sec) at 42 ℃. The reactions were placed on ice for 2 minutes (min) and pre-heated S.O.C. medium was added. The transformation mixtures were spread on agar plates and incubated at 37 ℃ overnight (ON). One colony from each variant was picked the following day and inoculated with 5 ml of growth medium. The culture was incubated at 37 ℃ for 7 hours (h) while shaking at 225 rpm. The pre-culture was diluted into fresh growth medium (1:500) and incubated ON at 37 ℃ whilst shaking at 225 rpm. The plasmid purification was performed as described in section 3.4, followed by quantification (section 3.5) and sequencing (section 3.7).

3.3 Site-directed mutagenesis

3.3.1 Variant plasmids prepared by site-directed mutagenesis

Seven of the *BRCA1* variants analysed in this study were prepared by site-directed mutagenesis using the WT plasmid (pcDNA3 GAL4 DBD:BRCA1 BRCT) as template and the Quik-Change II XL Site-Directed Mutagenesis Kit as described in the protocol. The custom ordered primers were delivered in dry form and dissolved in TE-Buffer following the manufacturer's instructions. The sequences of the primers are listed in Table 2.3.

3.3.2 Polymerase chain reaction (PCR) for site-directed mutagenesis

The site-directed mutagenesis reaction for each variant is shown in Table 3.1. The PCR reaction was run with the parameters listed in Table 3.2.

Table 3.1: Components of a site-directed mutagenesis reaction

Component	Quantity (µl)
Reaction buffer 10X	5
Plasmid DNA template (10 ng)	2
Forward primer (125 ng)	1.25
Reverse primer (125 ng)	1
dNTP mix	1
QuickSolution reagent	3
PfuUltra HF DNA polymerase (2.5 U/ µl)	1
mqH_2O	35.5

Table 3.2: PCR cycling parameters for site-directed mutagenesis

Segment	Cycles	Temperature (°C)	Time
Initial denaturation	1	95	1 min
Denaturation	18	95	50 sec
Annealing		60	50 sec
Extension		68	7 min, 18 sec
Final Extension	1	68	7 min

After the PCR, samples were placed on ice for 2 min to cool, and 10 U of DpnI was added to the reactions, which were then incubated at 37 °C for 1 h.

3.3.3 Transformation of site-directed mutagenesis products into XL10-Gold ultracompetent cells

For transformation, XL10-Gold ultracompetent cells were thawed on ice. For each transformation reaction, 45 µl cells and 2 µl β-mercaptoethanol were used. The cells were incubated on ice for 10 min and swirled gently every 2 min. For each variant, 2 µl of DpnI-treated DNA was added to a vial of cells and the transformation reactions were incubated on ice for 30 min. The cells were heat-shocked at 42 °C for 30 sec and subsequently placed on ice for 2 min. Pre-heated S.O.C. medium was added to cells, and the reactions were incubated at 37 °C for 1 hour while shaking at 225 rpm. The reaction volumes were plated on agar plates and incubated at 37 °C ON. The following day, ten colonies from each variant were re-plated on a new agar plate to avoid merging of the colonies. The plates were kept at 37 °C ON and then stored at 4 ˚C. Colonies for each variant were cultured separately in growth medium ON.

The plasmid DNA was purified by QIAGEN QIAprep Spin Miniprep Kit or QIA Filter Plasmid Maxi Kit (section 3.4). The plasmid DNA was subsequently quantified (section 3.5) and sequenced (section 3.7).

3.4 Plasmid purification

QIAGEN QIAprep Spin Miniprep Kit was used for screening of colonies after mutagenesis, while QIA Filter Plasmid Maxi Kit was used to prepare plasmid DNA for transfection. For plasmids purified by QIAGEN QIAprep Spin Miniprep Kit the protocol for Plasmid DNA Purification using microcentrifuges was followed. Prior to Miniprep, colonies were inoculated in growth medium ON at 37 ˚C and 225 rpm while shaking. For purification with Maxiprep, bacterial pre-cultures were prepared by inoculating a colony in growth medium for 7 h at 37 ˚C and 225 rpm. The pre-culture was diluted 1:500 in new medium and incubated ON at 37 ˚C and 225 rpm. For plasmids purified with QIA Filter Plasmid Maxi, the manufacturer's protocol for high-copy plasmids was followed with a few changes in centrifuge times: all centrifuge settings were set to 4 ˚C and 4600 rpm (the instruments´ maximum speed). The ON culture was initially centrifuged for 20 min. After elution, the DNA was precipitated in isopropanol and centrifuged for 1 h. The supernatant was discarded, and the DNA pellet was washed with 70% ethanol and centrifuged for 20 min. Supernatant was removed and the pellet was air-dried for 10 min. The plasmid DNA was dissolved in 100 µl of the kit elution buffer.

3.5 Measurement of the quantity and quality purified plasmid DNA

Purified plasmid DNA was quantified by absorbance at NanoDrop Microvolume UV-Vis Spectrophotometer. Both A260/280 and A260/230 ratios were noted, and samples were measured three times. A ratio of A260/280 ~ 1.8 for DNA and A260/230 ratio 1.8-2.2 for nucleic acids are in general accepted as "pure".

3.6 Agarose gel electrophoresis

For agarose gel electrophoresis, a 1% agarose gel was prepared using Elite 2-in-1 Agarose Tablets. One tablet was dissolved in 100 ml 1 X TBE buffer until dissolved (1-5 min) and heated in the microwave until clear and all particles were dissolved. The solution was cooled to approximately 60 °C before pouring. The gel was run at 75 voltage (V) with Quick-Load® 100 bp DNA Ladder. Samples and DNA ladder were loaded on the gel and the electrophoresis

was run until the tracking dye had migrated 60-70% the length of the gel. Images were taken with ChemiDoc MP Imaging system Using the setting GelGreen and auto-optimal exposure.

3.7 Sanger sequencing

Each sequencing reaction was set up as listed in Table 3.3 with the sequencing primers as described in Table 2.5. Each variant plasmid was sequenced with all six primers listed to cover the whole GAL4 DBD and BRCA1 BRCT domains as well as the flanking regions of the vector backbone. The thermal cycling parameters are shown in Table 3.4. After the PCR, reactions were stored at -20 °C and sequencing was carried out by capillary electrophoresis at the Department of Medical Genetics.

Table 3.3: Sanger sequencing reaction setup

Component	Amount for each sample (µl)
5X Big Dye Terminator Sequencing Buffer	2
Big Dye version 3.1	2
Primer (10 µM)	0.5
DNA (~ 1000 ng/µl)	2
mqH_2O	3.5

Table 3.4: Sanger sequencing PCR parameters

Step	Cycles	Temperature (°C)	Time
Initial denaturation	1	96	1 min
Denaturation		96	10 sec
Annealing	25	50	5 sec
Extension		60	4 min
Hold	1	4	∞

Sequences were aligned against the WT reference sequence using the SeqScape software (version 2.5). The entire amplified sequence was inspected for unintended changes, and the chromatograms were inspected for background noise.

3.8 Cell culture

3.8.1 Cell line, culture maintenance and subculturing

Human Embryonic Kidney cell line (HEK293FT) (Invitrogen™, R70007) was cultured and used in this study. The HEK293FT cell line is a fast-growing and highly transfectable clonal isolate derived from the HEK293T cell line [81].

HEK293FT cells were kept in Dulbecco's Modified Eagle´s Medium (DMEM) with 4.5g/L D-glucose. The cell line medium was supplemented with 10% FBS and 1% Penicillin Streptomycin. The cells were incubated in humidified air at 37 ˚C with 5% CO_2 in 75 cm^2 flasks. Approximately twice a week, the cells were split by washing with PBS and detaching from the flask using trypsin (0.5 g/l porcine trypsin). After incubating with trypsin for 5 min (37 ˚C, 5% CO_2), fresh medium was added. The cells were diluted 1:10 or 1:20 with fresh medium, and the remaining cells were either used for experiments or discarded.

3.8.2 Seeding cells for experiments

About 20 h before transfection, the HEK293FT cells were seeded. After splitting as described (3.8.1), 20 µl of cells were diluted 1:10 in PBS and the cell count was measured using a 60 µm sensor and an Automated Cell Counter. For experiments, either 3.5 x 10^5 or 1.65 x 10^5 cells were used, seeded in 12-well and 24-well plates, respectively. Medium was added to a total of 1 ml for 12-well plates and 0.5 ml for 24-well plates.

3.8.3 Transfection of cells

Transfection of HEK293FT cells was performed with jetPRIME. The amount of jetPRIME buffer and reagent were adjusted to the well size and amount of DNA: for 12-well and 24-well plates, respectively, 75 µl and 37.5 µl of JetPRIME buffer and 1.5 µg and 0.76 µg DNA was used. The DNA was diluted into jetPRIME buffer and vortexed for 10 sec. For 1.5 µg and 0.76 µg of DNA, 3 µl and 1.5 µl of jetPRIME reagent was used, respectively. The solutions were vortexed for 1 sec and incubated for 10 min at RT. The DNA-lipid complexes were added dropwise to the cells and the medium was replaced after four h from transfection time.

3.8.4 Mycoplasma testing of cell line

HEK293FT cells were tested for mycoplasma by using the Mycoplasma Detection Kit (ATCC) according to the protocol provided by the manufacturer. Cell cultures were 50-70% confluent when harvested. The cells were scraped in their medium and counted by an Automated Cell Counter to ensure that the cell number was below 10^6 (cell numbers above 10^6 can interfere with the analysis). Of the cell suspension, 1 ml was centrifuged at 13,000 rpm for 3 min at 4 °C. The supernatant was removed, cells were resuspended in 50 µl lysis buffer and incubated at 37 °C for 15 min and subsequently at 95 °C for 10 minutes. The samples were centrifuged at 13,000 rpm for 5 min at 4 °C and supernatant was kept and stored at -80 °C until further

analysed. The PCR reaction was set up according to Table 3.5, and lysis buffer, universal PCR mix, universal primers and positive control were provided in the kit. The PCR cycle parameters are shown in Table 3.6.

Table 3.5 Reaction setup for mycoplasma test

Component	Samples (µL)	Positive Control (µL)	Positive Control + Sample (µL)	Negative Control (µL)
Universal PCR Mix	20	20	20	20
Primers Mix	2.5	2.5	2.5	2.5
Test Sample	2.5	-	2.5	-
Positive Control	-	2.5	1.0	-
H_2O or TE	-	-	-	2.5
Total volume	25	25	26	25

Table 3.6 PCR cycling parameters for mycoplasma test

Segment 1	Initial Denaturation: 94 °C for 1.5 min		
Segment 2	Touchdown PCR Parameters:		
	Temperature (°C)	Time (seconds)	Cycles
Denaturation	94	30	
Annealing	70 → 60.5*	30	20
Elongation	72	45	
Segment 3	Continue cycling at a constant Annealing Temp:		
Denaturation	94	30	
Annealing	60	30	12
Elongation	72	45	
Segment 4	Final elongation: 72 °C for 4 min and hold at 4 °C		

*Temperature decreases 0.5 °C per cycle (e.g., 70 °C for 1 cycle, 69.5 °C for 1 cycle, etc., to 60.5 °C for 1 cycle).

The PCR products were analysed by agarose gel electrophoresis, as described in section 3.6. The samples were prepared using 10 µl of the PCR product and 1.5 µl Gel Loading Dye.

3.8.5 Fingerprinting of HEK293FT cells

To check for potential cell line cross contamination due to continuous cell line culturing, the genomic DNA from the cell lines were purified and subjected to fingerprinting. In principle, cells are harvested, and genomic DNA is purified, followed by PCR amplification and analysis of highly variable short tandem repeats (STR) markers generating a genetic fingerprint profile of the cell line. The profile is then compared to known standards (provided by manufacturer of the cell line).

To purify genomic DNA from cell lines for fingerprinting analysis, HEK293FT cells were first washed in PBS and trypsinated to detach from the flask. The cells were counted with an

Automated Cell Counter as the protocol specifies that the cell number was required to be below 5 x 10^6. Cells were centrifuged for 5 min at 300 g and the supernatant was removed. The cell pellet was resuspended in PBS to a final volume of 200 µl and 20 µl proteinase K was added. For the purification of DNA, QIAamp DNA Blood Mini Kit was used. The protocol for DNA Purification from Blood or Body Fluids from the QIAamp® DNA Mini and Blood Mini Handbook was followed. The DNA concentration and purity were measured by Nanodrop (section 3.5). Subsequently, fingerprinting analysis by PCR and electrophoresis were performed at Department of Pathology, Haukeland University Hospital.

3.9 Western blot analysis of BRCA1 protein variants in HEK293FT cells

3.9.1 Harvesting of cells and protein quantification

Forty-eight hours after transfection of 3.5 x 10^5 HEK293FT cells (section 3.8.3) with 1.5 µg *BRCA1* variant plasmids, cells were washed with PBS and scraped in RIPA buffer with protease inhibitor (inhibitor of serine and cysteine proteases). The lysates were frozen at -80 °C for at least ON. Transfected cell lysates were thawed and centrifuged at 13000 g for 10 min at 4 °C, and the supernatants were kept. Protein quantification was carried out using Pierce BCA Protein Assay Kit according to protocol provided. Absorbance was measured at 562 mm using a microplate reader and Gen 5 All-In-One Microplate Reader Software. All samples including standard BCA concentrations were measured in duplicates in a microplate, and cell lysate was diluted 1:2 to keep the concentrations within the linear range of the BSA standard. The total protein concentrations were calculated from the BSA standard curve.

3.9.2 SDS-PAGE and western blot analysis

For optimalisation of western blot analysis, different amounts of the WT protein were loaded, ranging from 2 to 20 µg. Subsequently, for the BRCA1 protein variants, 7 µg of protein lysate was used. A mixture of protein lysate, 1X of Lammeli Sample Buffer, 1X of NuPAGE Sample Reducing Agent and mqH_2O to a final volume of 20 µl was prepared, and the samples were denatured for 10 min at 70 °C. The samples and Precision Plus Protein Unstained Standard were loaded on Mini-Protean TGX Stain-Free Gels. The gels were run in the Mini-Protean Tetra system at 200 V for 30 min with 1X Tris/Glycine/SDS Buffer and activated for 45 sec and imaged using ChemiDoc MP Imaging system. The proteins were then transferred to a low-autofluorescence (LF) PVDF membrane using the Trans-Blot Turbo RTA Mini LF PVDF Transfer Kit. The transfer was performed with Trans-Blot Turbo Transfer System, following

the provided protocol for TGX Stain-Free Gels. After transfer for 3 min at 2.5 A and 25 V, the membranes were imaged in the ChemiDoc MP Imaging system using the Stain Free Blot setting. These images provided an image of the total protein loaded on stain-free blots, which was subsequently used for normalisation (section 3.9.3). The membranes were blocked with blocking buffer for 5 min prior to addition of antibody. For primary antibody incubation, membranes were co-incubated at 4 °C ON with GAL4 (DBD) diluted 1:500 and anti-β-actin (C4) diluted 1:200 in blocking buffer. Membranes were washed three times with PBS-T before addition of secondary antibody m-IgGk diluted 1:5000 in blocking buffer and incubated for 1 h at RT while rocking. In addition to the secondary antibody, 1X of Precision Protein StrepTactin-HRP Conjugate was added in the blocking buffer to visualise the ladder. The membranes were washed three times with PBS-T and twice with PBS prior to imaging. Clarity Western ECL Substrate was used for visualisation of the bands, and blots were imaged using ChemiDoc MP Imaging system in the Chemiluminescence auto-optimal setting.

