The BRCA2 DNA binding domain

MARSHALLPLAN SCHOLARSHIP

FINAL REPORT

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ABSTRACT

The tumor suppressor protein BRCA2 is a multi-domain protein required for efficient homologous recombination repair of DNA double-strand breaks (DSBs) and protection of stalled DNA replication forks. Germline mutations within BRCA2 predispose to breast, ovarian, pancreatic, and other tumors. Although the DNA binding domain (DBD) of BRCA2 is the most conserved part of BRCA2 among species, it has been shown to be not essential for cells to survive. In this project, we looked at the effects of the deletion of the BRCA2 DBD on mouse embryonic fibroblasts (EF) cells. We could not observe significant differences in proliferation or viability compared to wild-type cells. However, we could observe an increase in DNA damage as indicated by an elevated number in chromosomal aberrations in mutant cells. These results emphasize that the BRCA2 DBD is non-essential for survival in mouse EF cells but plays a role in DNA repair. We also started investigating another putative DNA binding domain, which could explain how BRCA2 is able to exert its function in the absence of the DBD.

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1. INTRODUCTION

1.1 The tumor suppressors BRCA1 and BRCA2

Germline mutations in the tumor suppressor genes *BRCA1* or *BRCA2* (Breast cancer early onset 1/2) are associated with an increased risk of developing cancer, especially breast and ovarian tumors. Studies have shown that by the age of 70, people carrying a mutation in *BRCA1* have a cumulative risk of 65% for breast and 39% for ovarian tumors. *Brca2* mutation carriers have corresponding cumulative risks of 45% and 11% (Antoniou *et al.*, 2003). The products of these genes play critical roles in double strand break (DSB) repair by homologous recombination (Tutt *et al*., 2002). The inability to correctly process this form of lesion leads to high levels of genomic instability in *Brca1* and *Brca2* germline mutation associated tumors (Moynahan & Jasin 2010). In normal cells genomic instability triggers cell-cycle arrest or apoptosis, thus additional somatic mutations that suppress their induction are required for tumorigenesis (Dasika *et al*., 1999). In the absence of functional BRCA1 or BRCA2, cells become reliant on other factors or pathways to repair DSBs. If these alternative pathways are compromised as well, cells become unable to repair lesions, leading to cell death. This concept, where a defect in either of two genes is compatible with life, but defects in both genes leads to cell death is called “synthetic lethality” and can be exploited for therapeutic approaches in cancer treatment. Therefore, BRCA1 and BRCA2 defective tumors are sensitive to treatment with poly(ADP-ribose) polymerase (PARP) inhibitors. (Bryant *et al.,* 2005; Farmer *et al*., 2005). The synthetic lethal interaction between PARP inhibition and loss of BRCA function is not yet fully understood, but it is suggested that PARP inhibition leads to an increase in DSBs or collapsed replication forks, that require the action of BRCA proteins to be resolved (van Wietmarschen & Nussenzweig, 2018).

Several Brca1 – and Brca2 “knock-out” mouse mutants have been generated by gene targeting. But unlike BRCA-deficient cells which are rapidly proliferating in situ, the deficiency of either of these proteins results in embryonic lethality in the mouse. The developmental failure was shown to be due to a proliferation defect associated with activation of the p53 pathway (Hakem *et al*., 1996; Liu *et al.,* 1996; Ludwig *et al*., 1997; Suzuki *et al.,* 1997). Similarly, mutations in Brca2 that truncate the protein prior to the BRC repeats, lead to embryonic lethality in mice homozygous for this mutation (Sharan *et al*., 1997). Together, these findings show the importance of homologous recombination for embryonic viability and development.
That BRCA2 loss triggers cell death was also shown in non-transformed human mammary epithelial cell lines. Loss of BRCA2 in these cells was shown to trigger DNA under-replication, leading to mitotic abnormalities, 53BP1 nuclear body formation in the ensuing G1 phase and G1 arrest. Loss of p53 partially rescued this phenotype (Feng and Jasin, 2017).

1.2 Homology directed repair of DNA breaks

DNA is constantly facing damage through exogenous agents and endogenous processes, leading to a variety of lesions as shown in Figure 1. Thus, a variety of DNA repair pathways have evolved to recognize and restore DNA structure and the information encoded within it (Hoeijmakers, 2001). DSBs can be generated by exogenous agents or endogenous processes, such as replication stress (Kass *et al*., 2016b). A DSB is considered to be the most deleterious type of DNA damage, as a single-unresolved DSB can cause cell death (Bennett *et al*., 1993). The two major mechanisms for DSB repair in vertebrates are non-homologous end-joining and homologous recombination (Chapman *et al*., 2012; Jasin & Rothstein, 2013). Non-homologous end-joining can occur during any stage in the cell cycle but is error-prone and results in small deletions or insertions. Homologous recombination repair in contrast, is primarily functional in the

S and G2 phases and is generally error-free because it uses a homologous DNA sequence, typically the sister chromatid, as a template for repair.

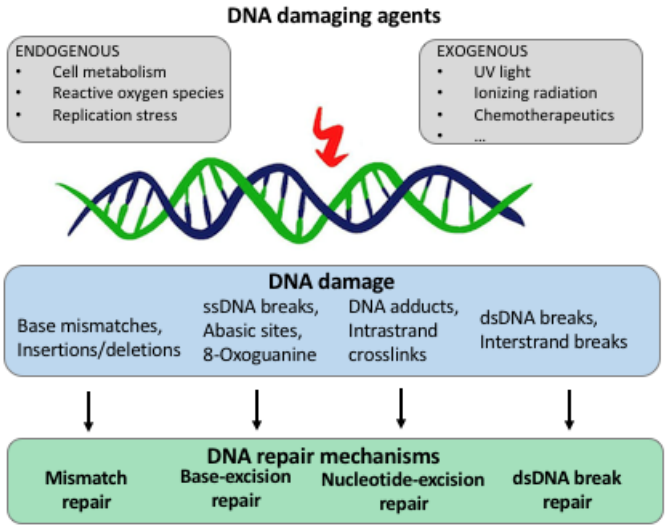

Figure 1 DNA repair mechanism. *A variety of exogenous or endogenous DNA damaging agents and processes lead to different types of DNA damage. Depending on the type of damage they can be repaired via a variety of pathways, such as mismatch repair (MMR), base-excision repair (BER), nucleotide-excision repair, homologous recombination (HR) or non-homologous end-joining (NHEJ).*

To initiate DNA repair via homologous recombination, BRCA1 co-localizes with the resection complex Mre11-RAD50-NBS1 (MRN) and directly interacts with the resection factor CtIP (Carney *et al.*, 1998; Sartori *et al*., 2007). Additionally, BRAC1 counteracts the NHEJ factor 53BP1 to facilitate DNA end resection and promote homologous recombination repair. The 3' single-strand ends are then stabilized by binding of replication protein A (RPA). BRCA1 also interacts with PALB2, which interacts with BRCA2. BRCA2 facilitates loading of the RAD51 recombinase. RPA is subsequently replaced by RAD51, which leads to the formation of RAD51 nucleoprotein filaments. These RAD51 filaments then mediate the search for homology within the sister chromatid, which is used as a template for repair (West, 2003; Sung & Klein, 2006). The products of homologous recombination are generally non-crossovers, and result in a single gene conversion (Jasin & Rothstein; 2013). A simplified model of homologous recombination repair is shown in Figure 2.

Consistent with their roles in homologous recombination repair, cells with complete loss of BRCA1 or BRCA2 function are highly sensitive to DNA lesions caused by chemotherapeutics (Bishop et al., 2000) and PARP inhibitors (Bryant *et al*.,2005; Farmer et al., 2005), that would normally be repaired through this pathway. Vice versa, tumors can become resistant to these agents by restoring homologous recombination.

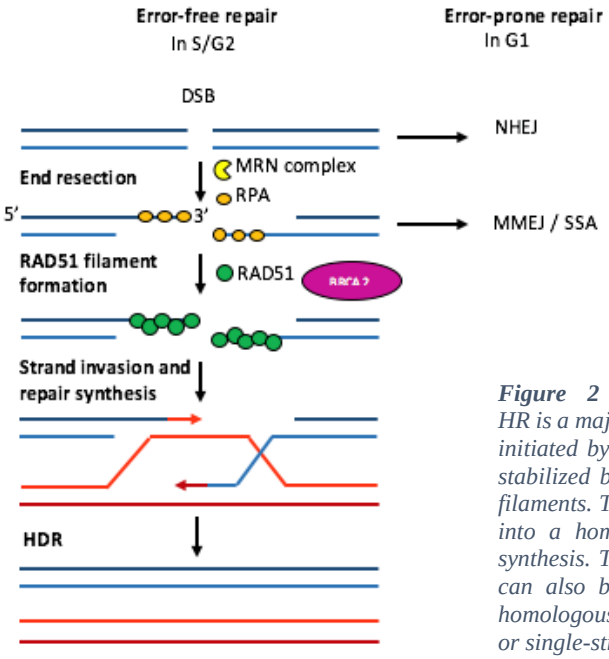

Figure 2 Basic scheme of homologous recombination (HR) repair. *HR is a major pathway for the error-free repair of DSBs in the S/G2 phase. HR is initiated by end-resection to generate 3' single strands. The ssDNA is initially stabilized by RPA, which is replaced by RAD51 recombinase to form RAD51 filaments. These filaments conduct the search for homology and strand invasion into a homologous DNA, usually the sister chromatid, followed by repair synthesis. The products of HR in DSB repair are usually non-crossovers. DSBs can also be repaired via error-prone DNA repair pathways, such as non-homologous end-joining (NHEJ), microhomology-mediated end joining (MMEJ) or single-strand annealing (SSA).*

1.3 Homologous recombination in meiosis

During meiosis, DSBs are deliberately created in the DNA of germ-line cells, to initiate genetic recombination between homologous parental chromosomes. The basic mechanism of meiotic recombination is similar to homologous recombination in somatic cells. However, there are a few key differences between these processes. During meiosis DSBs are deliberately introduced by the DNA endonuclease SPO11 (Keeney *et al*., 1997) and in addition to RAD51, another recombinase called DMC1, a meiosis specific paralog of RAD51, is involved (Bishop *et al.*, 1992; Yoshida *et al.*, 1998). Also, the search for homology occurs between homologous chromosomes, and leads to formation of at least one crossover per chromosome pair (Neale & Keeney, 2017).