3.9.3 Quantification and normalisation of western blot

The Western Blot results were normalised using two different approaches. In one method, the housekeeping gene β-actin was used as a loading control and for normalisation. In the second method, total protein in each sample was used for the same purpose. For both methods, the analyses were performed in ImageLab version 6.1. For the bands and lanes of interest, i.e. the BRCA1 protein bands, actin bands and the total protein lane, the intensities were adjusted by subtracting the background, resulting in an adjusted band intensity or adjusted lane intensity.

When normalising against actin, the actin protein bands resulting from western blots probed with anti-β-actin was used. The adjusted total band volume of the BRCA1 protein variant bands (achieved from western blots probed with anti-GAL4 (DBD)) were divided by the adjusted total band volume of the actin band of the corresponding variant, resulting in normalised intensities for each BRCA1 protein variant.

When normalising against total protein, the total protein in each lane on the stain free blot was used. A normalisation factor was calculated for each BRCA1 protein variant sample. The factor was calculated by dividing the adjusted total lane volume (the adjusted volume of all the proteins in the lane) of the WT by the adjusted total lane volume of the variant lane. The normalisation factor thus represents a ratio between the amount of total protein loaded for the

WT and the BRCA1 protein variant. The normalisation factor of a variant is then multiplied by the adjusted total volume of the BRCA1 protein variant band (achieved from western blots probed with anti-GAL4 (DBD)) of the sample to obtain a normalised intensity.

For both normalisation methods, the normalised BRCA1 protein variants were expressed as percentages of the amount of WT protein, and the WT was set to 100%. Western blot analysis was carried out in three biological replicates. Each replicate was divided between two gels, and each western blot contained a WT and EV protein sample. The protein expression of the variants on a blot was calculated relative to the WT band on the same blot.

3.10 Transactivation (TA) assay of BRCA1 protein variants

3.10.1 The principle of transactivation assay

The TA assay is based on a Dual-Luciferase Reporter Assay System. Cells are co-transfected with the three plasmids, i.e., pGAL4-e1b-Luc plasmid (Firefly), phRG-TK plasmid (Renilla) and GAL4 DBD:BRCA1 BRCT (*BRCA1* variant plasmid) which from now are referred to as Firefly, Renilla and *BRCA1* variant plasmids, respectively. The two reporter plasmids Firefly and Renilla encode firefly (*Photinus pyralis*) and *Renilla* luciferases (*Renilla reniformis*), respectively. The firefly and *Renilla* luciferases are enzymes that produce light as a by-product of catalysis. The amount of light produced in each reaction is measured by a microplate luminometer and allows a measurement of the enzyme activity. The signal from firefly luciferase correlates with the amount of fusion protein (BRCA1 protein variant) that is expressed and binds to the GAL4 promoter. The mechanism of transactivation of Firefly by the DBD:BRCA1 fusion protein is illustrated in Figure 3.1 A and the enzymatic reactions of the firefly and *Renilla* luciferases are illustrated in Figure 3.1 B.

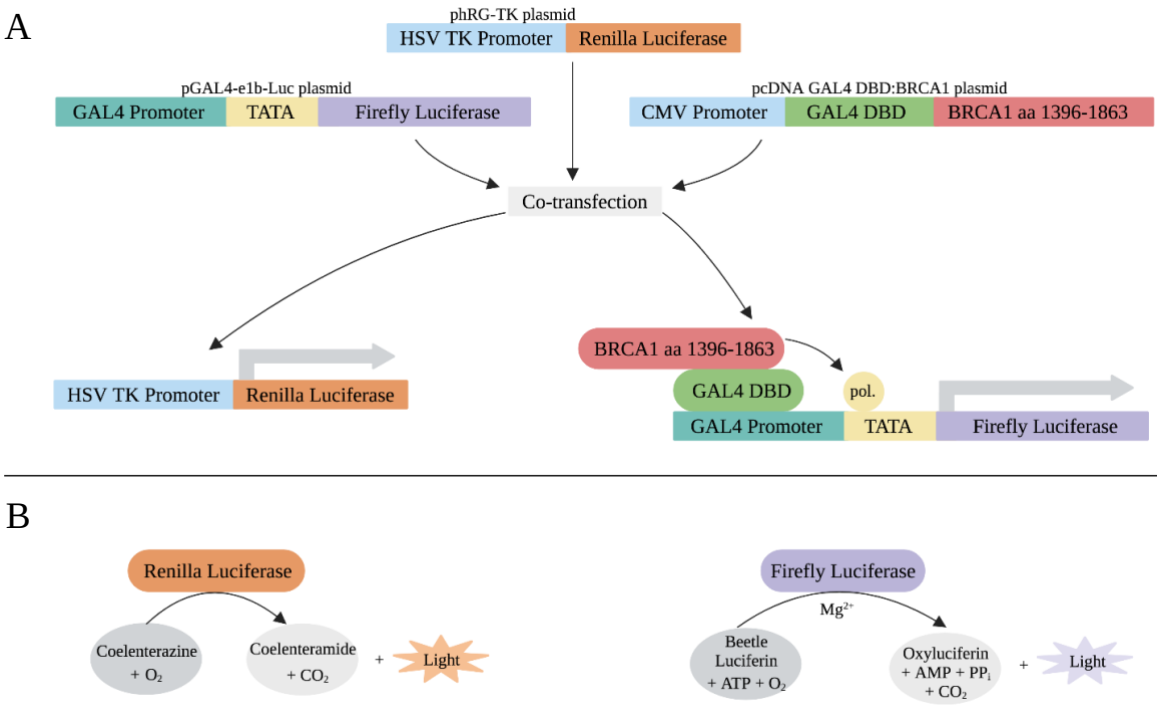

Figure 3.1 A schematic representation of the transactivation (TA) assay and the bioluminescent reactions of *Renilla* and firefly luciferases. In A, the WT (pcDNA3 GAL4 DBD:BRCA1) is co-transfected with the reporter plasmids encoding firefly (pGAL4-e1b-Luc) and *Renilla* luciferases (phRG-TK). Expression of the WT plasmid creates a fusion protein of the BRCA1 BRCT domain and GAL4 DBD. The latter binds to the GAL4-specific promoter in the pGAL4-e1b-Luc reporter plasmid and induces expression of firefly luciferase. The *Renilla* signal is used as an internal control used for normalization. The figure in A is modified from Langerud et al. [77]. In B, the enzymatic reactions of the luciferases *Renilla* and firefly produce light as a by-product of catalysis is shown. The light produced by *Renilla* and firefly luciferases are measured at 480 nm and 560 nm, respectively. Square boxes indicate DNA, rounded boxes indicate protein. The figure was made with Biorender.com.

While the firefly luminescence signal is dependent on transactivation by the DBD domain fused to the BRCA1 protein, the activity of *Renilla* luciferase is independent of the fusion construct. The *Renilla* luciferase is used as an internal control to normalise the firefly luciferase signal against variation caused by the cell count and the transfection efficiency.

The two enzymes catalyse two different bioluminescent reactions, illustrated in Figure 3.1 B. The substrate of firefly luciferase, beetle luciferin, is oxidized to oxyluciferin and luminescence measured at 560 nm. The *Renilla* luciferase oxidizes a different substrate, coelenterazine, to coelenteramide, which yields luminescence measured at 480 nm. The first buffer added in the reaction (LAR II), contains the substrate of firefly luciferase, and the firefly signal is measured. The second buffer (Stop & Glo) is subsequently added, which contains the substrate for *Renilla* luciferase along with a component that quenches the firefly signal.

3.10.2. TA Assay protocol

HEK293FT cells (1.65×10^5) were seeded according to section 3.8.2. The three plasmids (Firefly, Renilla and *BRCA1* variant (or WT or EV)) were co-transfected using jetPRIME buffer and reagent as described in 3.8.3. For each variant, 36 ng of both *BRCA1* variant plasmids (or WT or EV) variant and Firefly plasmid was used, along with 3.6 ng Renilla plasmid (total amount of DNA was 75.6 ng). For each sample (*BRCA1* variants, WT and EV plasmids), transfections were performed in three technical replicates, and in three biological replicates. For bleed-through test, one series of samples were transfected in the same manner but excluding the Renilla plasmid (total 72 ng DNA). After 24 h, cells were washed with PBS and harvested by addition of Passive Lysis Buffer (PLB) and plates were placed on a rocker for 15 min at RT. Cells were centrifuged for 10 min at 13000 rpm and 4 °C, and the supernatants were kept. The lysate was diluted 1:20 in PLB in a white microplate and protected from light. The transactivation reaction was measured on the microplate luminometer (Centro XS^3 LB 960). The instrument injectors were washed in two cycles of ten washes with mqH_2O. The background signal was measured for 2 sec. The injection speed for the reagents was set to medium. For LARII, 100 µl was dispensed, and the absorbance of firefly luminescence was measured at 560 nm for 5 sec. For Stop & Glo reagent, 100 µl was dispensed, and absorbance of *Renilla* luminescence was measured at 480 nm for 5 sec.

3.10.3 Analysis of TA assay data

Each measurement of TA activity yielded one signal for firefly luminescence and one signal for *Renilla* luminescence. The firefly luminescence signals were divided by their respective *Renilla* luminescence signals to normalise for transfection efficiency and cell number. Nine firefly/*Renilla* ratios were obtained for each variant (WT and EV had in total 18 datapoints as technical replicates were split in two for practical purposes). For the three technical replicates within each biological replicate, the average WT firefly/*Renilla* ratio was calculated and set to 100%. The three firefly/*Renilla* ratios for each of the BRCA1 protein variants were calculated as an average percentage of TA activity relative to WT activity.

Finally, based on the average TA activities of the three biological replicates, the average percentage of TA activity for each BRCA1 protein variant relative to WT, and standard deviations were calculated.

3.11 Analysis of *BRCA1* variants by Alamut Visual

BRCA1 variants were examined with Alamut Visual (version 2.15), a mutation analysis software, to retrieve data on minor allele frequency (MAF, the frequency with which a variant is observed in a healthy population) from the Genome Aggregation Database (gnomAD). In addition, variant classifications available in the database ClinVar were also accessed through Alamut. Within Alamut Visual, the built-in Protein Basic Local Alignment Search Tool (BLASTP) was used to obtain the a multiple sequence alignment of 13 orthologs for the original amino acids (the species included are human, chimpanzee, gorilla, orangutan, macaque, mouse, dog, cow, opossum, chicken, African clawed frog, Tetraodon puffer fish, and purple sea urchin), and the degree of conservation was assessed as low (conserved in 6 species or less), moderate (conserved in 7-10 species) or high (conserved in 11-13 species). In addition, the physicochemical properties of the original and new amino acids were compared (hydrophobic, polar, uncharged, positive/negative and or sulphur containing).

3.12 Statistical analysis

The statistical significance of the variants' transactivation activity was examined by Student's t-test in Excel. The t-test was two-tailed with unequal variance and analysed against the WT. The results were deemed significant if the p-value < 0.05.

4. Results

4.1. Preparation of variant plasmids and sequence verification

In order to functionally study the *BRCA1* variants, variant plasmids were prepared. The eleven *BRCA1* variant plasmids already made (Table 2.1) were transformed followed by colony culturing and plasmid purification (section 3.4). The eleven *BRCA1* variant plasmids were subsequently sequenced by Sanger sequencing (section 3.7), which followed by alignment to the reference sequence revealed that all variant plasmids harboured the expected substitution. In addition, the WT and EV plasmids were sequenced, which showed that the WT plasmid did not harbour unintended changes and that the EV did not contain the GAL4 DBD:BRCA1 BRCT fusion construct.

Seven *BRCA1* variant plasmids were made by site-directed mutagenesis (section 3.3), followed by transformation, and plasmid purification (section 3.4). After Sanger sequencing, the colonies harbouring the substitution of interest and no additional changes were grown in growth culture, and plasmids were purified (section 3.4) and re-sequenced (section 3.7). The sequencing results showed that all seven mutagenesis reactions were successful. There were no unintended changes, and the electropherograms of all sequences showed no signs of background noise.

4.2 Mycoplasma test and fingerprinting of the HEK293FT cell line

Prior to experiments with the HEK293FT cells, the cell line was tested for mycoplasma, as described (section 3.8.4) and analysed by agarose gel electrophoresis (section 3.6). The cells showed no sign of mycoplasma (Supplementary Figure 7.1). The HEK293FT cell line was also subjected to fingerprinting, which revealed that the STR-values of the analysis were in accordance with the DNA-profile (data not shown) of the HEK293FT cells (provided by the manufacturer).

4.3 Western blot analysis of GAL4 DBD:BRCA1 BRCT fusion protein variants expressed in HEK293FT cells

The pcDNA3 GAL4 DBD:BRCA1 BRCT (aa 1396–1863) produces a fusion protein consisting of the yeast DBD domain followed by the BRCT domain of BRCA1. The wild type fusion protein will be referred to as WT and variants as BRCA1 protein variants.

4.3.1 Initial optimalisation of protein loading

Prior to the investigation of the expression of different BRCA1 protein variants, the optimal loading quantity was assessed by titration of total protein lysate. HEK293FT cells were transfected with WT plasmid, lysed and subjected to SDS-PAGE electrophoresis and western blotting (section 3.9). Different amounts of total protein lysate (2-20 μg) were loaded, and the membrane was incubated with anti-GAL4 (DBD) (Figure 4.1).