2. BRCA2

2.1 Function of BRCA2

The role of BRCA2 in DNA damage repair via homologous recombination is to recruit RAD51 to DNA damage sites (Tarsounas *et al.* 2003, Yuan *et al*. 1999) and promote the formation of RAD51 filaments on single stranded DNA (ssDNA) that arises from end resection (Prakash et al, 2015). BRCA2 facilitates RAD51 filament assembly in two ways: 1) it helps RAD51 to overcome the inhibitory effect of RPA, a protein that binds to ssDNA with high-affinity and prevents RAD51 loading, and 2) it specifically promotes the assembly of RAD51 to ssDNA, through its preference to bind ssDNA instead of dsDNA (Jensen et al. 2010, Liu et al. 2010, Thorslund et al. 2010). The importance of *BRCA2* for maintaining genome integrity is demonstrated by early embryonic lethality in BRCA2 mutant mice (Connor et al., 1997; Ludwig et al., 1997; Sharan et al., 1997; Suzuki et al., 1997). In addition, BRCA2 is required for protection of newly synthesized DNA strands at stalled replication forks from degradation by MRE11 nuclease, by stabilizing the RAD51 filaments (Figure 3). Replication fork stalling can occur when a fork encounters a lesion or nucleotides become limiting. (Schlacher et al., 2011, 2012; Ying et al., 2012).

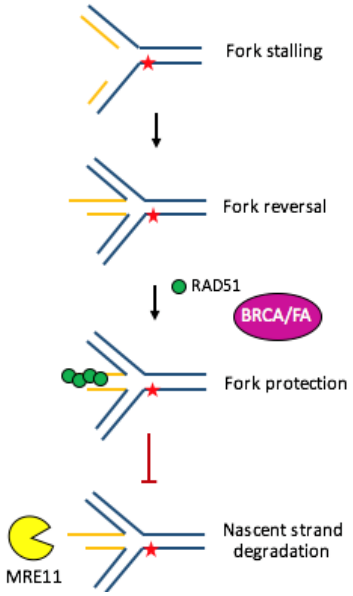

Figure 3 Fork protection of nascent DNA strands. *When the replication fork encounters a lesion (red star) or nucleotides become limiting, it can reverse itself. BRCA1 and BRCA2, as well as other Fanconi Anemia (FA) proteins stabilize RAD51 filaments at stalled forks and thereby protect the nascent strands from degradation by MRE11 and other nucleases.*

2.2 Domains & structure of BRCA2

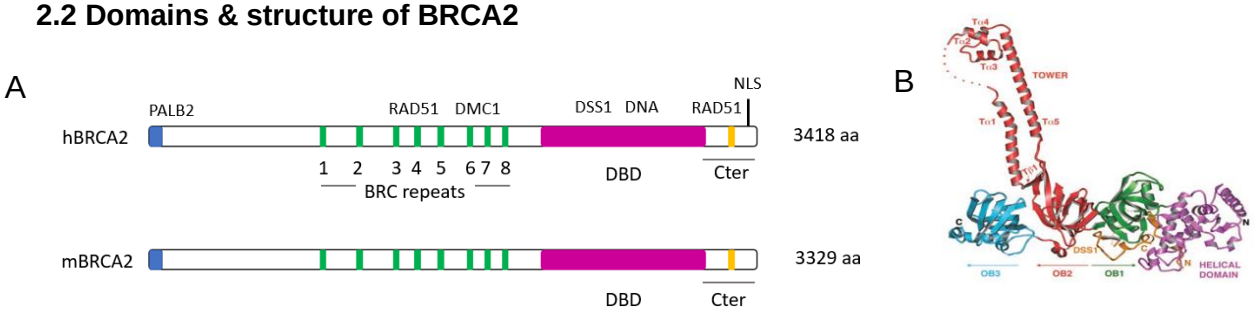

Figure 4 BRCA2 structure and domains*. (A) BRCA2 interacts with several proteins at different locations. It has a N-terminal PALB2 binding domain, BRC repeats that interact with RAD51, a DSS1 and DNA binding domain (DBD) and another RAD51 binding domain at the C-terminus. NLS = Nuclear Localization Signal (B) crystal structure of the DBD; The DBD consists out of three OB folds, a helical domain and a tower domain with a three-helix bundle. (Yang et al., 2002)*

Human BRCA2 is a large (3418 amino acids) polypeptide with multiple domains (Fig. 4A, Yang *et al.*, 2002). BRCA2 has many sites at which it interacts with different proteins as well as DNA to execute its function in homologous recombination. Upon binding to RAD51, DSS1 and ssDNA purified BRCA2 protein has been shown to undergo structural rearrangement. Its structural plasticity was proposed to be an important feature for its function in cells (Le *et al*., 2020; Sanchez *et al*., 2017; Sidhu *et al*., 2020).

At the amino (N) terminus, BRCA2 interacts with PALB2 (partner and localizer of BRCA2). PALB2 has also been identified as a breast cancer suppressor and is also linked to Fanconi anemia (Tischkowitz *et al.,* 2010). PALB2 interacts with both, BRCA1 and BRCA2 and was also shown to bind to DNA and RAD51 (Xia *et al*., 2006 & 2007; Buisson *et al.,* 2010; Dray *et al.,* 2010). In chicken DT40 cells, the DBD and the PALB2 interacting domain were found to have at least partially overlapping roles in focal accumulation of BRCA2 (Al Abo *et al*., 2014). Another study that investigated various BRCA2 domains using BRCA2 fusion peptides found that either the

PALB2 binding region or the DBD is sufficient for homologous recombination activity (Siaud *et al.,* 2011). However, loss of interaction between the two proteins was shown to affect homologous recombination and genome stability and increased cancer formation in mice (Hartford *et al.,* 2016).

Full length BRCA2 interacts with 5–6 RAD51 molecules (Jensen *et al*., 2010). Located in its central region, BRCA2 contains eight repeats of ~35 amino acids, the "BRC" repeats. The BRC repeats bind RAD51 and promote loading of multiple RAD51 monomers onto RPA coated ssDNA. At the same time BRCA2 prevents loading of RAD51 onto dsDNA substrates (Carreira *et al.* 2009; Jensen *et al.* 2010; Liu *et al.* 2010; Thorslund *et al*. 2010). Another RAD51 interacting domain is located in the C-terminal ~200 amino acid portion of BRCA2, encoded by exon 27. It helps to stabilize the RAD51 presynaptic filament (Sharan *et al.* 1997; Wong *et al*. 1997, O. R. Davies & Pellegrini, 2007) and is required for efficient homologous recombination (Moynahan *et al*., 2001). CDK phosphorylation of residue S3291 within the Cter at G2/M phase destabilizes Rad51 filaments and was found to link Rad51 disassembly to mitotic entry (Ayoub *et al.,* 2009). The C-terminal RAD51 interaction with RAD51 was also shown to be important for protection of nascent strands at stalled replication forks (Schlacher *et al*., 2012). Human BRCA2 was shown to diffuse as oligomeric complexes that sequester all detectable nuclear RAD51 (Reuter *et al.,* 2014). At repair sites BRCA2 and RAD51 were found to be arranged in separate locations, which indicates release structural rearrangements of BRAC2 leading to the release of RAD51 (Sanchez et al., 2017; Whelan *et al.,* 2018).

DMC1, a meiosis specific paralog of RAD51, was shown to interact with BRCA2 via BRC repeats 6-8 (Martinez *et al*., 2016). Previously it had also been reported to interact with BRCA2 via a Phenylalanine-Proline-Proline (PhePP) motif adjacent to the BRC repeats *in vitro* (Thorslund *et al.* 2007). However, mutation of a key residue within this site (Phe-2406) had no effect on meiotic progression and gametogenesis in the mouse (Biswas *et al.,* 2012).

DSS1, a small acidic 70 amino acid protein, interacts with BRCA2 via the helical domain, OB1 and OB2 (Yang *et al*., 2002). BRCA2-DSS1 interaction has been reported to promote homologous recombination in mammalian cells (Gudmundsdottir *et al*. 2004). Binding of DSS1 has been shown to mask a nuclear export signal (NES) in the DBD region, thereby promoting its retention in the nucleus (Jeyasekharan *et al*., 2013). Conversely, BRCA2 mutants abrogated for DSS1 binding display defects in homologous recombination (Siaud *et al.,* 2011).

The 736 amino acid C-terminal DNA binding domain (DBD) is the most evolutionarily conserved region of *BRCA2* (Yang *et al.*, 2002). The DBD is composed of a helical domain with a three-helix bundle (3HB), three oligonucleotide/ saccharide binding (OB) folds and a tower domain (Fig. 4B). The OB folds important for binding of ssDNA and can also be found in ssDNA binding proteins such as RPA. The helix-turn-helix (HTH) motif within the 3HB extending from OB2 has been implicated in dsDNA binding (Yang *et al.,* 2002 & 2005). In a study with BRCA2 fusion peptides, those where parts of the tower were deleted showed a defect in homologous recombination, which supports a role for the BRCA2 3HB in homologous recombination (Siaud *et al.,* 2011). Another N-terminal DNA binding domain was described in human BRCA2 (NTD). The NTD was proposed to bind to dsDNA/ssDNA junctions, thereby facilitating the loading and stabilization of RAD51 nucleoprotein filament onto RPA-coated ssDNA by the BRC repeats. However, the contribution of this NTD to DNA binding of BRCA2 is yet unknown(von Nicolai *et al*., 2016). Only recently, there have been indications that human BRCA2 has another putative DNA binding domain within the C-terminal region (CTRB). A mutation in this region was demonstrated to abolish BRCA2-DNA interaction (Patrick Sung, Keystone meeting presentation, September 2020).

2.3 Mutations and predisposition to cancer

The tumor suppressor BRCA2 is a large protein which is mutated in 50–60% of familial breast cancers. Hereditary monoallelic mutations in the tumor suppressor gene *BRCA2* increases the risk for breast cancer (Wooster *et al*. 1994). A recent study found the cumulative risk of developing breast cancer to be 69% by the age of 80 for BRCA2 mutation carriers (Kuchenbaecker, *et al.,* 2017). Germline mutations within this gene also increase the risk for ovarian and other tumors, such as prostate and pancreatic cancer, albeit at a lower frequency (Antoniou *et a*l., 2003; van Asperen *et al.,* 2005).

Biallelic *BRCA2* mutations lead to a subtype of Fanconi anemia (Fa-D1) (Ceccaldi *et al*., 2016). This rare genetic disorder is characterized by developmental issues and early childhood onset of tumors in the brain and kidney as well as hematological tumors (Howlett *et al*., 2002; Meyer *et al*., 2014). However, given the requirement of BRCA2 during embryogenesis in mice, it is required that at least one allele remains partially functional (Moynahan, 2002).

3. AIMS OF THIS PROJECT

Although the DBD is the most conserved region of BRCA2, it was shown to be not essential for cell survival. The human CAPAN1 pancreatic cancer cell line carries a truncating c.6174delT frameshift mutation. Intragenic deletion of this mutation and restoration of the open reading frame (ORF) lead to expression of BRCA2 isoforms that restored homologous recombination and thus conferred resistance to PARP inhibitors and DNA crosslink induction by cis-diaminedichloroplatinum(II) (cisplatin). Cisplatin creates intra- and inter-strand crosslinks which cause DNA damage and interfere with DNA repair mechanisms. Interestingly, most of the new BRCA2 species recovered from this experiment lacked the DBD, indicating that the BRCA2 DBD may be dispensable for homologous recombinatiomn under some conditions. However, these PARP inhibitor resistant clones showed genomic instability and abnormal karyotypes (Edwards *et al.,* 2008).

A recent study investigated the effect of deleting the DBD on dynamic activities of BRCA2. Their findings demonstrated that the deletion of the DBD had a significant effect on conformational changes in response to binding partners after DNA damage. In mouse ES cells, deletion of the BRCA2 DBD lead to an increase in sensitivity to DNA damaging agents and a reduction of homologous recombination activity by ~50%. Deletion of the BRCA2 DBD did not seem to affect BRCA2's localization to DNA damage sites, its diffusive activity or RAD51 accumulation. Analysis of purified human BRCA2 DBDΔ with scanning force microscopy (SFM) imaging showed that the DBD is relevant for conformational changes in response to ssDNA and RAD51. The DBD was also found to contribute to BRCA2's ability to interact with itself (Paul *et al*., 2021).

Travis White, a postdoc in the Jasin lab is currently investigating the effect of deleting the DBD in a non transformed setting. The Brca2 DBD encoding sequences (exons 16-26) were deleted in mouse embryonic stem (ES) cells and mice (Fig. 5). Consistent with findings from the study described above, homozygous *Brca2* DBDΔ mouse ES cells display a significant reduction in homologous recombination repair as well as increased sensitivity to olaparib and cisplatin compared to wild-type or heterozygous *Brca2* DBDΔ cells. Surprisingly however, mice carrying this mutation were found to be viable and did not show a phenotype that would indicate a defect in homologous recombination.

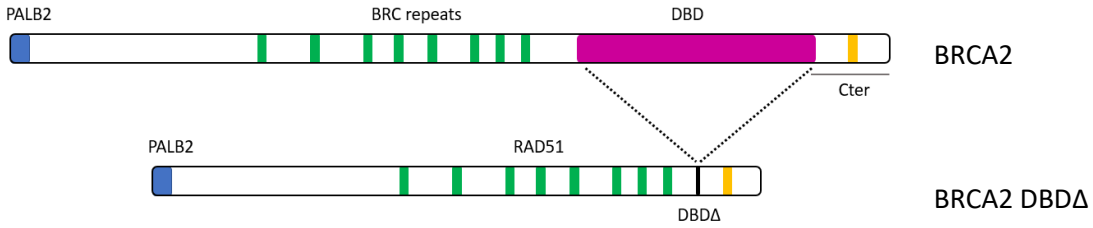

Figure 5 Scheme of BRCA2 DBDΔ*; exons 15-26 were deleted by CRISPRCas9 in mouse ES cells and in mice to lead to endogenously express a BRCA2 peptide lacking the DBD; also pCAGGs and PiggyBac vectors with mBRCA2 DBDΔ were used*

Deletion of the DBD cells lead to an increased sensitivity to DNA damaging agents in mouse ES cells. Here, we further examined the effect of DNA damaging agents on chromosomal stability by looking at chromosomal aberrations in *Brca2* DBDΔ mouse ES cells.

Given that deletion of the BRCA2 DBD did reduce its activity in homologous recombination-mediated DNA repair in mouse ES cells, it was surprising that this deletion did not lead to a

noticeable phenotype in the mouse. Here, we wanted to examine the effects that a deletion of the DBD has on mouse embryonic fibroblast (EF) cells. We used different assays to look at how the mutation affects cell viability and proliferation and whether these cells show signs of defective homologous recombination. Another question we were trying to answer in the course of this project was whether deletion of the Brca2 DBD has an effect on tumor formation in the mouse.

Also, recently a putative DNA binding domain has been described in the C-terminal region of human BRCA2 (Patrick Sung, Keystone Meeting presentation, September 2020). To evaluate whether the corresponding residues in mouse BRCA2 are important for DNA binding as well, we planned an experiment that will allow us to look at cell viability after a mutation in this domain.

4. RESULTS

4.1 Deletion of the BRCA2 DBD increases aberrations in mouse ES cells in response to DNA damaging agents

Previous experiments had demonstrated that homologous repair levels were reduced in DBDΔ mouse ES cells and that the mutant cells displayed increased sensitivity to DNA damaging agents such as cisplatin and olaparib (Paul *et al*., 2021).
Based on these findings, we here assessed whether treatment with these agents lead to an increase in chromosomal aberrations. Chromosomal aberrations in mouse ES cells were analyzed after replication disruption by PARP inhibitor olaparib or DNA crosslinking by cisplatin.
We did not observe a significant difference in untreated cells. Olaparib treated *Brca2*$^{DBD\Delta/DBD\Delta}$ cells however displayed an increase in aberrations such as chromosome breaks, acentric chromosomes, fusions, or radials compared to the wildtype *Brca2* cells (Fig 6). Cisplatin seemed to not have an effect on either of the cells at a concentration of 316nM cisplatin. A possible explanation could be, that we only treated the cells for 5h, which might have been too short to observe an effect. At a concentration of 1000nM the cells expressing the BRCA2 mutant displayed a higher instability.
The finding that the PARP inhibitor lead to elevated chromosomal instability in the *Brca2*$^{DBD\Delta/DBD\Delta}$ cells supports that these cells have a defect in homologous recombination repair.

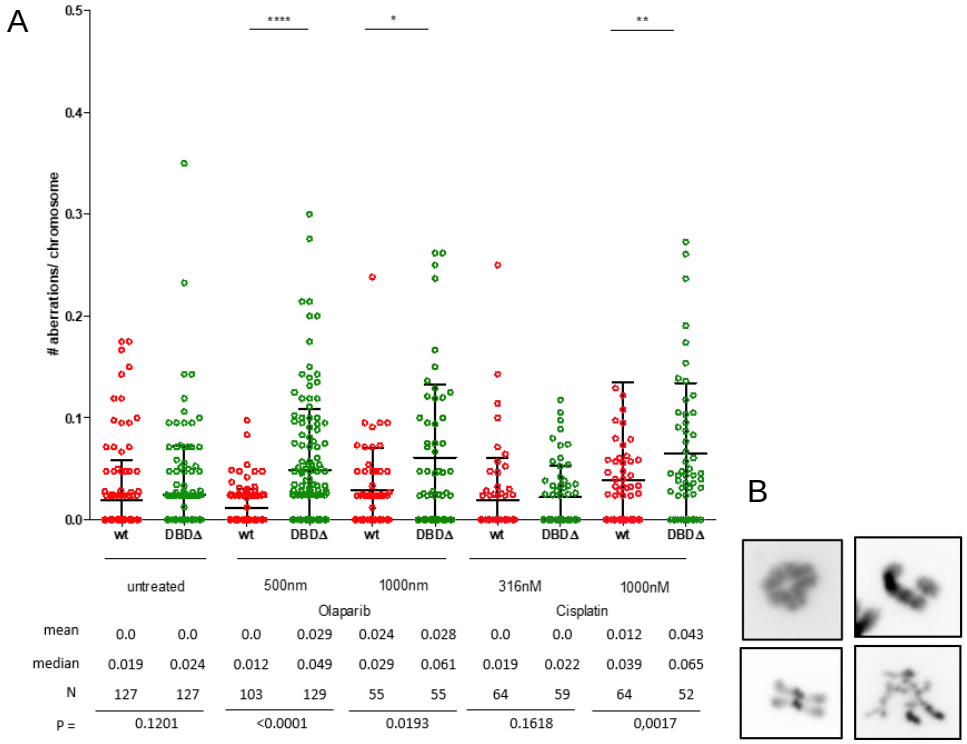

Figure 6 Effect of genotoxic agents on BRCA2 DBDΔ mouse ES clones. *(A) Mouse ES cells (wild-type or mutant) were treated with cisplatin (500 nM / 1000 nM) or olaparib (316 nM / 1000 nM), arrested in the metaphase and aberrations were analyzed. (B) The image shows some observed aberrations such as (from left to right): radials, break, fusion, decondensed chromosome*

4.2 Contribution of the BRCA2 DBD to homologous recombination repair function in mouse EF cells

As a previous experiments by Travis White in mouse ES cells had indicated, that deletion of the BRCA2 DBDΔ was able to support cell viability and proliferation in these cells to the same extent as wild-type BRCA2, we wanted to test if this was also the case in mouse EF cells.