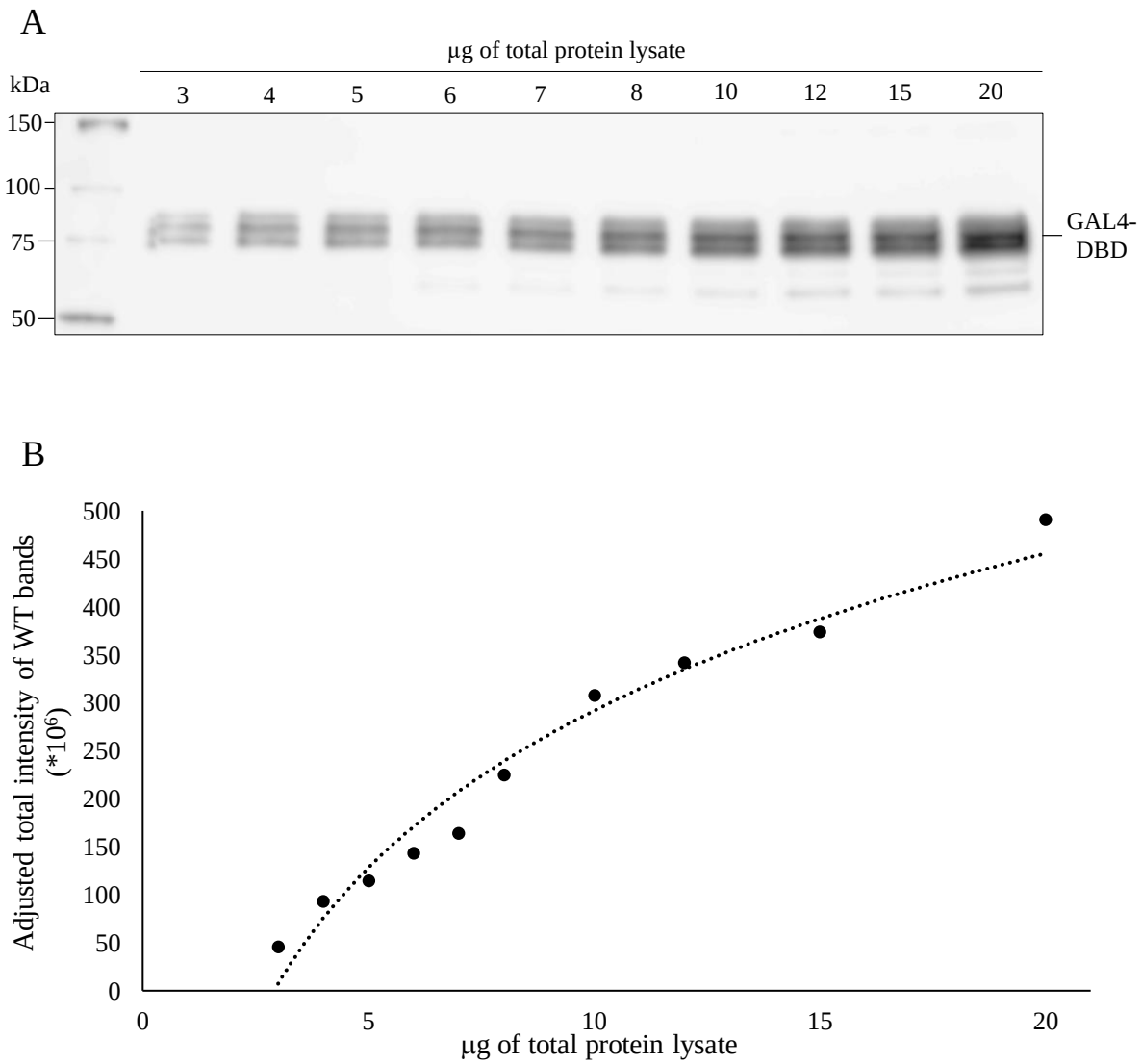

Figure 4.1: Western blot titration of the WT protein expressed in HEK293FT cells. HEK293FT cells were transfected with the WT plasmid, lysed after 48 hours and the total protein concentration was measured. In A, a range from 2-20 μg total protein lysate was analysed with SDS-PAGE and western blot using anti-GAL4 (DBD) antibody. In B, the adjusted total band intensity of the WT protein bands was plotted against the amount of loaded total protein lysate.

As expected, there was an increase in the WT protein band intensity in line with the increasing amount of total protein lysate loaded from left (3 ug) to right (20 ug) as shown in Figure 4.1A. In Figure 4.1 B, the adjusted total band intensities of the WT protein bands were plotted against the amount of total protein lysates loaded. Based on these findings, 7 µg total protein lysate appeared to be within the linear range of antibody binding, and an optimal loading quantity for subsequent analysis.

4.3.2 Western blot analysis of BRCA1 protein variants

To investigate the effect of the *BRCA1* missense variants on the protein expression level, HEK293FT cells were transfected with *BRCA1* variant plasmids, lysed and total protein concentration measured. For SDS-PAGE followed by western blot analysis (section 3.9), 7 µg of total protein lysate was used as determined by the initial titration experiment (section 4.3.1). The membranes were incubated with both anti-GAL4 (DBD) and anti-β-actin. In Figure 4.2, one representative image of the three biological replicates is shown. The (likely) benign and (likely) pathogenic controls are marked in green and red, respectively.

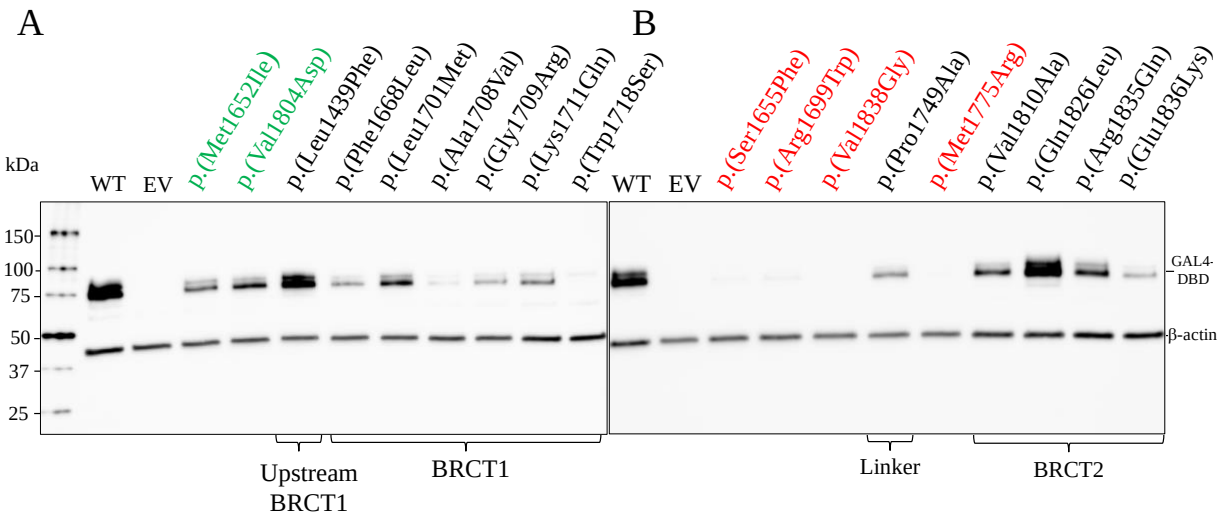

Figure 4.2: Western blot analysis of BRCA1 protein variants. The blots A and B represent one replicate of western blot, where HEK293FT cells were transfected with the *BRCA1* variants plasmids, EV and WT and 7 µg of total protein was analysed by SDS-PAGE followed by western blot. The anti-GAL4 (DBD) and β-actin were used (corresponding to the upper and lower bands, respectively). The (likely) benign and (likely) pathogenic controls are marked in green and red, respectively. The bottom brackets indicate the localisation of the variants of interest close to or in the BRCT domain of BRCA1.

The predicted molecular weight of the GAL4 DBD:BRCA1 BRCT WT fusion protein is 69 kDa. In accordance with this, bands at around 75 kDa and 80 kDa were seen for the WT and variant proteins in the blots when incubated with anti-GAL4 (DBD). As expected, all samples showed distinct actin bands at approximately 42kDa.

The BRCA1 protein variants p.(Ala1708Val) and p.(Trp1718Ser) exhibited a very faint or no protein band at all, similar to the (likely) pathogenic controls and EV. On the other hand, the BRCA1 protein variants p.(Leu1439Phe), p.(Leu1701Met), p.(Val1810Ala), p.(Gln1826Leu) and p.(Arg1835Gln) all showed bands intensities similar to, or even stronger than, the (likely) benign controls and the WT. The remainder of the BRCA1 protein variants, p.(Phe1668Leu), p.(Gly1709Arg), p.(Lys1711Gln), p.(Pro1749Ala) and p.(Glu1836Lys) showed intermediate band intensities.

4.3.3 Normalisation of western blot results

In order to correct for potential differences in loading quantities, the western blot results from incubation with anit-GAL4 (DBD) were normalised against β-actin and against total protein lysate using ImageLab version 6.1 (described in section 3.9.3). Representative stain-free blots with the total protein lysate used for normalisation are shown in Figure 4.3, where A and B corresponds to the blots A and B in Figure 4.2.

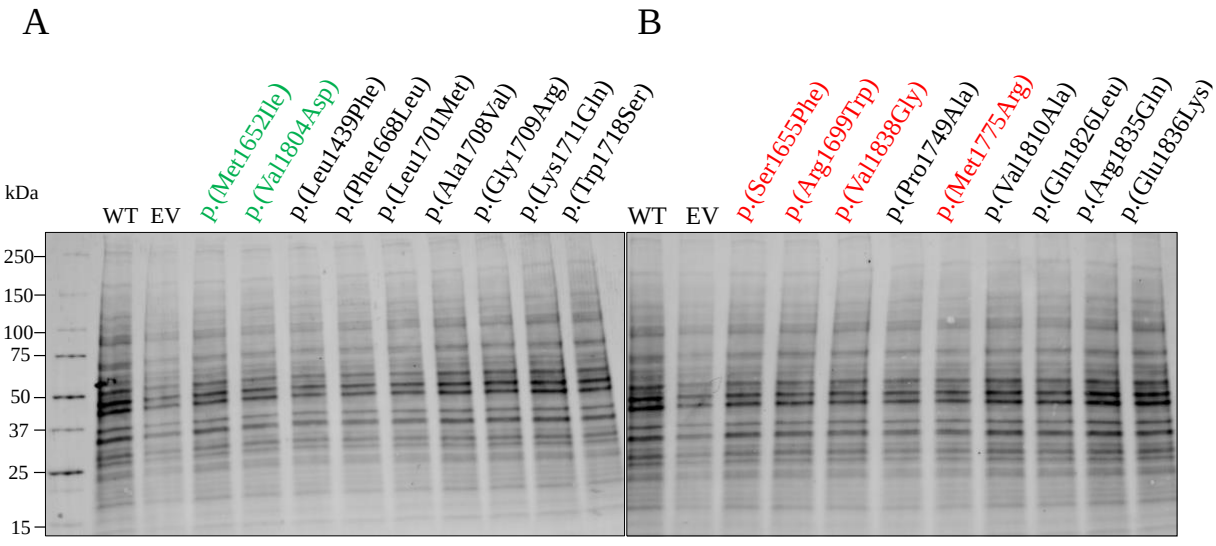

Figure 4.3 Total protein of HEK293FT cells transfected with *BRCA1* variant plasmids. Cells were transfected with the *BRCA1* variant plasmids, WT and EV and 7 µg of total protein lysate was loaded of each sample. The image was captured after the transfer of proteins to stain-free blots, before incubation with antibodies. The blots A and B here corresponds to blots A and B presented in Figure 4.2.

4.2.4 Relative expression of BRCA1 protein variants

After normalisation using both β-actin and total protein, relative protein expression level for each of the BRCA1 protein variants were calculated. The relative protein expression levels were expressed as a percentage of WT within each biological replicate (WT set to 100%). The averages and standard deviations were calculated based on the three biological replicates and are shown in Figure 4.4 A (relative protein expression normalised against β-actin) and 4.4 B (relative protein expression normalised against total protein).

In both cases, the relative protein expression levels for the (likely) benign controls are shown in green, while the (likely) pathogenic controls are shown in red (control variants are grouped to the left). Correspondingly, green and red dotted lines are drawn across the figures to provide a comparison of the protein variants to the controls. Note that these lines should not be used as absolute thresholds for benign and pathogenic cut offs, as they are drawn from a rather limited number of control variants used in this study.

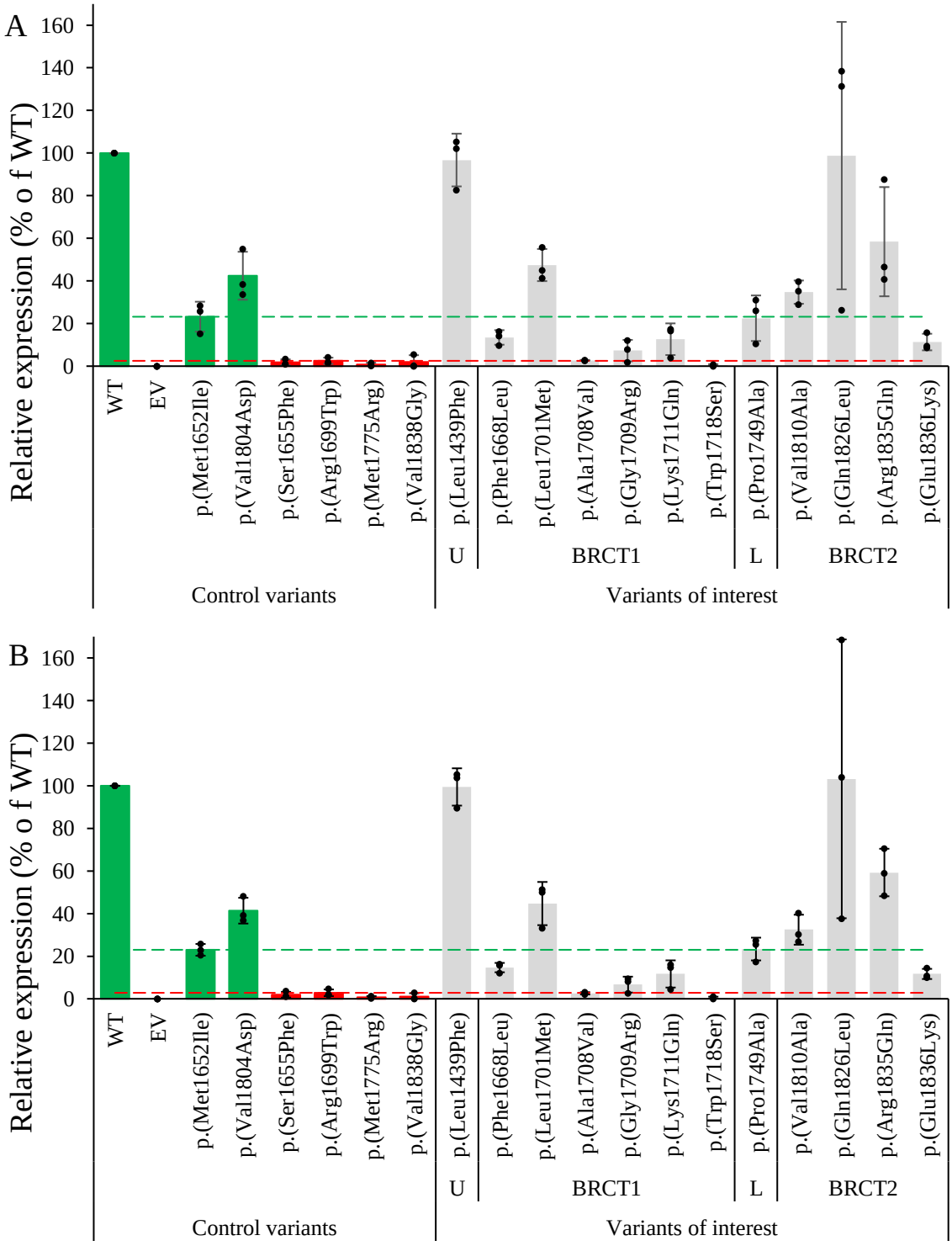

Figure 4.4: Relative expression of BRCA1 protein variants normalised against β-actin and total protein. HEK293FT cells were transfected with *BRCA1* variant plasmids, WT or EV plasmids and 7 µg of total protein lysate was analysed by SDS-PAGE and western blot using the anti-GAL4 (DBD) and anti-β-actin antibodies. The western blots were performed in three biological replicates, and the data was normalised against anti-β-actin in graph A and against total protein in graph B. The U denotes the region upstream of the BRCT domain, while L denotes the linker region connecting the BRCT1 and BRCT2 repeats. The height of each bar represents the mean relative protein expression level for each variant as a percentage of WT and one dot represents the value of one biological replicate. The error bars indicate standard deviation between the three replicates.