Cell proliferation was examined through a WST-1 based approach over a time course of four days. Here, the BRCA2 DBDΔ cells showed a slight but significant reduction in proliferation compared to the wild type cells (Fig. 7A). Cell cycle analysis (done by Travis White) in mouse ear fibroblasts showed no significant difference between wild type and homozygous BRCA2 DBDΔ cells (7B). However, there seem to be slightly more cells in G2 phase in the DBD mutant cells. This can also be observed for other DNA repair mutants and could be because spontaneous lesions arising during S-phase are resolved slower in these cells.

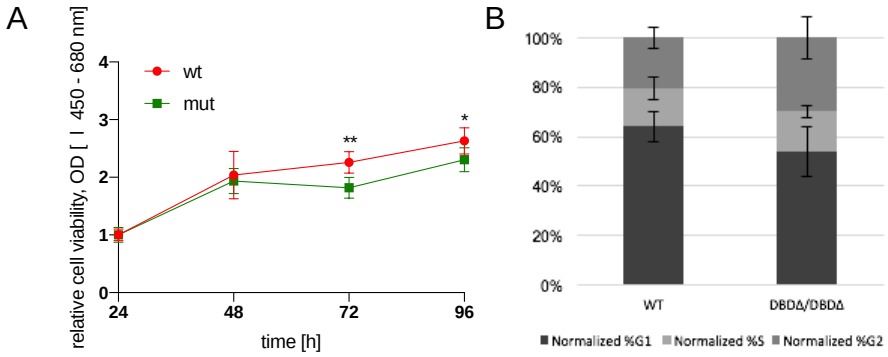

Figure 7 Brca2DBDΔ/DBDΔ mouse EF cells*. A) Proliferation; Two cell lines per genotype, combined genotypes, B) Cell cycle analysis in ear fibroblasts, n=3, by Travis White*

Proliferation of wild-type and Brca2$^{DBD\Delta/DBD\Delta}$ mouse EFs growing in the presence of increasing amounts of olaparib or cisplatin was also assessed using the WST-1 based assay. The mouse EF cells did not display a high sensitivity to cisplatin or olaparib and there was no significant difference between wild type or mutant cells with either treatment (Fig. 8A, B).

Treatment of mouse ES cells with either cisplatin or olaparib displayed a significant difference in chromosomal aberrations between wild-type and BRCA2 DBDΔ mutant cells. The experiment was repeated in mouse EF cells. In the untreated cells, the Brca2$^{DBD\Delta/DBD\Delta}$ mutants showed an increase in breaks and fragments compared to the wildtype. Like in the mouse ES cells, the Brca2$^{DBD\Delta/DBD\Delta}$ mouse EF cells also showed increased levels of aberrations when treated with olaparib (500nM). Treatment with cisplatin (316nM) increased the number of aberrations in both genotypes to the same extent (Fig. 8C). Aberration profiles cells and examples of aberrations as observed in the mouse EF cells are shown in Fig 8E.

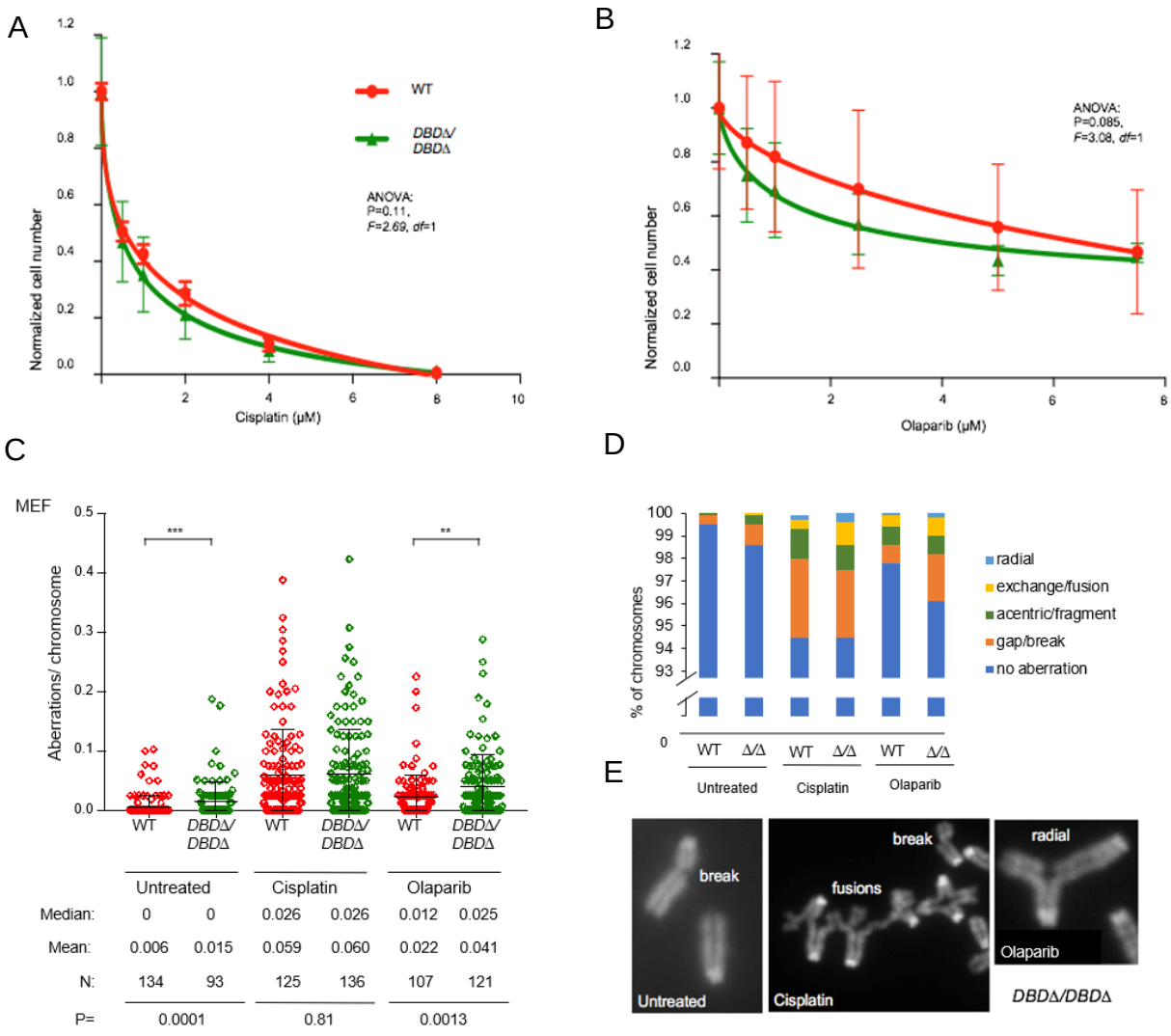

Figure 8 Effect of genotoxic agents on BRCA2 DBDΔ mouse EF clones*. (A) Treatment with cisplatin for 4 days, N=3 (B) olaparib, 4 days (C) Aberrations per chromosome in untreated cells or cells treated with cisplatin (316 nM) or olaparib (500 nM), N =3 (D) Aberration-profiles E) Example pictures of different aberrations as found in the mouse EF cells*

4.3 Deletion of the BRCA2 DBD abolishes DSS1 interaction, but not RAD51 or DMC1 binding

DSS1 interacts with BRCA2 via residues located in the helical domain and OB fold 1 and 2 within the DBD region (Yang *et al.,* 2002). As DSS1 binds mostly within the DBD, the BRCA2-DSS1 interaction should be impaired with the BRCA2 DBDΔ mutants. There are also some DSS1 interacting regions within exon 15, which are still present in the mutant version. To ensure that DSS1 could not bind to BRCA2 DBDΔ via the residual interacting residues in exon 15 and, we conducted co-immunoprecipitation experiments. We co-expressed the BRCA2 constructs and myc-tagged DSS1 in HEK293T cells and used the myc-tag to pull down the complex.

BRCA2 interacts with RAD51 and DMC1 in regions adjacent to the DBD (Fig. 4). The deletion of exons 16-26 in BRCA2 DBDΔ should impair binding of DSS1 to BRCA2 but not of RAD51 or DMC1. To ensure, that this was the case, we conducted co-immunoprecipitations with these proteins and BRCA2 wt and DBDΔ constructs. We used FLAG-tagged full-length mouse BRCA2 wild-type and BRCA2 DBDΔ constructs and co-expressed myc-DSS1, RAD51 or DMC1 in HEK293T cells. The complexes were pulled down with the help of either anti-myc beads in the case of DSS1 or anti-FLAG beads for RAD51 and DMC1.

These experiments confirmed that the deletion of the DBD abolishes BRCA2 interaction with DSS1 (Fig 9A), but its ability to interact with RAD51 and DMC1 is maintained (Fig.9B, C).

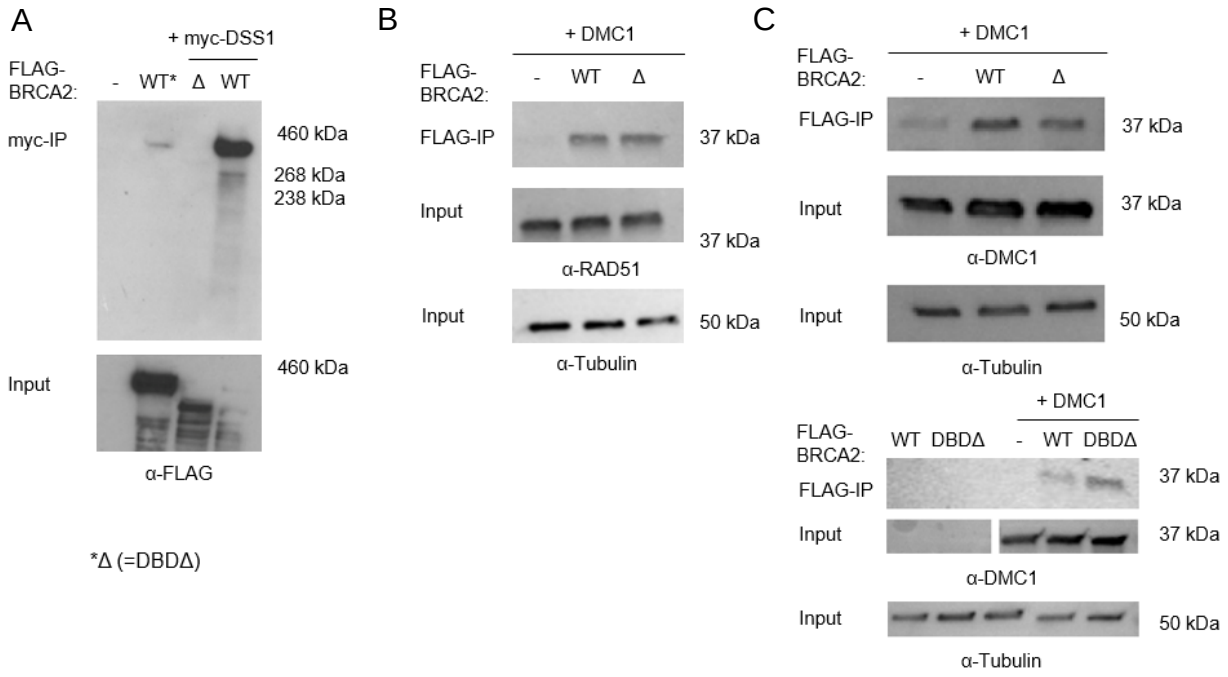

Figure 9 Effect of deleting the BRCA2 DBD on interaction with DSS1, RAD51, DMC1 (*A) Deletion of the DBD abolishes BRCA2-DSS1 interaction; WT* = negative control: wildtype BRCA2 + RAD51 (no myc-tag) (B) Deletion of the DBD does not impair BRCA2-RAD51 interaction, (C) Deletion of the DBD does not affect BRCA2 DMC1 interaction*

4.4 Effect of Brca2 DBDΔ deletion on tumor formation

The product of the retinoblastoma (*Rb*) gene is a tumor suppressor protein that plays a role in the negative control of progression of the cell cycle from G_1-S phase (Weinberg, 1995). Mutations in this gene, or in upstream regulators, lead to a predisposition to retinoblastoma, osteosarcoma and other cancers (Giacinti and Giordano, 2006). Mouse embryos with homozygous mutations in the *Rb* gene (*Rb+/-*) die in midgestation. Rb heterozygous mice (*Rb+/-*) are predisposed to develop tumors in the pituitary gland and the thyroid gland as well as neuroendocrine tumors (Hinds *et al.,* 1992; Lee *et al.,* 1992; Clarke *et al.,* 1995; Lee *et al.,* 1994, Nikitin *et al*., 1994). This predisposition can be exploited to accelerate tumor formation in mouse models to observe effects of other mutations.

A Rb tumor model will be used to study the effect of deleting the BRCA2 DBD on tumor formation in mice. Mice heterozygous for *Rb* ($Rb^{+/-}$) and for *Brca2* DBDΔ ($Brca2^{DBD\Delta/+}$) were crossed and

Brca2 $^{+/+}$; *Rb* $^{+/-}$ and *Brca2* $^{DBD\Delta/\ DBD\Delta}$; *Rb* $^{+/-}$ mice were obtained. Their pituitary glands were removed at the age of 60 days and 90days (Nikitin and Lee, 1996). The glands were fixed, stained with Ki67, a marker for proliferation (Scholzen and Gerdes, 2000) and compared for the formation of early atypical proliferates (EAP), which are an indicator for tumor formation.

We could not detect any EAPs in the glands (example pictures shown in Fig. 14), however also our positive control, a gland from a *Brca2lex1/lex2* mouse did not show any EAPs as expected. These mice express a BRCA2 peptide deleted for the C-ter ~200 amino acids encoded by exon 27, which is important for DNA repair via homologous recombination and fork protection function of BRCA2. Carla Abreu, a postdoc in the Jasin lab, had previously shown that this mutation led to EAPs in mice, however they were from a 126/B6 background, whereas in this study we used mice with a B6 (93.75% B6) background. The experiment will be repeated in a 126/B6 background to evaluate if there is an experimental problem or if there are differences between the used mouse strains.

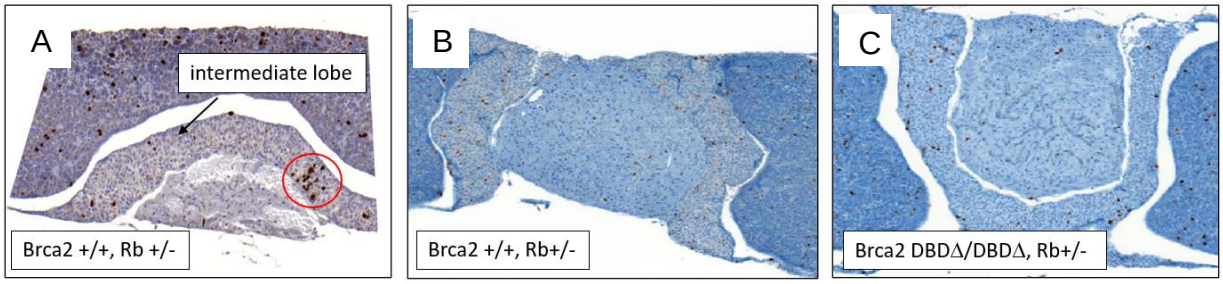

Figure 14 Early Atypical Proliferates in mouse pituitary glands *(A) example of a pituitary gland section from a Rb$^{+/-}$ in 129/B6, by Carla Abreu, red circle: EAP (B) a gland from wt Brca, Rb+/- in B6 (C) a gland from Brca2 DBDΔ, in B6*

4.5 Contribution of the BRCA2 CTRB to DNA binding

It was recently discovered that human BRCA2 has another putative DNA binding domain located in the C-terminal RAD51 binding (CTRB) region (Patrick Sung, Keystone meeting presentation, September 2020). We inserted the corresponding mutation in mouse *Brca2* into a wildtype *mBrca2* PiggyBac vector. We deleted the DBD from wild-type and CTRB mutated vectors, to generate the *Brca2* DBDΔ and DBDΔ + CTRB PiggyBac vectors. Loss of functional BRCA2 was shown to trigger cell death. We will use these constructs to study the contribution of the C-terminal DNA binding domain to BRCA2 function and test whether BRCA2 is able to support RPE-1 cell viability when deleted for both DNA binding domains.

FLAG-tagged mouse BRCA2 wild-type and DBDΔ cDNAs will be stably integrated and expressed in the human Doxycyclin (Dox)-inducible Cas9 cell line RPE-1 (McKinley *et al.,* 2017). Two guide RNAs were designed that target endogenous human *BRCA2*, but not mouse *Brca2*. The guide RNAs will be introduced in the cells via lentiviral transduction. Addition of Dox to the cells leads to Cas9 mediated disruption of endogenous *BRCA2*. Disruption of *BRCA2* after Dox induced expression of Cas9 in these cells leads to cell lethality.

The lentivirus does not only carry the two guides but also a gene encoding GFP (Fig. 10A). To assess whether the *mBRCA2* constructs are able to complement loss of endogenous *BRCA2*, we

will look at GFP-positive cells with FACS analysis. A scheme of our general approach is shown in figure 10B.

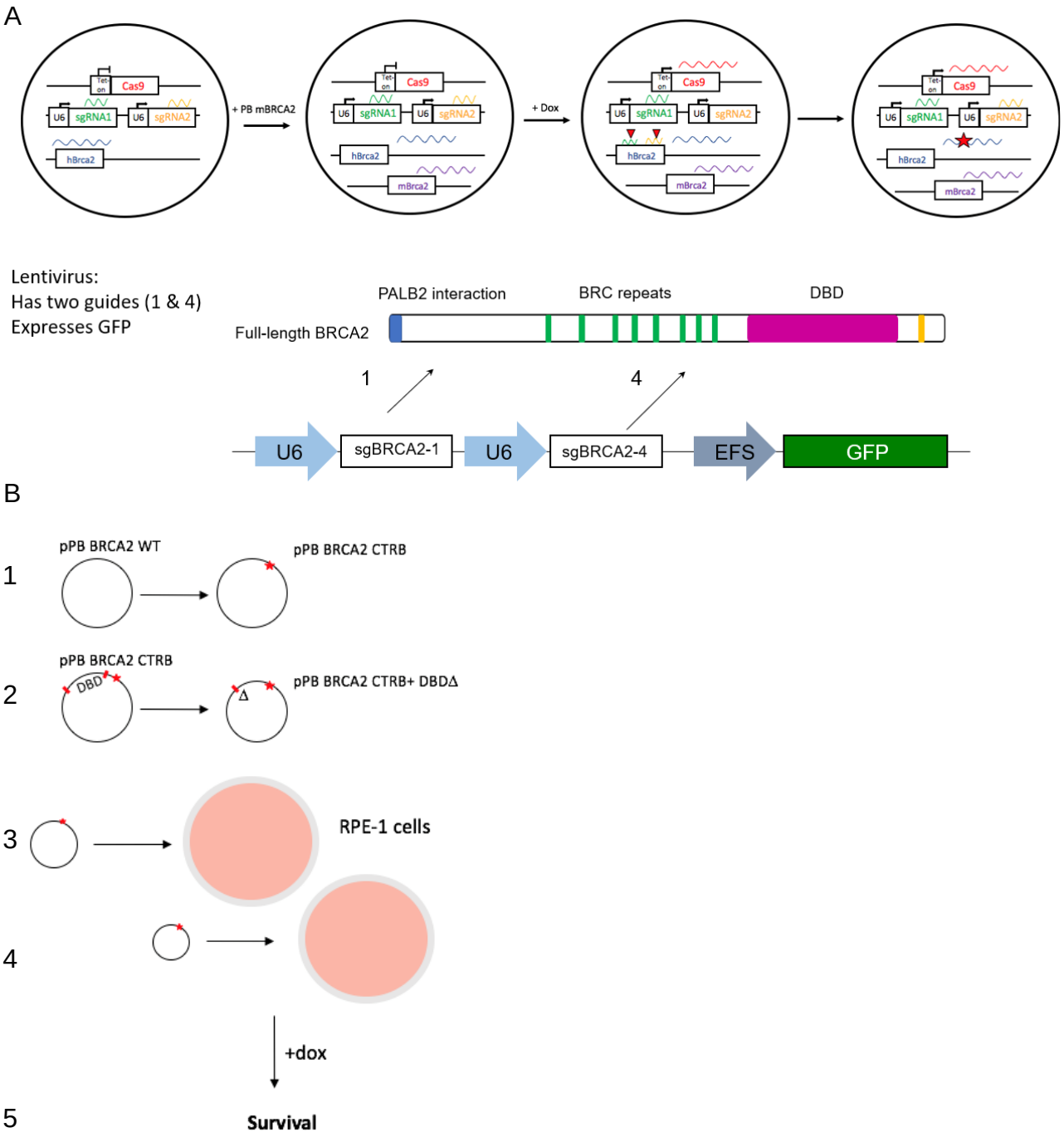

Figure 10 Contribution of the CTRB to BRCA2 DNA binding *Experimental scheme (A) RPE-1 conditional BRCA2 cells, adapted from McKinley et al., 2017 (B) Brca2 DBDΔ and Brca2 DBDΔ + CTRB genes will be stably integrated into the RPE-1 genome. Survival after Dox- induced disruption of endogenous BRCA2 will be assessed by measuring GFP-expression.*