Comparison of Figure 4.4 A and B revealed that both normalisation methods resulted in similar relative protein expression levels of the BRCA1 protein variants (% of WT) (more details are provided in Supplementary Table 7.1).

In summary, the relative expression of the BRCA1 protein variants p.(Leu1439Phe), p.(Leu1701Met), p.(Val1810Ala), p.(Gln1826Leu) and p.(Arg1835Gln) showed protein expression levels similar to the WT or benign controls. On the other hand, the BRCA1 protein variants p.(Ala1708Val) and p.(Trp1718Ser) showed limited or no protein, similar to the pathogenic controls. The remaining BRCA1 protein variants, p.(Phe1668Leu), p.(Gly1709Arg), p.(Lys1711Gln), p.(Pro1749Ala) and p.(Glu1836Lys) harboured intermediate relative protein expression levels between the control groups, ranging from 7% to 23% (for both normalisation methods). For both normalisation methods, there seemed to be a trend that the variants with higher expression levels presented the largest standard deviations.

4.4 Transactivation Assay

4.4.1 Initial bleed-through test

The *Renilla* signal is measured after the initial firefly signal has been measured and quenched, as explained in section 3.10.1. To ensure that the Firefly signal is properly quenched and does not affect the subsequent detection of the *Renilla* signal, a bleed through test was first performed.

Two series of samples were prepared, one with all three plasmids (*BRCA1* variant plasmid/WT/EV in combination with both Firefly and Renilla plasmids, and one bleed through series transfected without the Renilla plasmid (only *BRCA1* variant plasmid/WT/EV in combination with Firefly). The variant p.(Leu1439Phe) was included in the bleed-through test, as this variant had shown to have a very high TA activity in a previous study performed by our group (unpublished data). The results from the transactivation assay for the three samples WT, EV and p.(Leu1439Phe) analysed transfected with and without Renilla is plotted in Figure 4.5.

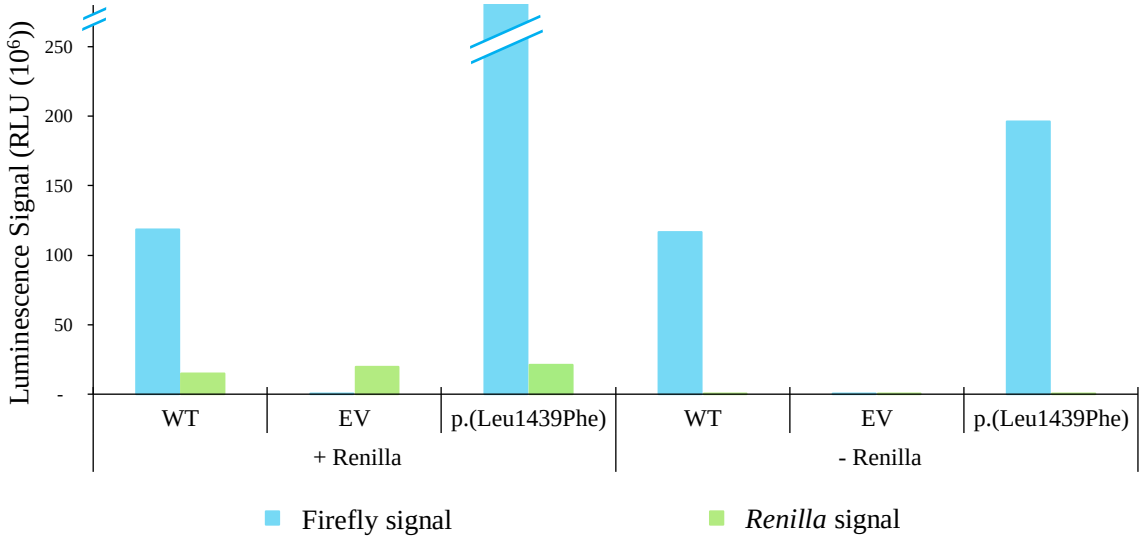

Figure 4.5: Test of bleed-through test of firefly signal in transactivation assay. HEK293FT cells were co-transfected with the WT/EV/p.(Leu1439Phe) in combination with Firefly (pGAL4-e1b-Luc) and with Renilla (phRG-TK) (marked as + Renilla). A parallel set of samples were prepared without Renilla (phRG-TK) (marked as – Renilla). The broken graph signifies that the signal exceeded the range of the microplate luminometer.

As expected, the firefly luminescence signal is consistent in all samples with and without Renilla plasmid, including EV, for which no signal is detected. The samples transfected with Renilla plasmid all showed similar *Renilla* luminescence signals levels. In accordance with the previous study (unpublished data), the variant p.(Leu1439Phe) harboured a high TA activity, higher than the WT. For the sample transfected with Renilla and p.(Leu1439Phe), the light emitted during the measurement of the signal exceeded the range of the instrument, resulting in overload (marked by a broken graph).

The bleed through samples transfected without Renilla plasmid consistently show little to no *Renilla* luminescence signal, independent of the strength of the firefly luminescence signals. Even for the variant p.(Leu1439Phe) with a higher firefly signal than the WT, the *Renilla* luminescence signal is barely noticeable. This indicates that there is no bleed through of the firefly luminescence disturbing the measurement of *Renilla* signal.

4.4.2 Transactivation activity (TA) of BRCA1 protein variants

To examine the TA activity of the *BRCA1* variant plasmids, EV and WT plasmid were transfected into HEK293FT cells along with the Firefly and Renilla plasmids as described in section 3.10.2.

After normalisation, the average TA activity of each variant was expressed as a percentage of WT activity (set to 100%). The results of the TA assay are shown in Figure 4.6. The control variants are grouped to the left whereas the variants of interest are listed by increasing amino acid number. The TA activities of (likely) benign controls are shown in green whilst the (likely) pathogenic controls are shown in red, and green and red dotted lines are drawn across the figures correspondingly. As mentioned in the section on relative protein expression (section 4.2.4), the lines are drawn from a limited number of controls, thus they should not be used as absolute thresholds for benign and pathogenic cut offs.

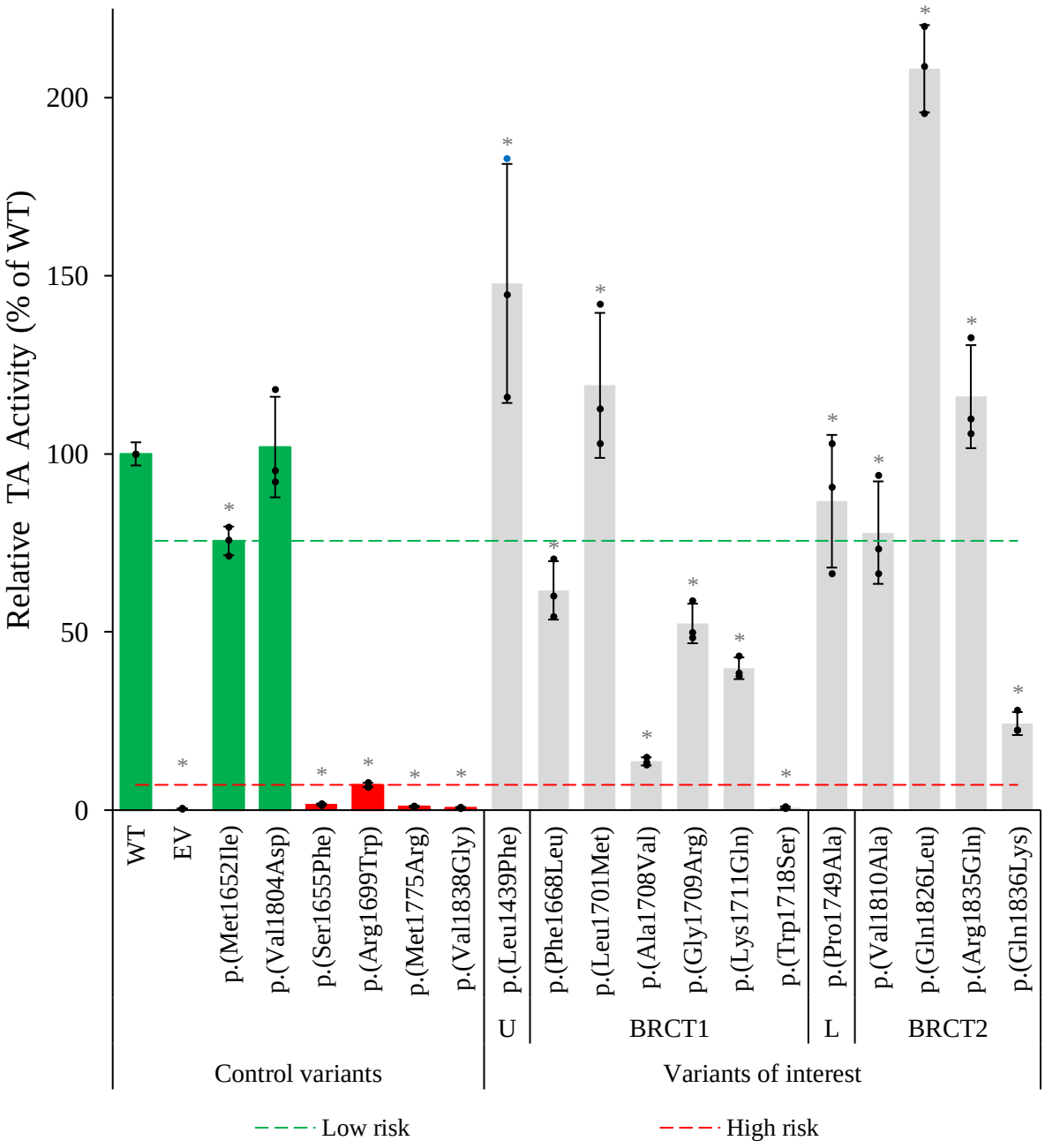

Figure 4.6 The transactivation activity of BRCA1 protein variants measured by dual-luciferase reporter assay. HEK293FT cells were co-transfected with *BRCA1* variant plasmids (or WT or EV) in combination with both Firefly (pGAL4-e1b-Luc) and Renilla (phRG-TK) plasmids. The cells were passively lysed followed by analysis of transactivation activity and normalisation. The TA analysis was performed in three biological replicates, where each replicate consists of three technical replicates. The relative TA activity of each variant was expressed as a percentage of WT activity (set to 100%). The three dots for each bar represent the average TA activity for each biological replicate. For the variant p.(Leu1439Phe), only seven data points exist as two out of three measurements in one of the biological replicates exceeded the range of the microplate luminometer (the dot representing this defective replicate is marked in blue).The mean relative TA activity from all three biological replicates is represented by the height of the bars and the standard deviations is represented by the error bars. The asterisk (*) represents statistical significance (p-value < 0.05). The U denotes the region upstream of the BRCT protein domain, while L denotes the linker region connecting the BRCT1 and BRCT2 repeats.

The BRCA1 protein variants p.(Leu1439Phe), p.(Leu1701Met), p.(Pro1749Ala), p.(Val1810Ala), p.(Gln1826Leu) and p.(Arg1835Gln) harboured similar or higher TA activities than the WT and the benign controls (ranging from 77.9-208.1%). On the contrary, the BRCA1 protein variant p.(Trp1718Ser) had no TA activity (0.8%), similar to that of the pathogenic controls. The remaining BRCA1 protein variants, p.(Phe1668Leu), p.(Ala1708Val), p.(Gly1709Arg), p.(Lys1711Gln), p.(Glu1836Lys) have intermediate TA activity (ranging from 13.7% to 61.7%), which lies between the TA activity of the (likely) benign and (likely) pathogenic controls, at 75.6% and 7.1 %, respectively.

4.5 Summary of functional results and *BRCA1* variant analysis by Alamut Visual

A summary of all data obtained in this study is listed in Table 4.1, including the relative protein expression levels and TA activities. The (likely) benign controls are shown in green, whilst (likely) pathogenic controls are shown in red. The physicochemical properties of the original and new amino acids are also listed. Additionally, as described in section 3.11, the MAF (from gnomAD) are retrieved from Alamut Visual, along with the degree of amino acid conservation of the altered residue based on alignment of *BRCA1* orthologues (13 orthologues in total). The level of conservation was defined as conserved for 11 to 13 species, moderate for 7 to 10 species and low for less than 7 species. Variant interpretations from ClinVar are included with the number of reports in parentheses, and the interpretations are based on the five-tier ACMG classification system (class 1: benign, class 2: likely benign, class 3: variant of uncertain significance (VUS), class 4: likely pathogenic and class 5: pathogenic). The interpretation of the variants at medical genetics' laboratories at Norwegian hospitals is included [68].

Table 4.1 Overview of *BRCA1* missense variants studied in this work. Benign (green) and pathogenic (red) control variants are grouped to the top of the table, while variants are listed in order of altered amino acid position. MAF, entries in ClinVar and Classification in "*BRCA1* Norway" is listed where available.

BRCA1 variant		Relative protein expression normalised against		TA activity (%)	Physicochemical properties of amino acids		MAF (%)	Degree of conservation (of 13)	ClinVar (number of entries)	Classification in "*BRCA1* Norway" [68]
Nucleotide substitution	Amino acid substitution	Total protein (% WT)	Actin (% of WT)		Original	New				
c. 4956G>A	p.(Met1652Ile)	23.0	23.2	75.6	Hydrophobic, sulphur	Hydrophobic	1.82	Moderate (7/13)	Class 1 (31) Class 2 (3) Class 3 (3)	Class 2 (1)
c. 5411T>A	p.(Val1804Asp)	41.4	42.4	101.9	Hydrophobic	Polar, negative charge	0.0095	Low (5/13)	Class 1 (5) Class 2 (5) Class 3 (2)	Class 2 (1)
c. 4964C>T	p.(Ser1655Phe)	1.9	1.8	1.6	Polar, uncharged	Hydrophobic, aromatic	-	High (12/13)	Class 3 (1) Class 4 (3) Class 5 (5)	Class 4 (1)
c. 5095C>T	p.(Arg1699Trp)	2.8	2.5	7.1	Polar, positive charge	Hydrophobic	0.0024	High (13/13)	Class 5 (34) Class 4 (1)	Class 5 (2)
c. 5324T>G	p.(Met1775Arg)	0.7	0.8	1.0	Hydrophobic, sulphur	Polar, Positive charge	0.0014	High (12/13)	Class 5 (17) Class 3 (1)	-
c. 5513T>G	p.(Val1838Gly)	1.0	2.0	0.7	Hydrophobic	Hydrophobic	-	High (11/13)	Class 4 (1) Class 3 (1)	Class 4 (3)
c. 4315C>T	p.(Leu1439Phe)	99.40	96.8	147.8	Hydrophobic	Hydrophobic, aromatic	0.0012	Low (6/13)	Class 2 (1) Class 3 (3)	Class 2 (1) Class 3 (3)
c. 5002T>C	p.(Phe1668Leu)	14.6	13.5	61.7	Hydrophobic, aromatic	Hydrophobic	0.0004	High (12/13)	-	Class 3 (1)
c. 5101C>A	p.(Leu1701Met)	44.7	47.5	119.2	Hydrophobic	Hydrophobic, sulphur	0.0032	High (13/13)	Class 3 (3)	-

Table 4.1 continued

Nucleotide substitution	Amino acid substitution	Total protein (% WT)	Actin (% of WT)	TA activity (%)	Original	New	MAF (%)	Degree of conservation (of 13)	ClinVar (number of entries)	Classification in "*BRCA1* Norway"
c. 5123C>T	p.(Ala1708Val)	2.6	2.7	13.7	Hydrophobic	Hydrophobic	0.0028	High (13/13)	Class 3 (14)	Class 3
c. 5125G>A	p.(Gly1709Arg)	6.7	7.3	52.4	Hydrophobic	Polar, positive charge	-	High (11/13)	Class 3 (5)	Class 3
c. 5131A>C	p.(Lys1711Gln)	11.7	12.6	39.8	Polar, positive charge	Polar, uncharged	-	High (12/13)	Class 3 (1)	Class 3
c. 5153G>C	p.(Trp1718Ser)	0.5	0.5	0.8	Hydrophobic, aromatic	Polar, uncharged	-	High (13/13)	Class 3 (2) Class 4 (2)	Class 4
c. 5245C>G	p.(Pro1749Ala)	23.4	22.5	86.7	Hydrophobic	Hydrophobic	0.0004	High (13/13)	Class 3 (2)	-
c. 5429T>C	p.(Val1810Ala)	32.4	34.7	77.9	Hydrophobic	Hydrophobic	0.0004	High (13/13)	Class 3 (7)	Class 3
c. 5477A>T	p.(Gln1826Leu)	103.3	98.8	208.1	Polar, uncharged	Hydrophobic	0.0025	Moderate (8/13)	Class 2 (1) Class 3 (2)	Class 2
c. 5504G>A	p.(Arg1835Gln)	59.3	58.4	116.1	Polar, positive charge	Polar, uncharged	0.0032	High (12/13)	Class 3 (10)	Class 3
c. 5506G>A	p.(Glu1836Lys)	11.7	11.3	24.3	Polar, negative charge	Polar, positive charge	0.0008	High (11/13)	Class 2 (1) Class 3 (5)	-

WT: wild type
TA: Transactivation
MAF: Minor Allele Frequency

5. Discussion

Pathogenic and likely pathogenic variants of *BRCA1* are known to cause HBOC, and affected individuals have an increased risk of developing breast, ovarian, pancreatic and prostate cancer [8]. On the other hand, benign and likely benign *BRCA1* variants are thought not to increase the risk of cancer. The increasing practice of genetic testing in medical genetics laboratories has led to a rise in the number of *BRCA1* VUS. These variants, for which there is often limited or conflicting evidence, create a challenge for physicians and genetic counsellors, as they cannot be used to make clinical decisions [65]. Functional studies of *BRCA1* VUSs can guide the assessment of the pathogenicity of such variants.

In this study, 11 *BRCA1* variants classified as VUS and one *BRCA1* variant classified as likely pathogenic have been studied. All variants are missense variants located within or close to the BRCT domains of BRCA1. The effect of the variants on protein expression level and on transactivation activity were examined, with the aim of generating new information about the VUSs and clarifying the cancer risk associated with the variants.

5.1 Comparison of normalising BRCA1 protein bands against total protein and actin

Traditionally, different housekeeping genes like actin, tubulin and GAPDH have been used for normalisation of western blot data to correct for potential loading differences. The housekeeping protein actin is widely used for this matter, including the laboratory at Department of Medical Genetics, Haukeland University hospital. However, normalisation against total protein is an emerging technique, which we therefore wanted to test. In order to compare the two different normalisation approaches (actin and total protein), three western blot replicates of BRCA1 protein variants were normalised with both methods.

As shown in Figure 4.4, normalisation against actin and total protein showed similar results with respect to relative protein expression levels for the in total 18 different *BRCA1* variants analysed (including controls). The minor differences in relative protein expression levels observed between the two normalisation methods indicate that both methods are reliable for the calculation of relative protein expression. Additionally, both normalisation methods presented similar levels of standard deviations; with a tendency of increased standard deviations for the

BRCA1 protein variants with high relative protein expression levels, as for p.(Leu1439Phe) and p.(Gln1826Leu). Although the differences were limited, the standard deviations of normalisation against actin showed higher standard deviations compared to normalisation against total protein for 12 of the 18 variants (Supplementary Table 7.1).

Normalisation against total protein does not depend on a single protein and is therefore more commonly recommended in the literature [82-87]. Moreover, it is reported that many common housekeeping proteins like actin, tubulin and GAPDH actually vary in expression [88-92]. The evaluation of the total protein is also useful as it reveals defects such as smeared bands, degraded protein samples and air bubbles from transfer. The three replicates performed in this study to compare normalisation against actin and total protein are however not sufficient to determine the best method, and normalisation against total protein should be further tested before possibly becoming the standard method at Department of Medical Genetics, Haukeland University hospital. For simplicity, further referral to relative protein expression levels in the discussion will refer to the normalisation against total protein.

5.2 Protein expression of *BRCA1* missense variants

The BRCT domain (aa 1646-1859) is highly conserved, even across distantly related species. Thus, the BRCT region is likely to be important for the function of BRCA1, and variants located within or close to this domain could potentially affect protein folding, stability and protein expression levels [93]. Western blot analysis of the *BRCA1* variants were performed to investigate the potential effect of the missense variants on the protein expression levels.

The theoretical molecular weight of the GAL4:DBD BRCA1 BRCT fusion protein is 69 kDa, of which the GAL4 DBD and BRCA1 BRCT domains are predicted to constitute 17 kDa and 52 kDa, respectively. The western blot analysis of all samples excluding EV produced visible protein bands of similar band patterns with the anti-GAL4 (DBD) antibody, which binds to the yeast specific GAL4 DNA-binding domain (DBD). The blots in Figure 4.2 show one band at 78-80 kDa and one at 75 kDa, close to the theoretical size of 69 kDa. The band sizes do not correspond to the individual sizes of the DBD domain (17 kDa) and BRCT domain (52 kDa), and therefore the 78-80 kDa and 75 kDa bands most likely represent the BRCA1 fusion protein and a corresponding degradation product, respectively. For EV, there is no band in the western blot probed with the anti-GAL4 (DBD) antibody, which indicates that there is no unspecific

binding of the anti-GAL4 (DBD) antibody to other proteins in the cell lysate. The benign controls have visible bands, proposing stable folded fusion proteins. The pathogenic controls have no or barely visible bands, suggesting that the BRCT domain of these variants are destabilised, which leads to degradation of these variants by the proteasome system of the cells. Another possibility could be that these variants were not expressed at gene level, which would be interesting to investigate by a gene expression (qPCR) assay (see section 5.7).

The BRCA1 protein variants p.(Leu1439Phe), p.(Leu1701Met), p.(Pro1749Ala), p.(Val1810Ala), p.(Gln1826Leu) and p.(Arg1835Gln) all showed high relative protein expression levels (23-103%), similar to the benign controls (23-41%) and WT (100%). This suggests that the substitutions in these positions are not deleterious for the overall stability of the BRCA1 protein variants. In contrast, the BRCA1 protein variants p.(Ala1708Val) and p.(Trp1718Ser) harboured low or non-detectable relative protein expression levels (1-3%), comparable to the protein expression levels seen for the pathogenic controls (1-3%). It appears that these missense changes disrupt their protein folding and stability, possibly making them prone to proteasomal degradation, which therefore lead to impaired protein expression levels. The BRCA1 protein variants p.(Phe1668Leu), p.(Gly1709Arg), p.(Lys1711Gln) and p.(Glu1836Lys) displayed relative protein expression levels between the benign and pathogenic controls (7-15%), creating a challenge in the evaluation of protein stability.

For a selection of the variants studied in this work, protein expression levels had been analysed previously by a former master student in our group (Nikara Pedersen), using the same anti-GAL4 (DBD) antibody. The inter-laboratory controls p.(Ala1708Val), p.(Gly1709Arg), p.(Lys1711Gln) and variant p.(Phe1668Leu) had all similar low and intermediate protein expression levels comparing this study with the previous study by Pedersen, ranging from 3-15% and 0-16%, respectively. The last inter-laboratory control p.(Gln1826Leu) exhibited relative protein expression levels higher than WT both in this study and in the earlier master's thesis.

Similarly, the variant p.(Leu1439Phe) exhibited WT-like levels in this study and higher than WT in the previous study. For the variants p.(Val1810Ala) and p.(Arg1835Gln), the observed expression levels in this work (32% and 59%, respectively) were higher than the previous analysis in our laboratory (18% and 34%, respectively), but in both analyses the variants were

in the intermediate expression level subgroup. In total, there is therefore good concordance between the findings of this study and the previous analysis in our laboratory.

The protein expression of the inter-laboratory controls p.(Lys1711Ser), p.(Glu1826Leu) and p.(Arg1835Gln) were previously analysed (not quantified) by Langerud et al. [77]. The variants p.(Lys1711Ser) and p.(Glu1826Leu) showed similar protein expression levels in this work compared to the previous studies in both HEK293F and MDA-MB-231 cell lines, but p.(Arg1835Gln) had a much stronger band than what was obtained in this work. When analysed by Jarhelle et al., the band for p.(Gly1709Arg) showed a distinct band, similar to the band intensity found for the inter-laboratory control in this study [78].

5.3 The transactivation of BRCA1 protein variants

5.3.1 Control variants and establishment of threshold levels

In order to investigate the functional consequences of the missense variants on the protein, TA assay was performed as described in section 3.10. The bleed-through test showed that the level of signal bleed-though was negligible.

As expected, the pathogenic variants p.(Ser1655Phe), p.(Arg1699Trp), p.(Met1775Arg) and p.(Val1838Gly) all harboured low TA activities, ranging from 1.0%-7.1%. The pathogenic control p.(Ser1655Phe) has previously been analysed by our laboratory and by Langerud et al., which provided similar results (4% and 5%, respectively), close to the 1.6% found in this work [77]. Langerud et al. also analysed p.(Arg1699Trp), and found a slightly higher TA activity (14%) than what obtained in this work (7.1%). The variant p.(Met1775Arg) harboured a TA activity of 1%, and is well documented as pathogenic and is a widely used control for TA assays [70, 80, 94-97]. The low activities observed for all the pathogenic controls are in line with their high degree of conservation.

The benign control p.(Met1652Ile) harboured a TA activity of 75.6%, similar to the 75-80% reported by Langerud et al. [77]. When studied previously by our group, p.(Met1652Ile) showed somewhat higher TA activity (104%). The consistently high TA activity is in accordance with the moderate degree of conservation and high frequency of this variant in the healthy population (1.82%). The benign control p.(Val1804Asp) harboured a WT-like TA activity (101.9%), and was the only variant not statistically significant compared to the WT. concordant with moderate

degree of conservation and with the findings by Langerud et al. Both benign controls were analysed by Lee et al. to have no folding defect, normal peptide binding activity, normal binding specificity and normal TA activity [98].

Based on data described above, the mentioned six control variants therefore appear as suitable controls for the TA assay, and were used for defining thresholds of high, intermediate and low activities when analysing the variants of interest. The boundary of high TA activity was set at the TA activity level observed for the lowest benign control variant (75.6%, p.(Met1652Ile)), thus all variants with activities above this value were categorised as (likely) benign (low risk). Correspondingly, the lower cut off level of TA activity was set to the highest TA activity of the pathogenic controls (7.1%, p.(Arg1699Trp)), and variants with activities below this value were considered as (likely) pathogenic (high risk). Variants harbouring TA activity levels between the upper and lower thresholds were considered as VUSs. As no gain-of-function mutation in the BRCT domain are known to be associated with high-risk, an upper boundary for benignity was not set [77]. Importantly, these thresholds are not absolute and are based on a limited set of data. Only six control variants were included in this thesis due to time limitation. Ideally, more control variants should be included to define a more definite threshold. According to Brnich et al., a minimum 11 total pathogenic and benign variant controls are recommended [71].

5.3.2 BRCA1 protein variants with high TA activity (77.9% - 208.1%)

In total, six variants exhibited high TA activities, further discussed below.

The variant **p.(Leu1439Phe)** showed a high TA activity (147.8%), in accordance with the previous result from our group (167%). One of the biological replicates of this sample was composed of only one measurement instead of three, which likely contributed to the high standard deviation of this variant. The change from leucine to phenylalanine could potentially be damaging, as phenylalanine is large and aromatic, in contrast to the smaller and hydrophobic leucine. However, both amino acids are hydrophobic and Leu1439 is poorly conserved and located almost 200 amino acids upstream of the BRCT domain. In total, the high TA activities suggest the variant to be of (likely) benign nature, in line with the entry of p.(Leu1439Phe) as a likely benign in ClinVar. This supports the reclassification of p.(Leu1439Phe) to likely benign.

According to the saturation genome editing assay performed by Findlay et al. (2018), the variant **p.(Leu1701Met)** is functional [79]. This is in line with the high TA activity (119.2%) found in

this study. This suggests that the substitution from leucine to methionine does not interfere with the transactivation activity of the BRCT domain, although Leu1701 is highly conserved. The high TA activity is likely due to the hydrophobic properties of both leucine and methionine. The three entries in ClinVar for p.(Leu1701Met) are VUS, but the high TA activity along with the saturation genome editing support a reclassification to likely benign.