To integrate the *mBrca2* constructs into the RPE-1 genome, we used the PiggyBac transposon system, which inserts a desired sequence in a “cut and paste” mechanism into the host genome

(Fig 11A). We used a PiggyBac vector containing the wild-type and DBDΔ *mBrca2* sequence already present in our lab to introduce the wt and mutant m*Brca2* into the RPE-1 genome. We then introduced the mutation into the CTRB by In-Fusion cloning as shown in Fig 11B. The wt *BRCA2* PiggyBac vector was cut with *AvrII* and *SpeI*. Two inserts were amplified from the wild-type Brca2 vector. One of the primers of each inserts was designed such that it carried the desired mutation and the other primer overlapped with the vector. The inserts and cut vector were then fused to yield the vector containing the mutation.

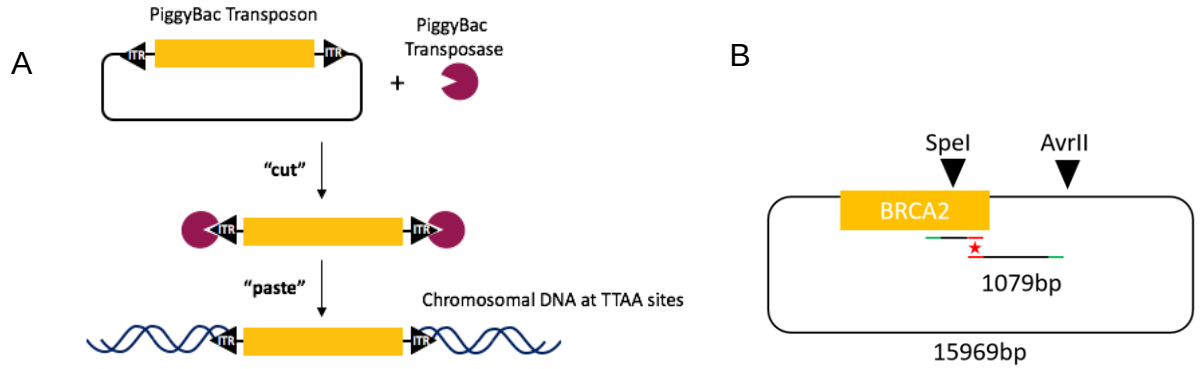

Figure 11 PiggyBac Brca2 vectors *A) Scheme of the PiggyBac (PB) transposase system. The PB transposase recognizes transposon-specific inverted terminal repeat (ITR) sequences flanking the transposon vector. It operates in a "cut- and paste" mechanism to move the genes of interest between the two ITRs from the original site and integrate it into the host genome. B) Scheme of in-fusion cloning to insert the mutation into the Cter region of mBrca2 in a PiggyBac vector. Inserts A and B were amplified from the wild-type PiggyBac vector. The wild-type vector was cut with restriction enzymes and fusion of the linearized vector and the inserts by In-Fusion cloning lead to the desired BRCA2 construct carrying the mutation; green: overlap insert/vector, red star: CTRB mutation*

The correct construction of the vector was verified by PCR (Fig. 12A) and by sequencing.

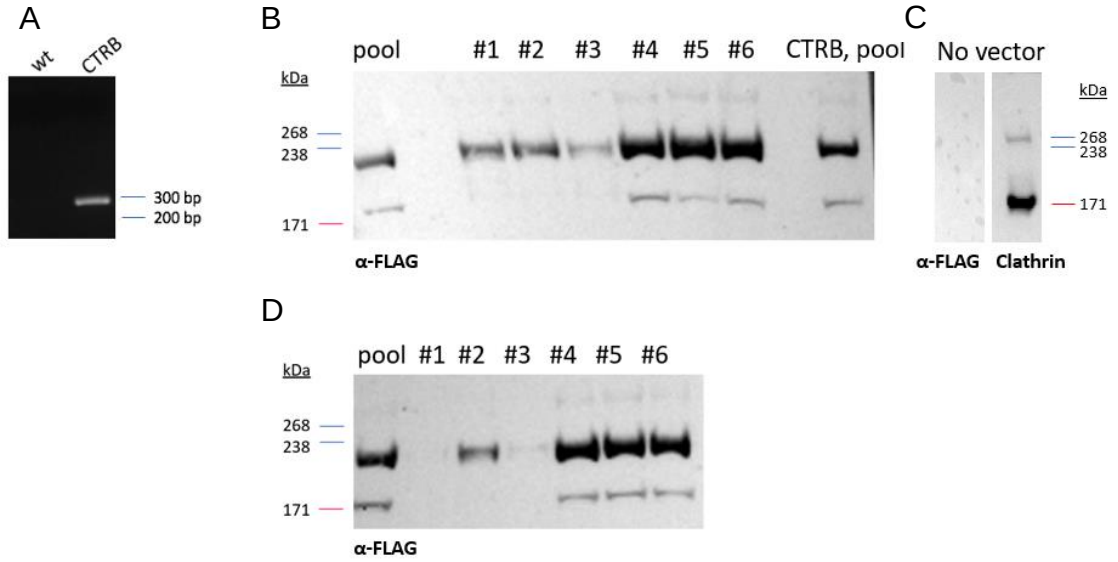

***Figure 12 PiggyBac Brca2 constructs**: (A) The Brca2 vector with the CTRB mutation produces a 308 bp band, the wild-type vector does not produce a band because one of the primers only binds to the CTRB sequence but not the WT one (B) RPE-1 + wt pb pool and single clones, CTRB mutation pool (C) RPE-1 cells, no PiggyBac (D) RPE-1 +DBDΔ pb pool and single clones*

The wt, DBDΔ and the CTRB constructs were integrated into RPE-1. Expression of the BRCA2 constructs was then tested by western blot, probing for the FLAG-tag of the protein. The analysis showed that none of the expressed proteins showed the expected protein sizes (Fig 12 B, D). All cells showed expression of a FLAG-tagged protein at a size of ~ 230kDa, which is too small for wild-type BRCA2 (size: 374 kDa) and DBDΔ BRCA2 (298 kDa). RPE-1 cells without PiggyBac showed no expression of FLAG tagged protein (Fig 12C).

4.6 PCR detection of BRCA2 constructs from genomic DNA

As the RPE-1 cells did not show BRCA2 expression of the right size, we wanted to look at integration of the PiggyBac constructs in the RPE-1 cells. Therefore, we designed primers to detect the FLAG-tag and the 5' end of BRCA2. The FLAG PCR produces a 352 bp product. The other primer sets detect the wild-type BRCA2 sequence or DBD deletion at the 3' end of BRCA2. The wildtype forward primer binds in exon 21-22 which is deleted in the DBDΔ BRCA2 and together with the reverse primer in exon 27 produces a band of 1139 bp. The DBDΔ forward primer binds in exon 15 and uses the same reverse primer as the wildtype one. It produces a 346 bp band. The FLAG tag was detected in all of the clones, as was the wildtype BRCA2 PCR product. The BRCA2 DBDΔ product was not amplified from any clone. This suggests, that in fact BRCA2 wildtype was integrated into the cells and not the DBDΔ version of BRCA2. Figure 13 shows the primer locations and the PCR products.

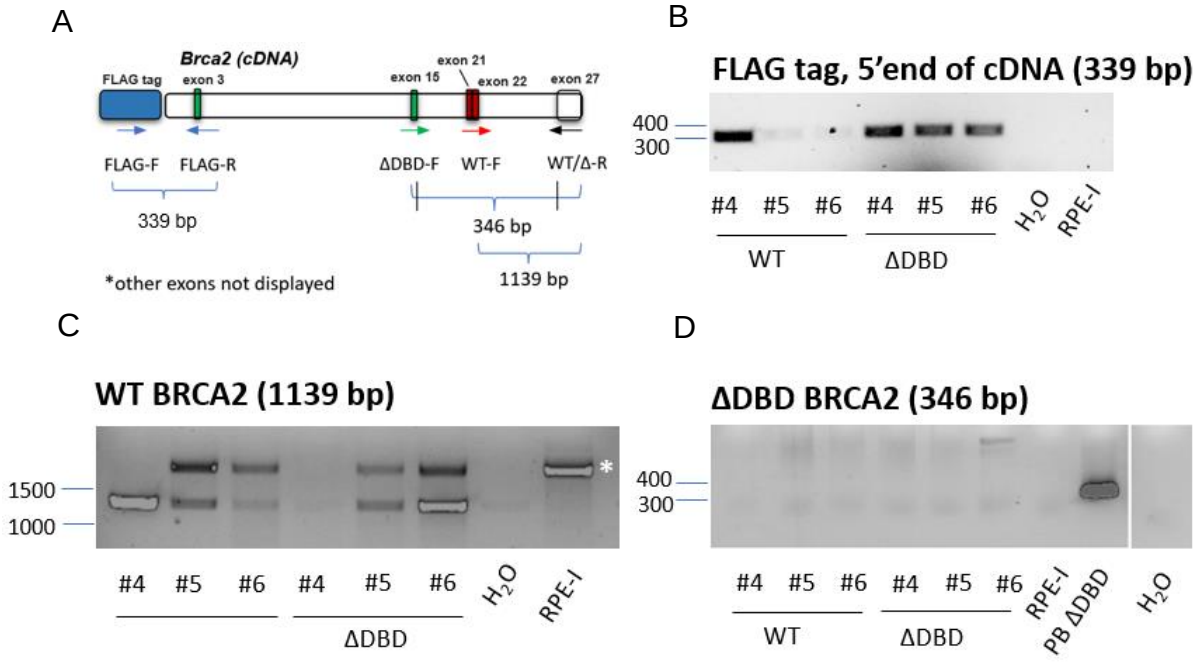

***Figure 13 PCR from genomic DNA from RPE-1 PiggyBac BRCA2 cells** (A) PCR schemes for FLAG, wt and DBDΔ BRCA2 detection (B) FLAG PCR in clones 4-6 from RPE-1 with wt and DBDΔ BRCA2 PiggyBac vector (C) WT PCR, *unspecific band in RPE-1 cells (D) DBDΔ PCR*