The only variant included in this study that is located in the linker region between BRCT1 and BRCT2 is **p.(Pro1749Ala).** This variant harboured a high and TA activity (86.7%), although the original amino residue is highly conserved. In general, an amino acid change to or from proline could be damaging as it is the only cyclic amino acid with high conformational rigidity. Proline residues are frequently located in turn and loop structures of proteins and are believed to play a pivotal role during early stages of protein folding [99]. A study by Fernandes et al. extensively looking at missense changes across the BRCT linker region also found that p.(Pro1749Ala) to displayed high TA activity, while other altered residues in the linker appeared to have low TA activities [80]. Other missense substitutions of the proline residue in position 1749 to serine, threonine, leucine, glutamine and arginine harboured low TA activities and were interpreted as having compromised function [80, 98]. It appears from this study and that of by Fernandes et al., that p.(Pro1749Ala) is one of the few variants in the linker with benign characteristics. It was also characterised as functional also by the aforementioned study Findlay et al. and in a study by Wan et al. combining clinical phenotypes with saturation genome editing [79, 100]. In summary, the evidence is supportive of a reclassification to likely benign.

Similar to previous analysis by our group (68%) and the benign controls in this work, the TA activity for **p.(Val1810Ala)** was measured 77.9%, although Val1810 is highly conserved. Valine is substituted to alanine, and the two amino acids have similar characteristics (hydrophobic), with the major difference between these two being two methyl groups. The minor change in physicochemical properties could explain the high TA activity, and the variant was also reported as functional by Findley et al. [79]. These results sustain a reclassification to likely benign.

The variant **p.(Gln1826Leu)** was included in this study as an inter-laboratory control. In line with previous work performed by Langerud et al., and by our group, this variant showed TA activities higher than the WT (208%, 140% and 164%, respectively) [77]. This variant thus

performs well as an inter-laboratory control for high TA activity. The missense alteration replaces the polar Gln1826 with the hydrophobic leucine residue. However, Gln1826 is only moderately conserved, and replacement with a hydrophobic residue appears to improve the stability and or function of the BRCT domain. Additionally, p.(Gln1826Leu) was regarded as functional by Findlay et al. [79]. At the beginning of this thesis, this variant was classified as likely benign by Oslo University Hospital (OUH), but as VUS at University Hospital of North Norway (UNN) and Trondheim University Hospital (TUH). Through the national project aiming to reach consensus between medical genetic laboratories regarding classification of *BRCA1* variants ("*BRCA1* Norway"), the variant was reclassified to a likely benign variant by all hospitals, which is consistent with the work presented in this study [19].

The inter-laboratory control **p.(Arg1835Gln)** showed a high TA activity (208.1%) in line with the previous finding by our group and by Langerud et al., (108% and 115%, respectively) [77]. The consistent results confirm the variant as a suitable inter-laboratory control. The results from Findley et al. indicate that the change from the positive arginine to the polar, but uncharged, glutamine in residue 1835 causes an intermediate effect on the protein function [79]. Arginine in position 1835 has a high degree of conservation in line with the entries in ClinVar reporting the variant as a VUS. Despite the intermediate interpretation and classifications as VUS, the variant continually harboured TA activities similar to a low risk variant, which supports reclassification to likely benign.

5.3.3 BRCA1 protein variants with intermediate TA activity (61.7 – 13.7%)

Five variants harboured intermediate TA activities and will be further discussed below.

Despite the high degree of conservation for Phe1668, the variant **p.(Phe1668Leu)** had a TA activity at 61.7%, similar to previous analysis in our group showing a TA activity of 81%. However, in the previous study, based on a TA activity of 81%, this variant belonged to subgroup of high TA activity. Both phenylalanine and leucine have hydrophobic side groups, which could be a plausible explanation of the high activity of the variant, and it was also classified as functional by Findlay et al. [79]. Though p.(Phe1668Leu) appears to lean towards likely benign, it should remain as VUS as it harboured both intermediate and high TA activity measured by our group.

The inter-laboratory variant **p.(Ala1708Val)** harboured an intermediate TA activity of 13.7%. Similar results were observed by Langerud et al., where the p.(Ala1708Val) had a TA activity of 16% in MDA-MB-231, which was similar to the pathogenic control p.(Arg1699Trp) (14%) in the same study [77]. When analysed previously by our group, the variant harboured an intermediate activity of 16%, thus p.(Ala1708Val) consistently falls in the lower range of the intermediate scale. The low TA activity was somehow unexpected, as there are only minor differences in the side groups of the alanine and valine. However, the high degree of conservation for Ala1708 is in line with the low TA activity and entries in ClinVar as VUS. On one hand, earlier studies have suggested p.(Ala1708Val) to have pathogenic characteristics. A study looking at a combination of genetic, in silico, and histopathologic analyses found the variant to be likely pathogenic [101]. Additionally, Lee et al. reported the variant to have a folding defect and compromised binding sensitivity, binding activity and TA activity [86]. On the other hand, Findlay et al. reported p.(Ala1708Val) as functional [98], in accordance with homologous recombination (HR) assays; the variant displayed 47% of WT activity in homologous recombination assay by Lu et al., and almost a WT-like homologous recombination activity when analysed by Petitalot et al. [94, 102]. These conflicting results, especially between the different assays TA and HR, reveals that the variant should remain a VUS.

Previous analysis by Jarhelle et al. at UNN, revealed WT-like TA activities for the inter-laboratory control **p.(Gly1709Arg)** [78]. This contrasts to the intermediate TA activity of 52.4% measured in this work, and in the former analysis in our lab (43%) as well as by Langerud et al. (50 %) [77]. This reveals discrepancy for p.(Gly1709Arg), and the variant should be retested at UNN. The findings of intermediate TA activities, corresponding to a classification as VUS, is in concordance with its reports in ClinVar, as well as the high degree of conservation. The glycine to arginine substitution leads to a change from a small and hydrophobic to a larger and positively charged amino acid, respectively. Despite the change in amino acid properties and conserved Gly1709, the variant was described to be functional by Findlay et al. [79]. p.(Gly1709Arg) has consistent intermediate activity and therefore cannot be reclassified based on these results.

Consistent results of intermediate TA activity levels of approximately 40% was observed for the inter-laboratory control variant **p.(Lys1711Gln)** in this study, in the previous analysis performed in our lab, and by Langerud et al. [77]. These consistent results suggest the variant

to be a suitable inter-laboratory control for intermediate variants. In line with the intermediate TA activity, the Lys1711 residue is conserved, and the variant is reported as a VUS in ClinVar. In addition, the charge of the amino acid is changed from positive to neutral when replacing lysine with glutamine, potentially affecting the structure and function, although polarity is conserved. Despite the change in charge, Findlay et al. categorised the variant as functional [79]. The variant has a stable TA activity around 40%, and this intermediate functional result does not clarify the variants pathogenicity, suggesting it should remain status as VUS.

The variant **p.(Glu1836Lys)** showed an intermediate TA activity of 24.3 %. One might claim that activities in the lower range are expected as Glu1836 is conserved and glutamic acid is changed to lysine, which is a change from negative to positive charge. The variant has five entries in ClinVar as VUS, and one entry as likely benign. However, the likely benign entry is from 2013 and likely outdated. In the structure of the BRCT domain, the glutamic acid in position 1836 makes a hydrogen bond with arginine in position 1699, which is not possible if the residue is changed to lysine [103]. Findlay et al. regarded the variant as functional, which is in line with the results of Petitalot et al., who reported p.(Glu1836Lys) to be functional in HR assay [79, 94]. A study from Lee et al. combining cross-validation of structural and functional assays reports that the variant does not have a folding defect, but had compromised binding activity and compromised binding specificity [98]. In line with our study, Lee et al. also report of an uncertain level of transactivation (approximately 50%). As the results on this variant are conflicting, the variant should remain status as a VUS.

5.3.4 BRCA1 protein variant with low TA activity (< 7.1%)

The variant **p.(Trp1718Ser)** has not been studied by our group or a Norwegian laboratory previously, but is classified as likely pathogenic in Norway. In this study, the variant showed a TA activity level of 0.8% in the range of the pathogenic controls, strongly indicating that this missense variant impairs the functionality of the BRCT domain. The tryptophan in position 1718 is highly conserved, which initially suggests that a change could have structural and functional consequences. Furthermore, the amino acids tryptophan and serine differ considerably both in size and chemical properties, as tryptophan is aromatic while serine is smaller and polar. Correspondingly, the variant was deemed intermediate by Findlay et al. [79]. In a study by Lee et al., the variant showed compromised transactivation activity (approximately 12%), in addition to compromised binding specificity, binding activity and severe folding defect [98]. It is entered as a VUS in ClinVar twice and as likely pathogenic

twice. In Norway, the variant is classified as class 4, which is in line with its activity in this study [68]. In total, the low TA activity and pathogenic behaviours found by Lee et. al confirms this variant as likely pathogenic.

5.3.5 TA activities of the inter-laboratory controls

The organisation into high, intermediate or low TA activity were similar between laboratories (HUH, OUH and UNN) for four of the five of the inter-lab controls. The only variant that displayed inconsistency between laboratories was p.(Gly1709Arg), for which our laboratory twice reported of intermediate TA activities (43% and 52%) whilst Langerud et al. [77] reported 50% (low risk) and UNN reported of a WT-like activity. Both this study and that of Langerud et al. included six control variants (Langerud et al. had three benign and three pathogenic). This can partially explain the different cut off values, as 50% was interpreted as low risk by Langerud et al. while 43% and 52% were subgrouped as intermediate in our laboratory. This reflects the necessity of more control variants to precisely determine the threshold values for the cut-off borders, in order to be able to precisely discriminate between low, intermediate and high risk variants.

Nevertheless, the similar results for the other inter-laboratory controls provides the knowledge that although the TA assays are performed by different laboratories, the results are comparable and provide reliable and replicable results.

5.4 Comparison of protein expression and TA activity

In general, the TA activity of the BRCA1 protein variants was reflected in their relative protein expression with respect to the benign and pathogenic controls. All variants with high TA activity (p.(Leu1439Phe), p.(Leu1701Met), p.(Pro1749Ala), p.(Val1801Ala), p.(Gln1826Leu) and p. (Arg1835Gln)) also showed high levels of protein expression. Similarly, the BRCA1 protein variants with intermediate protein expression (p.(Phe1668Leu), p.(Gly1709Arg), p.(Lys1711Gln) and p.(Glu1836Lys)) similarly harboured intermediate TA activity. Consistency between protein expression and TA activity was also found for the variant with no protein expression (p.(Trp1718Ser)) and almost no TA activity. The only exception from this consistency was the p.(Ala1708Val) variant, which showed low protein expression level and intermediate TA activity. Although not comparable, the benign threshold at protein level (23%) is considerably lower than the equivalent cut off in the TA assay (75%). As p.(Gly1709Arg)

had protein expression level of 7% harboured TA activity of 52.4%, it appears that protein expression levels do not necessarily correlate to the level of protein activity.

5.5 Localisation of the variant in the BRCT domain

The localisation of the missense variant within (or close to) the BRCT domain did not appear to have a connection to protein expression and TA activity. Apart from the variant localised upstream of the BRCT domain (p.(Leu1439Phe)), there was no clear association between pathogenicity and the position of the variants in the BRCT1, linker or BRCT2 repeats. Both BRCT1 and BRCT2 domains had variants of low, intermediate and high protein expression and/or TA activity (including control variants). The variant located in the linker (p.(Pro1749Ala)) appears to be one of the few variants in the linker with benign characteristics [80].

5.6 Limitations of the study

In general, for both western blotting and transactivation assay, more control variants should ideally be included to better assess the cut off values for low and high risk thresholds. This would provide more secure grouping of the variants as (likely) benign and (likely) pathogenic [71].

In addition, western blot analysis is not a quantitative method, although it was used in a quantitative manner here. Even though the data was normalised, some variants presented with rather large standard deviations. Additionally, the stability of the fusion protein harbouring only the BRCT domain of BRCA1, might not reflect the native BRCA1 full length protein.

The TA assay provides valuable information on the functionality of the BRCT domain of BRCA1. However, there are a few limitations. Firstly, and perhaps most importantly, the assay is performed with only a smaller part of the *BRCA1* gene, and does not include the N-terminal RING. The RING domain binds the obligatory partner of BRCA1, BARD1, and together they have important roles in HR like DNA end resection and D-loop formation [31]. It would therefore be valuable to investigate the activity of the full-length BRCA1 in the TA assay. Secondly, only variants near or within the BRCT domain can be analysed by TA assay, limiting the assays' range. It is however possible that variants located outside of the BRCT domain can

be close to and influence the BRCT domain in the three-dimensional protein structure, and thereby affect the TA activity of the BCRT domain.

Despite these limitations, the TA assay is a reliable and sensitive assay that reflects the function of variants in the BRCT domain, making it well suited to assess the effect of missense variants in the BRCT domain of BRCA1.

5.7 Concluding remarks

In this study, 18 *BRCA1* missense variants located close to or in the BRCT domain of BRCA1 were examined; in total six (likely) benign or (likely) pathogenic controls, five inter-laboratory controls (all VUSs) and seven variants of interests (six VUS and one likely pathogenic).

The relative protein expression levels were normalised against both actin and total protein, which gave similar results for all the variants.

The TA assay showed similar activities between laboratories for four of the five inter-laboratory control variants, indicating that the results are comparable, and the assay is reliable.

Of the 11 VUS included in this study, evidence was provided to support reclassification for six. The five variants p.(Leu1439Phe), p.(Leu1701), p.(Pro1749Ala), p.(Val1810Ala) and p.(Arg1835Gln) presented benign characteristics, and are thus proposed reclassified to class 2 as (likely) benign. In line with the results presented here, p.(Gln1826Leu) was recently reclassified as likely benign [68]. Five variants were suggested to remain as VUS: p.(Phe1668Leu), p.(Ala1708Val), p.(Gly1709Arg) p.(Lys1711Gln) and p.(Glu1836Lys) due to scarce, conflicting or intermediate functional results. Further research, like HR assay and clinical data are needed in order to reclassify these variants. The two variants p.(Phe1668Leu) and p.(Gly1709Arg) leaned towards benign characteristics, and could possibly be reclassified if additional (likely) benign variants were included as controls. The pathogenicity of the variant p.(Trp1718Ser) was confirmed as likely pathogenic, and the recent classification of the variant as a VUS in ClinVar should be reconsidered.

There seems to be no association between the localisation of the missense variants in the BRCT1, linker or BRCT2 repeats and the functional activity for these variants.

Lastly, we highlight that the results presented here are supportive evidence based on functional analysis. The variants need to be assessed in combination with additional information like clinical data and family history before considering reclassification.

5.8 Further perspectives

As mentioned previously, the TA assay is performed with only a part of the BRCA1 protein (aa 1396–1863) expressed, excluding the N-terminal region containing the RING domain (1-109) which is known to be important for the native structure of the full-length protein. Therefore, performing TA assay with the full-length BRCA1 protein would be highly informative. Although not described in this thesis, several attempts were made to subclone a construct where the full-length *BRCA1* was inserted into the pcDNA3 GAL4 DBD WT plasmid by seamless cloning. Unfortunately, the seamless cloning procedure was not successful, and the assay was therefore carried out using only the C-terminal part of the BRCA1 protein. A successful seamless cloning experiment followed by TA assay would provide highly valuable information on TA activity of the full length BRCA1 protein in comparison to the activity of only a part of the protein, and is therefore a future prospect for the group.

In this study, the *BRCA1* variants were expressed in HEK293FT cells, and it would be highly interesting to repeat the experiments in a more tissue-relevant cell line. The triple negative breast cancer cell like MDA-MB-231 cell line was included in a transfection test procedure at an early stage in this thesis, but the HEK293FT cells exhibited a much higher transfection efficiency and was thus preferred. Further experiments in a breast or ovarian cancer cell line would increase the validity of the results presented here.

In addition to using a related cell line, qPCR analysis of the *BRCA1* variants would shed light on the variant's effects on mRNA level. Especially for variants of low relative protein expression levels and low TA activity, qPCR would be essential to determine whether the observations are due to reduced transcription or reduced protein stability. Pilot experiments of a gene expression assay by qPCR has therefore been performed with primers and probe designed to bind the yeast specific DBD domain in the fusion construct. Although the DBD probe bound well to the WT *BRCA1* cDNA, the specificity was not optimal as it also appeared

to bind cDNA generated from cells transfected with EV and untransfected cells. Thus, it is desired to further optimise of the qPCR assay to remove the background noise.

The variant interpretation in this study was mostly based on the relative protein expression levels and the TA activity observed. BRCA1 is a multifunctional protein, and several additional functional studies would be of interest, particularly analysing the HR activity of the variants.

6. References

1. Hanahan, D. and R.A. Weinberg, *The hallmarks of cancer.* Cell, 2000. **100**(1): p. 57-70.
2. Hanahan, D. and R.A. Weinberg, *Hallmarks of cancer: the next generation.* Cell, 2011. **144**(5): p. 646-74.
3. Hanahan, D., *Hallmarks of Cancer: New Dimensions*. Cancer Discov, 2022. **12**(1): p. 31-46.
4. Sung, H., et al., *Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries.* CA Cancer J Clin, 2021. **71**(3): p. 209-249.
5. Norway, C.R.o., *Cancer incidence, mortality, survival and prevalence in Norway.* , in *Cancer in Norway*. 2020. p. 126.
6. Wang, L.H., et al., *Loss of Tumor Suppressor Gene Function in Human Cancer: An Overview.* Cell Physiol Biochem, 2018. **51**(6): p. 2647-2693.
7. Pecorino, L., *Molecular Biology of Cancer*. 4th ed. 2016, New York, United States of America: Oxford University Press. 375.
8. Yoshida, R., *Hereditary breast and ovarian cancer (HBOC): review of its molecular characteristics, screening, treatment, and prognosis.* Breast Cancer, 2021. **28**(6): p. 1167-1180.
9. Hodgson, A. and G. Turashvili, *Pathology of Hereditary Breast and Ovarian Cancer.* Front Oncol, 2020. **10**: p. 531790.
10. Petrucelli, N., M.B. Daly, and T. Pal, *BRCA1- and BRCA2-Associated Hereditary Breast and Ovarian Cancer*, in *GeneReviews(®)*, M.P. Adam, et al., Editors. 1993, University of Washington, Seattle

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11. Kuchenbaecker, K.B., et al., *Evaluation of Polygenic Risk Scores for Breast and Ovarian Cancer Risk Prediction in BRCA1 and BRCA2 Mutation Carriers.* J Natl Cancer Inst, 2017. **109**(7).
12. Hall, J.M., et al., *Linkage of early-onset familial breast cancer to chromosome 17q21.* Science, 1990. **250**(4988): p. 1684-9.
13. Miki, Y., et al., *A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1.* Science, 1994. **266**(5182): p. 66-71.
14. Lin, D., et al., *A novel model to characterize structure and function of BRCA1.* Cell Biol Int, 2018. **42**(1): p. 34-44.
15. Clark, S.L., et al., *Structure-Function Of The Tumor Suppressor BRCA1.* Comput Struct Biotechnol J, 2012. **1**(1).
16. Hu, Q., et al., *Mechanisms of BRCA1-BARD1 nucleosome recognition and ubiquitylation.* Nature, 2021. **596**(7872): p. 438-443.
17. Joo, W.S., et al., *Structure of the 53BP1 BRCT region bound to p53 and its comparison to the Brca1 BRCT structure.* Genes Dev, 2002. **16**(5): p. 583-93.
18. Leung, C.C. and J.N. Glover, *BRCT domains: easy as one, two, three.* Cell Cycle, 2011. **10**(15): p. 2461-70.
19. Henderson, B.R., *Regulation of BRCA1, BRCA2 and BARD1 intracellular trafficking.* Bioessays, 2005. **27**(9): p. 884-93.
20. Mark, W.Y., et al., *Characterization of segments from the central region of BRCA1: an intrinsically disordered scaffold for multiple protein-protein and protein-DNA interactions?* J Mol Biol, 2005. **345**(2): p. 275-87.

21. Paull, T.T., et al., *Direct DNA binding by Brca1.* Proc Natl Acad Sci U S A, 2001. **98**(11): p. 6086-91.
22. Brázda, V., et al., *Strong preference of BRCA1 protein to topologically constrained non-B DNA structures.* BMC Mol Biol, 2016. **17**(1): p. 14.
23. Shiozaki, E.N., et al., *Structure of the BRCT repeats of BRCA1 bound to a BACH1 phosphopeptide: implications for signaling.* Mol Cell, 2004. **14**(3): p. 405-12.
24. Huyton, T., et al., *The BRCA1 C-terminal domain: structure and function.* Mutat Res, 2000. **460**(3-4): p. 319-32.
25. Wu, Q., H. Jubb, and T.L. Blundell, *Phosphopeptide interactions with BRCA1 BRCT domains: More than just a motif.* Prog Biophys Mol Biol, 2015. **117**(2-3): p. 143-148.
26. Mohammad, D.H. and M.B. Yaffe, *14-3-3 proteins, FHA domains and BRCT domains in the DNA damage response.* DNA Repair (Amst), 2009. **8**(9): p. 1009-17.
27. Sy, S.M., M.S. Huen, and J. Chen, *PALB2 is an integral component of the BRCA complex required for homologous recombination repair.* Proc Natl Acad Sci U S A, 2009. **106**(17): p. 7155-60.
28. Yu, X., et al., *The BRCT domain is a phospho-protein binding domain.* Science, 2003. **302**(5645): p. 639-42.
29. Gerloff, D.L., et al., *BRCT domains: A little more than kin, and less than kind.* FEBS Lett, 2012. **586**(17): p. 2711-6.
30. Clapperton, J.A., et al., *Structure and mechanism of BRCA1 BRCT domain recognition of phosphorylated BACH1 with implications for cancer.* Nat Struct Mol Biol, 2004. **11**(6): p. 512-8.
31. Tarsounas, M. and P. Sung, *The antitumorigenic roles of BRCA1-BARD1 in DNA repair and replication.* Nat Rev Mol Cell Biol, 2020. **21**(5): p. 284-299.
32. Yoshida, K. and Y. Miki, *Role of BRCA1 and BRCA2 as regulators of DNA repair, transcription, and cell cycle in response to DNA damage.* Cancer Sci, 2004. **95**(11): p. 866-71.
33. Powell, S.N. and L.A. Kachnic, *Roles of BRCA1 and BRCA2 in homologous recombination, DNA replication fidelity and the cellular response to ionizing radiation.* Oncogene, 2003. **22**(37): p. 5784-91.
34. Deng, C.X., *BRCA1: cell cycle checkpoint, genetic instability, DNA damage response and cancer evolution.* Nucleic Acids Res, 2006. **34**(5): p. 1416-26.
35. Hashizume, R., et al., *The RING heterodimer BRCA1-BARD1 is a ubiquitin ligase inactivated by a breast cancer-derived mutation.* J Biol Chem, 2001. **276**(18): p. 14537-40.
36. Mallery, D.L., C.J. Vandenberg, and K. Hiom, *Activation of the E3 ligase function of the BRCA1/BARD1 complex by polyubiquitin chains.* Embo j, 2002. **21**(24): p. 6755-62.
37. Zhang, W., et al., *BRCA1 regulates PIG3-mediated apoptosis in a p53-dependent manner.* Oncotarget, 2015. **6**(10): p. 7608-18.
38. Jasin, M. and R. Rothstein, *Repair of strand breaks by homologous recombination.* Cold Spring Harb Perspect Biol, 2013. **5**(11): p. a012740.
39. Radhakrishnan, S.K., N. Jette, and S.P. Lees-Miller, *Non-homologous end joining: emerging themes and unanswered questions.* DNA Repair (Amst), 2014. **17**: p. 2-8.
40. Krejci, L., et al., *Homologous recombination and its regulation.* Nucleic Acids Res, 2012. **40**(13): p. 5795-818.
41. Wright, W.D., S.S. Shah, and W.D. Heyer, *Homologous recombination and the repair of DNA double-strand breaks.* J Biol Chem, 2018. **293**(27): p. 10524-10535.
42. Venkitaraman, A.R., *Linking the cellular functions of BRCA genes to cancer pathogenesis and treatment.* Annu Rev Pathol, 2009. **4**: p. 461-87.

43. Greenberg, R.A., *Recognition of DNA double strand breaks by the BRCA1 tumor suppressor network.* Chromosoma, 2008. **117**(4): p. 305-17.
44. Sartori, A.A., et al., *Human CtIP promotes DNA end resection.* Nature, 2007. **450**(7169): p. 509-14.
45. Bunting, S.F., et al., *53BP1 inhibits homologous recombination in Brca1-deficient cells by blocking resection of DNA breaks.* Cell, 2010. **141**(2): p. 243-54.
46. Zhang, F., et al., *PALB2 links BRCA1 and BRCA2 in the DNA-damage response.* Curr Biol, 2009. **19**(6): p. 524-9.
47. Savage, K.I. and D.P. Harkin, *BRCA1, a 'complex' protein involved in the maintenance of genomic stability.* Febs j, 2015. **282**(4): p. 630-46.
48. Prakash, R., et al., *Homologous recombination and human health: the roles of BRCA1, BRCA2, and associated proteins.* Cold Spring Harb Perspect Biol, 2015. **7**(4): p. a016600.
49. Pascal, J.M., *The comings and goings of PARP-1 in response to DNA damage.* DNA Repair (Amst), 2018. **71**: p. 177-182.
50. Helsedirektoratet. *Nasjonal handlingsprogram med retningsinjer for diagnostikk, behandling og oppfølging av gynekologisk kreft*. 2016 31.05.2022; 3.:[Available from: https://www.helsedirektoratet.no/retningslinjer/gynekologisk-kreft-handlingsprogram.
51. Carvalho, M.A., F.J. Couch, and A.N. Monteiro, *Functional assays for BRCA1 and BRCA2.* Int J Biochem Cell Biol, 2007. **39**(2): p. 298-310.
52. Rosen, E.M., *BRCA1 in the DNA damage response and at telomeres.* Front Genet, 2013. **4**: p. 85.
53. Chai, Y.L., et al., *The second BRCT domain of BRCA1 proteins interacts with p53 and stimulates transcription from the p21WAF1/CIP1 promoter.* Oncogene, 1999. **18**(1): p. 263-8.
54. Zhang, H., et al., *BRCA1 physically associates with p53 and stimulates its transcriptional activity.* Oncogene, 1998. **16**(13): p. 1713-21.
55. Ouchi, T., et al., *BRCA1 regulates p53-dependent gene expression.* Proc Natl Acad Sci U S A, 1998. **95**(5): p. 2302-6.
56. Ongusaha, P.P., et al., *BRCA1 shifts p53-mediated cellular outcomes towards irreversible growth arrest.* Oncogene, 2003. **22**(24): p. 3749-58.
57. MacLachlan, T.K., R. Takimoto, and W.S. El-Deiry, *BRCA1 directs a selective p53-dependent transcriptional response towards growth arrest and DNA repair targets.* Mol Cell Biol, 2002. **22**(12): p. 4280-92.
58. Fabbro, M., et al., *BRCA1-BARD1 complexes are required for p53Ser-15 phosphorylation and a G1/S arrest following ionizing radiation-induced DNA damage.* J Biol Chem, 2004. **279**(30): p. 31251-8.
59. Anderson, S.F., et al., *BRCA1 protein is linked to the RNA polymerase II holoenzyme complex via RNA helicase A.* Nat Genet, 1998. **19**(3): p. 254-6.
60. Scully, R., et al., *BRCA1 is a component of the RNA polymerase II holoenzyme.* Proc Natl Acad Sci U S A, 1997. **94**(11): p. 5605-10.
61. Humphrey, J.S., et al., *Human BRCA1 inhibits growth in yeast: potential use in diagnostic testing.* Proc Natl Acad Sci U S A, 1997. **94**(11): p. 5820-5.
62. Monteiro, A.N., A. August, and H. Hanafusa, *Evidence for a transcriptional activation function of BRCA1 C-terminal region.* Proc Natl Acad Sci U S A, 1996. **93**(24): p. 13595-9.
63. Chapman, M.S. and I.M. Verma, *Transcriptional activation by BRCA1.* Nature, 1996. **382**(6593): p. 678-9.
64. Helsedirektoratet. *Nasjonalt handlingsprogram med retningslinjer for diagnostikk, behandling og oppfølging av pasienter med brystkreft*. 2019 23.03.2022 20.05.2022];

Available from: https://www.helsedirektoratet.no/retningslinjer/brystkreft-handlingsprogram.
65. 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, 2015. **17**(5): p. 405-24.
66. ENIGMA, *ENIGMA BRCA1/2 Gene Variant Classification Criteria* 2017: https://enigmaconsortium.org/.
67. Starita, L.M., et al., *Variant Interpretation: Functional Assays to the Rescue.* Am J Hum Genet, 2017. **101**(3): p. 315-325.
68. Hovland, H.N., et al., *BRCA1 Norway: comparison of classification for BRCA1 germline variants detected in families with suspected hereditary breast and ovarian cancer between different laboratories.* Fam Cancer, 2022.
69. Karczewski, K.J., et al., *The mutational constraint spectrum quantified from variation in 141,456 humans.* Nature, 2020. **581**(7809): p. 434-443.
70. Woods, N.T., et al., *Functional assays provide a robust tool for the clinical annotation of genetic variants of uncertain significance.* NPJ Genom Med, 2016. **1**: p. 16001-.
71. Brnich, S.E., et al., *Recommendations for application of the functional evidence PS3/BS3 criterion using the ACMG/AMP sequence variant interpretation framework.* Genome Med, 2019. **12**(1): p. 3.
72. Sruthi, C.K. and M. Prakash, *Amino acid impact factor.* PLoS One, 2018. **13**(6): p. e0198645.
73. Sosnay, P.R. and G.R. Cutting, *Interpretation of genetic variants.* Thorax, 2014. **69**(3): p. 295-7.
74. Møller, P., et al., *Genetic epidemiology of BRCA1 mutations in Norway.* Eur J Cancer, 2001. **37**(18): p. 2428-34.
75. Heramb, C., et al., *BRCA1 and BRCA2 mutation spectrum - an update on mutation distribution in a large cancer genetics clinic in Norway.* Hered Cancer Clin Pract, 2018. **16**: p. 3.
76. O'Neill, S.C., et al., *Distress among women receiving uninformative BRCA1/2 results: 12-month outcomes.* Psychooncology, 2009. **18**(10): p. 1088-96.
77. Langerud, J., et al., *Trans-activation-based risk assessment of BRCA1 BRCT variants with unknown clinical significance.* Hum Genomics, 2018. **12**(1): p. 51.
78. Jarhelle, E., et al., *Characterization of BRCA1 and BRCA2 variants found in a Norwegian breast or ovarian cancer cohort.* Fam Cancer, 2017. **16**(1): p. 1-16.
79. Findlay, G.M., et al., *Accurate classification of BRCA1 variants with saturation genome editing.* Nature, 2018. **562**(7726): p. 217-222.
80. Fernandes, V.C., et al., *Impact of amino acid substitutions at secondary structures in the BRCT domains of the tumor suppressor BRCA1: Implications for clinical annotation.* J Biol Chem, 2019. **294**(15): p. 5980-5992.
81. ThermoFisher. *293FT Cell Line*. [cited 2022 14.05]; Available from: https://www.thermofisher.com/order/catalog/product/R70007.
82. Neris, R.L.S., A.M.C. Dobles, and A.V. Gomes, *Western Blotting Using In-Gel Protein Labeling as a Normalization Control: Advantages of Stain-Free Technology.* Methods Mol Biol, 2021. **2261**: p. 443-456.
83. Vigelsø, A., et al., *GAPDH and β-actin protein decreases with aging, making Stain-Free technology a superior loading control in Western blotting of human skeletal muscle.* J Appl Physiol (1985), 2015. **118**(3): p. 386-94.
84. Gilda, J.E. and A.V. Gomes, *Stain-Free total protein staining is a superior loading control to β-actin for Western blots.* Anal Biochem, 2013. **440**(2): p. 186-8.