5. DISCUSSION

Tumors with inactivating mutations in tumor suppressor genes such as *BRCA1* or *BRCA2* show increased sensitivity to PARP inhibitors and platinum-based chemotherapeutics because of synthetic lethality. However, a substantial part of BRCA1/2-deficient patients do not respond to this treatment or acquire resistance over time. (Li, Liu, Wu *et al.,* 2020). One mechanism by which tumors become resistant to these therapies is restoration of functional homologous recombination- mediated DNA repair. Most *BRCA1* and *BRCA2* mutations found in tumors are single-nucleotide mutations or short indels that are frameshift mutations. Secondary reversion mutations, however, can restore the *BRCA* reading frame through deletion of the initial disease-causing mutations (Noordermeer, van Attikum, 2019; Gornstein *et al.,* 2018).

Recently, the BRCA2 DBD has been subject to various studies, but its exact function remains elusive. A study using CAPAN-1 cells showed, that deletion of the BRCA2 DBD can restore a partially functional BRCA2 and confers PARP inhibitors and cisplatin resistance to these formerly sensitive cells (Edwards *et al.*, 2008). Although, to our knowledge, no reversion BRCA2 peptides have been found in patients that show deletion of the entire DBD as in the CAPAN-1 cells, this demonstrates, that cells BRCA2 has some remaining activity without the DBD.
Another, recent study investigated the importance of the BRCA2 DBD in mouse ES cells and found that loss of the DBD impairs cell survival and led to an increased sensitivity to DNA damaging agents (Paul *et al.,* 2021). Conversely with this finding, we here observed an increase in chromosomal aberrations after treatment with the DNA crosslinking agent cisplatin or the PARP inhibitor olaparib in mouse ES cells.

Part of this project aimed to evaluate the effect of deleting the DBD in mouse EF cells. We found that the deletion had little impact on mouse EF cell viability and sensitivity to DNA damaging agents. We found however, that DBD mouse EF cells displayed more chromosomal aberrations than wild-type cells as observed in metaphase spread analysis. Treatment with the PARP inhibitor olaparib lead to a bigger increase in chromosomal aberrations in the mutant BRCA2, but cisplatin treatment increased the number in wild-type and mutant BRCA2 cells to the same extent.

Mice, homozygous for *Brca2* DBDΔ were viable and did not show any phenotype. It would have been interesting to evaluate, whether these mice showed an increased risk to tumors. A tool to look at tumor formation in mice, is by using mice with only one functional copy of Rb. Here, we used $Rb^{+/-}$, $Brca2^{DBD\Delta/\ DBD\Delta}$ mice to look at formation of early atypical proliferates (EAPs). We did not see any of these proliferates in wildtype or mutant mice, but also not in the positive control. Previously these experiments in our lab were done in mice with a mixed 129/B6 background, whereas here the mice were from a B6 background. We were wondering if this could explain why we did not see any EAPs even in the positive control and are currently breeding mice with a mixed background for further investigation.

Given how conserved the DBD is from fungi to humans and the importance of DNA binding activity for homologous recombination repair of DNA, it is surprising that deletion of the DBD seems to only have a minor effect on BRCA2's function. It is possible, that BRCA2 interaction with DNA in the absence of the DBD is mediated through interacting proteins. Some studies proposed that homozygous $Brca2^{DBD\Delta}$ cells may rely upon the BRCA1-PALB2 interaction to localize to sites of DNA damage (Siaud *et al.,* 2011; Al *Abo et al*., 2014). However, it remains to be elucidated whether this is also true in mouse or human cells. Other studies proposed, that BRCA2 bound RAD51 molecules could provide a platform for BRCA2 DNA interactions (Jensen *et al.,* 2010; Reuter *et al*., 2014; Sanchez *et al.,* 2017). It is also thinkable, that additional functional domains

in BRCA2 could substitute for the DBD. For example, its remaining ability to interact with DNA could be explained by a recently described putative DNA binding domain in the C-terminal region (CTRB) in human BRCA2. To test this hypothesis in the mouse, we planned to introduce this mutation into mouse Brca2 and observe the effect on its ability to complement loss of endogenous BRCA2 in RPE-1 cells. As we were not able to express the correct Brca2 constructs in the RPE-1 cells, we could not go on with the FACS experiment as planned. Thus, the existence and importance of this putative DNA binding domain in mouse Brca2 remains to be elucidated.

6. METHODS

6.1 Mouse Care

The care and use of mice were performed with the approval of the Memorial Sloan Kettering Cancer Center (MSKCC) Institutional Animal Care and Use Committee in accordance with institutional guidelines.

6.2 Genotyping

Genomic DNA from mouse tissue was extracted using the Qiagen Puregene Corekit A, following the manufacturers protocol. Cells were lysed in the provided detergent, supplemented with proteinase K at 55ºC for 12h. Proteins and other contaminants were removed by salt precipitation and finally, the DNA was precipitated in Isopropyl alcohol. The pellet was washed 1x with 70% EtOH and let dry at RT. The dry DNA pellet was then dissolved 50µl of the Puregene Hydration solution. The DNA was stored at 4ºC.

6.3 PCRs for genotyping

DBDΔ

A multiplex PCR was designed for detection of the wild type and the DBDΔ allele. The forward primer was designed to bind downstream of the DBD and then 2 reverse primers were used, one binding within the DBD, the other upstream of the DBD (sequences shown in table 1). A scheme of the reaction is given in figure 17. Wild-type alleles produce PCR products from primers A and B, which binds in the DBD region. Primer C binds behind the DBD, so DBDΔ alleles will amplify products with primers A and C.

Table 1 ***primer sequences for Brca2 wt / DBDΔ genotyping***

Primers	Sequence (5'-3')	Length [bp]	T_m [ºC]
mB2ds15-641F	TGCACTTTTACGTCTGGGCTCTA	23	60
mB2ds15-1078R	TCACTATAGAACAGTTAACTGGCCCA	26	59
mPIR2-572R	ACTCCTCCTCCTCCTTCTGAACA	23	60

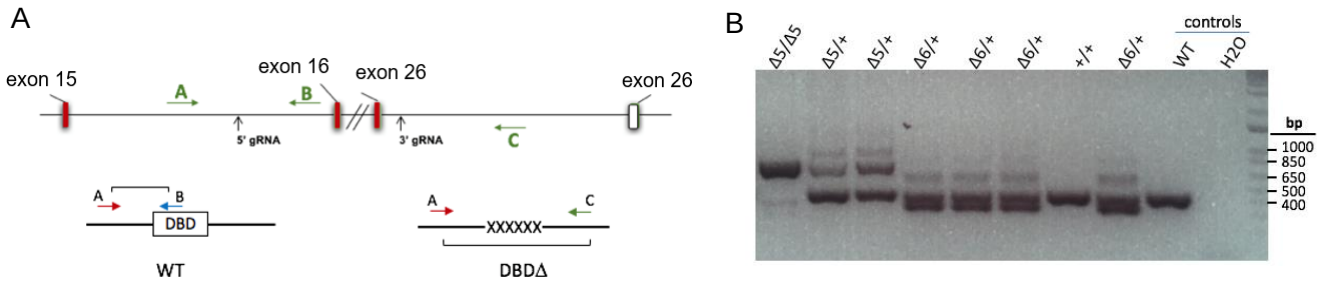

Figure 17 Brca2 DBDΔ PCR scheme*. (A) Scheme of the Brca2 DBDΔ genotyping (B) Brca2 genotyping of lines 5 and 6*

Depending on the size of the deletion, the wild-type fragment can be bigger or smaller than the DBDΔ one. In lines 4 and 5, the wild-type fragment is shorter than the mutant. Line 6 has a bigger deletion and thus the mutant product is shorter than wildtype. The sizes of the PCR products for the different lines are given in table 2.

Table 2 Brca2 DBDΔ genotyping: Sizes of PCR products

ΔDBD line	WT allele (PCR primers A and B)	ΔDBD allele (PCR primers A and C)
4	438 bp	760 bp
5	438 bp	769 bp
6	438 bp	348 bp

For every reaction 6.25µl polymerase mix (Thermofisher, DreamTaq Green PCR Master Mix, Cat # K1081), 4.25µl dH_2O and 0.5µl of each primer were used. 0.5µl DNA (diluted 1:10) were added to the PCR mix.

Rb

Genotyping for Rb was performed using three primers (sequences in table 3) and the same reaction mix as for DBDΔ genotyping. RbX3 is the primer common to both the WT and mutant allele. Wild type produces a 380 bp product and mutant a 410 bp band.

Table 3 primer sequences for Rb genotyping

Primers	Sequence (5'-3')	Length [bp]	T_m [ºC]
Rbx3	AATTGCGGCCGCATCTGCATCTTTATCGC	29	67
RI3	CCCATGTTCGGTCCCTAG	18	61
PGK3	GAAGAACGAGATCAGCAG	18	51

6.4 Mouse embryonic fibroblasts

For mouse EF isolation, E13.5 mouse embryos were separated from the placenta and embryonic sac, then minced following the removal of head and liver, and then dissociated in 0.05% trypsin at 37°C for 45 min in a 6-cm dish. The dissociated cells were re-suspended by pipetting, pelleted at 400 g for 10 min, and plated to a 6-cm dish coated with 0.1% gelatin for 1-2 days.

6.5 Cell culture

HEK293T and mouse EF cells were cultured in an incubator (37ºC and 50% humidity) in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% FBS (Fetal Bovine Serum, Gibco™, Thermo Fisher Scientific, Cat #26140079), 1% Penicillin-Streptomycin (Gibco™, Thermo Fisher, Cat # 15140122), non-essential amino acids (Gibco™, Thermo Fisher Scientific, MEM Non-Essential Amino Acids Solution (100X), Cat # 11140050) and L-Glutamine (Gibco™, Thermo Fisher Scientific, L-Glutamine (200 mM), Cat # 25030081).

The inducible knockout RPE-1 cells were cultured in Tetracycline free media: DMEM, 10% Tet-free FBS (Tet System Approved FBS, Clontech, Cat. #631101), 1% Penicillin-Streptomycin, 1% NEAA, 1% L-Glutamine.

6.6 RAD51, DSS1, DMC1 pCAGGs vectors

To generate cell lines expressing *hRAD51*, *DSS1* and *hDMC1* we used vectors with a pCAGGs backbone. pCAGGS-BSKX-H3/Not expresses cDNAs from a human cytomegalovirus enhancer/chicken β-actin promoter. The expression plasmids were previously described in the following publications: *hRAD51* (Brouwer *et al*., 2018), *hDMC1* (Prakash *et al*., 2020), *hDSS1* (Siaud *et al.,* 2011). The vectors for *hRAD51* and *hDMC1* contain an N-terminal 3xFLAG tag, *DSS1* contains an N-terminal c-*myc* tag.

The h*BRCA2*-WT PiggyBac transposon plasmid was generated in our lab as previously described (Feng *et al*., 2017). To generate the mBrca2-WT and -DBD∆ plasmids, the h*BRCA2*-WT plasmid was digested with PacI and NotI and the cDNA amplified from mouse ES cell mRNA was cloned into the backbone. A 3xFLAG tag was fused to the 5' end of the cDNA with overlap PCR.

6.7 Immunoprecipitation and western blot analysis

$5x10^6$ HEK293T cells were plated and ~24h later, 6 µg of the indicated expression vectors were transfected into HEK293T cells using lipofectamine 2000 (Fisher scientific Cat # 11668019). Two days after the transfection, cells were spun at 1000 rpm for 5 min at 4º C. Cell pellets were lysed in NETN buffer (100 mM NaCl, 20 mM Tris-Cl pH 8.0, 0.5 mM EDTA, 0.5% (v/v) Nonidet P-40 (NP-40)) with protease inhibitor cocktail (Roche Cat # 11873580001) by freeze-thawing the cells 3x and then spun at 16000 rpm for 10 min at 4ºC to collect the supernatant. Protein concentration was determined using a Bradford protein assay (Bio-Rad). 1 mg extract was used for each immunoprecipitation assay and all the steps were carried out at 4º C. The FLAG- (for RAD51 and DMC1) (Sigma Cat # A2220) or MYC-beads (for DSS1) (Sigma Cat # A7470) were blocked with 4% BSA in PBS-T (50 mM Tris [pH 7.5], 150 mM NaCl, 0.05% Tween 20) for 1 h and then washed three times with PBS-T. To pre-clear the extract, 25 µl of pre-washed mouse IgG agarose beads (Sigma Cat # A0919) were incubated with 1 mg of extract for 60 min followed by centrifugation at 14000 rpm for 10 min. 80 µl of the supernatant were transferred into a new Eppendorf and used for the input in the western blot. The rest of the supernatant was transferred to the eppendorf containing 25 µl of anti-FLAG M2 beads for RAD51 and DMC1 or anti-MYC beads for DSS1 and incubated for 2 h or over-night. The anti-FLAG M2 or anti-MYC beads with the extracts were spun at 14000 rpm for 3 min, supernatant was discarded, and beads were washed three times with PBS-T. After the third wash, the beads were resuspended in 50µl 2x SDS sample buffer (NEB Cat # B7703), 4µl of DTT (30x) were added to each sample and incubated for 10min at 70°C for BRCA2 or 95°C for RAD51, DMC1, and DSS1 and then spun at 8000 rpm for 3 min. To perform western blotting, input and pulldown were loaded on a precast SDS PAGE gel (BRCA2: NuPAGE Novex 3-8% Tris-Acetate Protein Gels, Thermo Fisher, Cat # EA03752BOX; DSS1, DMC1 and RAD51: Mini Protean TGX Precast gel 12%, BioRad, Cat # 4561043), transferred onto a nitrocellulose membrane (BRCA2)/ PVDF membrane (DSS1, RAD51, DMC1), and blocked with 5% milk in PBS-T (50 mM Tris [pH 7.5], 150 mM NaCl, 0.05% Tween 20) for 1 h.

For immunodetection, the following antibodies were used: anti-RAD51 (Cat # PC-130) from Millipore; anti-DMC1 (Cat # sc-22768) from Santa Cruz Biotechnology; anti-M2-FLAG- HRP (Cat # A8592) from Sigma; anti-MYC tag mouse (Cat # 2276S) from Cell Signaling Technology.

6.8 Analysis of metaphase spreads

Primary mouse embryonic fibroblasts (EFs) were plated on 10cm dishes at a density of 5 x 10^5 cells/plate and grown in regular media (DMEM high glucose, 10% FBS, 1% NEAA, 1% Penicillin/Streptomycin) or media supplemented with cisplatin (200nM) or olaparib (200nM). After 24h, the cells were arrested in G2-M phase with 0.1 µg/µl colcemid (KaryoMAX® Colcemid™ Solution in PBS, Fisher Scientific (Thermo) Cat. # 15212-012) for 1h if untreated or 5h if treated with genotoxic agents. The cells were then trypsinized and centrifuged. The cell pellets were re-suspended in media and 6 ml of hypotonic buffer (75 mM KCl) were added, while vortexing. Then 1 ml of cold fixing solution (MeOH, HoAc; 3:1) is added to the media. The cells are centrifuged and re-suspended in 6 ml of cold fixing solution. The chromosomes were stained with DAPI (ProLong® Gold antifade reagent with DAPI, Invitrogen, Cat # P36935) and analyzed under the microscope for chromosomal abnormalities. Aberrations were indicated by gaps/breaks, acentric chromosomes, exchanges/fusions and radial structures.

Metaphase spreads for mouse ES cells were prepared similarly. However, cells were treated with cisplatin (316nM, 1000nM) or olaparib (500nM, 1000nM) for only 5h instead of 24h.

6.9 Proliferation & cell viability assays of DBDΔ mouse EF cells

Primary BRCA2-WT and DBDΔ mouse EF cells were seeded in triplicates at a cell density of 2.5*10^3 cells/well and transfected in four 96-well plates (one for every time point). Cells were incubated from 24 to 96 h. After 24, 48, 72 and 96h WST-1 proliferation agent (Roche Applied Science, Vienna, Austria) was added to one of the plates according to the manufacturer's recommendations. Colorimetric changes were measured using a SpectraMax M3, SoftMax® Pro 5 (Molecular Devices) at a wavelength of 450 nm with a reference wavelength at 620 nm. To test cell viability, a similar setup to the one above was used. After 24h the media was complemented with media containing either cisplatin or olaparib at different concentrations. The IC50 for each treatment was calculated by curve fit in Excel and plotted in GraphPad Prism.

6.10 Introduction of a mutation into the CTRB of m*Brca2*

The inserts A and B (as shown in Fig 18 B) were amplified from the pCAGGS PiggyBac BRCA2 vector. The primers were designed and ordered from Integrated DNA Technologies. The PCR was performed using the iProof™ High-Fidelity PCR Kit (Bio-Rad, Cat. # 172-5330).

The Brca2 PiggyBac vector was digested with AvrII (New England Biolabs, Cat. # R0174S) and RsrII (New England Biolabs, Cat. # R0501S) in Cutsmart® buffer (New England Biolabs, Cat. # B7204S).

The right size of the amplified inserts and the linearized vector were verified by gel electrophoresis. The bands containing the DNA fragments were then cut from the gel and extracted from the gel using the PureLink® Quick Gel Extraction Kit (Invitrogen, Cat. # K210012) following the manufacturers protocol. DNA concentration was quantified by NanoDrop.

Cloning was performed with the help of In-Fusion® HD Cloning System (Takara Bio, Cat. # 639645) according to the provided user manual. Following the cloning reaction, competent *E.coli* cells (Stellar™ compentent cells, Cat. # 636766) were transformed with the reaction mixture.

The mutation was verified by PCR and sequencing. One of the primers binds to the mutated sequence but not to the wild-type and therefore only the mutant produces a PCR product.

The plasmid was isolated from the cells using the Maxi prep kit (Invitrogen, Cat. # K2100-17) according to the manufacturers protocol.

6.11 PCR detection of FLAG and BRCA2 wt/DBD in RPE-1 cells

Primers (sequences shown in table 4) were designed as shown in figure 13 A.

Table 4 primer sequences for FLAG, wt and DBDΔ BRCA2 detection in RPE-I cells

Primers	Sequence (5'-3')	Length [bp]	T_m [ºC]
3xFLAG F	ACAAGGATGACGATGACAAGGGT	23	60
mB2cDNA-274R	AAACTCTACCACTGGACC	18	52
mB215F2	AGAAAGGCGACATCTCCGTCT	21	59
mB2ex21-22F	ACCCAGATCACCTTGAGGCTT	21	60
mPIR195R	GTCAGTCCCATCTGTACCTTTGTC	24	58

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