85. Ghosh, R., J.E. Gilda, and A.V. Gomes, *The necessity of and strategies for improving confidence in the accuracy of western blots.* Expert Rev Proteomics, 2014. **11**(5): p. 549-60.
86. Oh, K., *Technical Considerations for Contemporary Western Blot Techniques.* Methods Mol Biol, 2021. **2261**: p. 457-479.
87. Rivero-Gutiérrez, B., et al., *Stain-free detection as loading control alternative to Ponceau and housekeeping protein immunodetection in Western blotting.* Anal Biochem, 2014. **467**: p. 1-3.
88. Dittmer, A. and J. Dittmer, *Beta-actin is not a reliable loading control in Western blot analysis.* Electrophoresis, 2006. **27**(14): p. 2844-5.
89. Ferguson, R.E., et al., *Housekeeping proteins: a preliminary study illustrating some limitations as useful references in protein expression studies.* Proteomics, 2005. **5**(2): p. 566-71.
90. Lin, J. and C. Redies, *Histological evidence: housekeeping genes beta-actin and GAPDH are of limited value for normalization of gene expression.* Dev Genes Evol, 2012. **222**(6): p. 369-76.
91. Ruan, W. and M. Lai, *Actin, a reliable marker of internal control?* Clin Chim Acta, 2007. **385**(1-2): p. 1-5.
92. Thellin, O., et al., *Housekeeping genes as internal standards: use and limits.* J Biotechnol, 1999. **75**(2-3): p. 291-5.
93. Szabo, C.I., T. Worley, and A.N. Monteiro, *Understanding germ-line mutations in BRCA1.* Cancer Biol Ther, 2004. **3**(6): p. 515-20.
94. Petitalot, A., et al., *Combining Homologous Recombination and Phosphopeptide-binding Data to Predict the Impact of BRCA1 BRCT Variants on Cancer Risk.* Mol Cancer Res, 2019. **17**(1): p. 54-69.
95. Anantha, R.W., et al., *Functional and mutational landscapes of BRCA1 for homology-directed repair and therapy resistance.* Elife, 2017. **6**.
96. Towler, W.I., et al., *Analysis of BRCA1 variants in double-strand break repair by homologous recombination and single-strand annealing.* Hum Mutat, 2013. **34**(3): p. 439-45.
97. Quiles, F., et al., *Functional and structural analysis of C-terminal BRCA1 missense variants.* PLoS One, 2013. **8**(4): p. e61302.
98. Lee, M.S., et al., *Comprehensive analysis of missense variations in the BRCT domain of BRCA1 by structural and functional assays.* Cancer Res, 2010. **70**(12): p. 4880-90.
99. Krieger, F., A. Möglich, and T. Kiefhaber, *Effect of proline and glycine residues on dynamics and barriers of loop formation in polypeptide chains.* J Am Chem Soc, 2005. **127**(10): p. 3346-52.
100. Wan, Q., et al., *Clinical phenotypes combined with saturation genome editing identifying the pathogenicity of BRCA1 variants of uncertain significance in breast cancer.* Fam Cancer, 2021. **20**(2): p. 85-95.
101. Chenevix-Trench, G., et al., *Genetic and histopathologic evaluation of BRCA1 and BRCA2 DNA sequence variants of unknown clinical significance.* Cancer Res, 2006. **66**(4): p. 2019-27.
102. Lu, C., et al., *Patterns and functional implications of rare germline variants across 12 cancer types.* Nat Commun, 2015. **6**: p. 10086.
103. Williams, R.S. and J.N. Glover, *Structural consequences of a cancer-causing BRCA1-BRCT missense mutation.* J Biol Chem, 2003. **278**(4): p. 2630-5.

7. Supplementary data

7.1 The sequence of the WT GAL4 DBD:BRCA1 BRCT fusion protein.

The GAL4 DBD sequence is marked in grey, with the start codon written in red front. The following BRCA1 BRCT (aa 1396-1863) sequence is written in normal text, with the positions of the variants (original amino acids) marked in bold and underlined. The sequence was kindly provided by N. Iversen and J. Langerud.

AAGCAAGCCTCCTGAAAGATGAAGCTACTGTCTTCTATCGAACAAGCATGCGATA
TTTGCCGACTTAAAAAGCTCAAGTGCTCCAAAGAAAAACCGAAGTGCGCCAAGT
GTCTGAAGAACAACTGGGAGTGTCGCTACTCTCCCAAAACCAAAAGGTCTCCGCT
GACTAGGGCACATCTGACAGAAGTGGAATCAAGGCTAGAAAGACTGGAACAGCT
ATTTCTACTGATTTTTCCTCGAGAAGACCTTGACATGATTTTGAAAATGGATTCTT
TACAGGATATAAAAGCATTGTTAACAGGATTATTTGTACAAGATAATGTGAATAA
AGATGCCGTCACAGATAGATTGGCTTCAGTGGAGACTGATATGCCTCTAACATTG
AGACAGCATAGAATAAGTGCGACATCATCATCGGAAGAGAGTAGTAACAAAGGT
CAAAGACAGTTGACTGTATCGCCGGAATTCCAGAGGGATACCATGCAACATAAC
CTGATAAAGCTCCAGCAGGAAATGGCTGAACTAGAAGCTGTGTTAGAACAGCAT
GGGAGCCAGCCTTCTAACAGCTACCCTTCCATCATAAGTGACTCTTCTGCC**C**TTG
AGGACCTGCGAAATCCAGAACAAAGCACATCAGAAAAAGCAGTATTAACTTCAC
AGAAAAGTAGTGAATACCCTATAAGCCAGAATCCAGAAGGCCTTTCTGCTGACA
AGTTTGAGGTGTCTGCAGATAGTTCTACCAGTAAAAATAAAGAACCAGGAGTGG
AAAGGTCATCCCCTTCTAAATGCCCATCATTAGATGATAGGTGGTACATGCACAG
TTGCTCTGGGAGTCTTCAGAATAGAAACTACCCATCTCAAGAGGAGCTCATTAAG
GTTGTTGATGTGGAGGAGCAACAGCTGGAAGAGTCTGGGCCACACGATTTGACG
GAAACATCTTACTTGCCAAGGCAAGATCTAGAGGGAACCCCTTACCTGGAATCTG
GAATCAGCCTCTTCTCTGATGACCCTGAATCTGATCCTTCTGAAGACAGAGCCCC
AGAGTCAGCTCGTGTTGGCAACATACCATCTTCAACCTCTGCATTGAAAGTTCCC
CAATTGAAAGTTGCAGAATCTGCCCAGAGTCCAGCTGCTGCTCATACTACTGATA
CTGCTGGGTATAATGCAATGGAAGAAAGTGTGAGCAGGGAGAAGCCAGAATTGA
CAGCTTCAACAGAAAGGGTCAACAAAAGAATGTCCAT**G**GTGGTGT**C**TGGCCTGA
CCCCAGAAGAATTTATGCTCGTGTACAAG**T**TTGCCAGAAAACACCACATCACTTT
AACTAATCTAATTACTGAAGAGACTACTCATGTTGTTATGAAAACAGATGCTGAG
TTTGTGTGTGAA**C**GGACA**C**TGAAATATTTTCTAGGAATTG**C**G**G**GAGGA**A**AATGG
GTAGTTAGCTATTTCT**G**GGTGACCCAGTCTATTAAAGAAAGAAAATGCTGAATG
AGCATGATTTTGAAGTCAGAGGAGATGTGGTCAATGGAAGAAACCACCAAGGT**C**
CAAAGCGAGCAAGAGAATCCCAGGACAGAAAGATCTTCAGGGGGCTAGAAATCT
GTTGCTATGGGCCCTTCACCAACA**T**GCCCACAGATCAACTGGAATGGATGGTACA
GCTGTGTGGTGCTTCTGTGGTGAAGGAGCTTTCATCATTCACCCTTGGCACAGGT
G**T**CCACCCAATTGTGGTTG**T**GCAGCCAGATGCCTGGACAGAGGACAATGGCTTCC
ATGCAATTGGGC**A**GATGTGTGAGGCACCTGTGGTGACCC**G**A**G**AGTGGG**T**GTTGG
ACAGTGTAGCACTCTACCAGTGCCAGGAGCTGGACACCTACCTGATACCCCAGAT
CCCCCACAGCCACTACTGA

7.2 Comparison of the mean relative protein expression levels and standard deviations of western blot data normalised with actin and total protein.

The (likely) benign and (likely) pathogenic controls are marked in green and red, respectively.

Table 7.1 Relative expression of BRCA1 protein variants normalised against anti-β-actin

Normalization	Actin		Total protein	
Variant	Mean (% of WT)	Standard deviation	Mean (% of WT)	Standard deviation
WT	100	-	100	-
p.(Met1652Ile)	23.2	7.0	23.0	2.8
p.(Val1804Asp)	42.4	11.3	41.4	6.0
p.(Ser1655Phe)	1.8	1.4	1.9	1.3
p.(Arg1699Trp)	2.5	1.5	2.8	1.6
p.(Met1775Arg)	0.8	0.8	0.7	0.5
p.(Val1838Gly)	2.0	3.2	1.0	1.6
p.(Leu1439Phe)	96.8	12.4	99.4	8.8
p.(Phe1668Leu)	13.5	3.4	14.6	2.2
p.(Leu1701Met)	47.5	7.5	44.7	10.1
p.(Ala1708Val)	2.7	0.2	2.6	0.5
p.(Gly1709Arg)	7.3	5.0	6.7	3.7
p.(Lys1711Gln)	12.6	7.4	11.7	6.4
p.(Trp1718Ser)	0.5	0.4	0.5	0.5
p.(Pro1749Ala)	22.5	10.7	23.4	5.3
p.(Val1810Ala)	34.7	5.5	32.4	7.0
p.(Gln1826Leu)	98.8	62.8	103.3	65.4
p.(Arg1835Gln)	58.4	25.6	59.3	11.2
p.(Glu1836Lys)	11.3	3.9	11.7	2.4

7.3 Mycoplasma test of the HEK293FT cell line

The PCR products of the mycoplasma test were analysed by agarose gel electrophoresis.

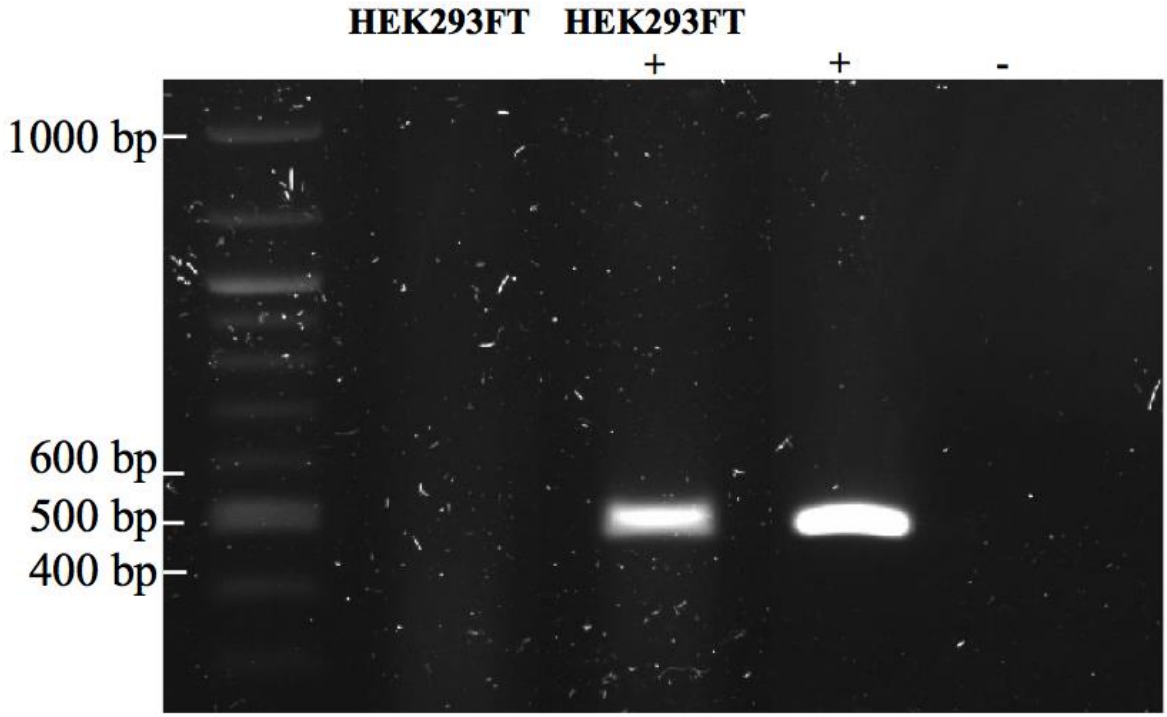

Figure 7.1. Mycoplasma test of the cell line HEK293FT. The cell line HEK293FT was tested for mycoplasma using the Universal Mycoplasma Detection Kit from ATCC. In contrast to the positive control showing a band of the expected size (464 bp), no bands were visible for the cell line or the negative control, indicating that there was no mycoplasma contamination in the cell culture. The + and – signs indicate positive and negative controls, respectively.