Understanding BRCA1(-complexes) in DNA repair and cancer

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Chapter 1

General introduction

The more you know about the human body, the more complicated and incredible it becomes. Two cells fusing to generate a zygote can be the start of a whole new human being. Our adult bodies produce, on average, 25 million cells per second. The DNA content of one cell is over two meters long when stretched out. During all those cell divisions, all that DNA needs to be replicated. These examples are just a few of the countless processes within us that need to be executed correctly to ensure life. However, no process is flawless, and luckily, our body is equipped with a wide variety of back-up pathways, repair mechanisms and subsequent checks to catch the slip-up when errors occur. However, sometimes these pathways fail and cancer, for example, is a consequence of one cell escaping or deceiving the multiple control mechanisms in place.

CANCER

Cancer is among the leading causes of death and the number of new cancer patients is expected to rise as our life expectancy increases. Breast cancer is one of the most common cancers in most countries around the world. There are different types of breast cancer, depending on which cell type is growing abnormally and whether the cancer has invaded the surrounding breast tissue or not. Around 5%-10% of all breast cancer cases are caused by hereditary mutations in tumour suppressor genes such as BRCA1 and BRCA2 (1,2). Additionally, mutations in other genes, for example RAD51, can phenocopy BRCA1/2-mutated tumours, a condition called BRCAness (3,4). The risk to develop breast cancer is around 12% for the general population by the age of 70 and with a BRCA1 mutation this risk increases to 55%-72%. Mutations in BRCA1 also dramatically increase the risk for ovarian cancer from 1%-2% to 39%-44% (5). Furthermore, many cancers of different origin harbour somatic *BRCA1* mutations.

BRCA1 is important to maintain genomic stability through different mechanisms and BRCA1 mutated tumours display gross chromosomal instability (6,7). BRCA1 mutations are spread throughout the 1863 amino acid long protein and consist of missense mutations, large deletions, insertions, nonsense mutations and splice-site variants. Frameshift mutations are the most common in BRCA1, followed by missense and nonsense mutations (8,9). Hotspots for pathogenic BRCA1 mutations are in the N-terminal RING domain, C-terminal BRCTs and the largest exon of the protein, namely exon 11 (Figure 1) (https://enigmaconsortium.org) (10-12). Increased genetic testing in the last decades has also revealed so-called variants of unknown significance (VUS), mutations which are not yet classified as pathogenic or benign.

My thesis is directed at understanding the functional role of BRCA1 in tumour prevention. In the remainder of this chapter, I will give an overview of the current knowledge on BRCA1 biology and the importance of BRCA1 in genome maintenance.

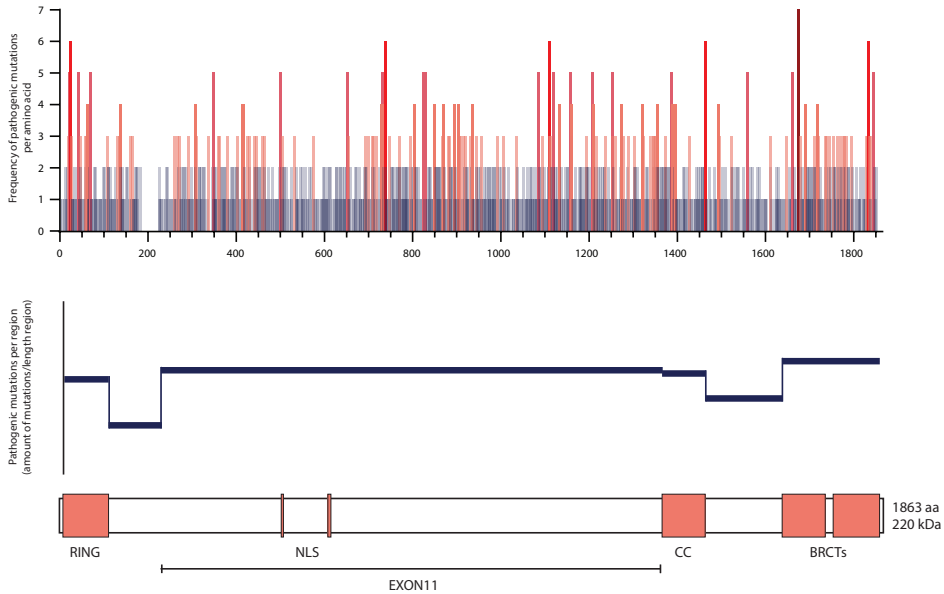

Figure 1. Mutations in BRCA1. Schematic representation of BRCA1, including the location and size of the different domains of BRCA1. The blue line above indicates the incidence of BRCA1 mutations with a pathogenic clinical significance described by the ENIGMA consortium (Spurdle *et al.* 2012) for each BRCA1 domain. The incidence was calculated by the amount of mutations in a certain domain divided by the length of that domain. The graph on top indicates the frequency of BRCA1 mutations with a pathogenic clinical significance for each BRCA1 amino acid described by the ENIGMA consortium (Spurdle *et al.* 2012). Blue indicates a frequency of ≤ 2, red indicates a frequency of ≥ 3, the darker the red the higher the frequency. The amino acids in the graph above align with the domains of BRCA1 in the schematic representation below.

DSB REPAIR

The key function of BRCA1 is to maintain genome stability. The genome is constantly threatened by endogenous and exogenous sources which can lead to different DNA lesions. Examples of exogenous sources causing DNA damage are UV irradiation, ionizing irradiation, and various chemicals. Hydrolysis and oxidation, but also processes like replication and transcription are examples of endogenous causes of DNA damage. When the lesions are not repaired properly, genomic instability can arise, which is one of the hallmarks for cancer (13). Our cells can protect the genome by activation of the DNA damage response (DDR) which includes detecting the different DNA lesions, activating cell cycle checkpoints and repairing the lesions. DNA double-strand breaks (DSBs) are the most cytotoxic DNA lesions which can be repaired by different mechanisms, with BRCA1 playing a key role in the most faithful DSB repair mechanism: homologous recombination (HR). Besides HR, cells can repair DSBs through non-homologous end-joining (NHEJ),alternative end-joining (alt-EJ) or single-strand annealing (SSA) (Figure 2).

Non-homologous end-joining

NHEJ is the canonical DSB repair pathway, active throughout all phases of the cell cycle. The broken DNA ends are bound by Ku70/Ku80, whereafter DNA-PKcs is activated and several enzymes required for DNA end-processing are recruited and activated (14). Additionally, the Shieldin complex is recruited to DBSs in a 53BP1- and RIF1-dependent manner, where it inhibits end-resection and thereby HR (15-20). The broken ends are ligated together by the LIG4-XRCC4-XLF complex, assisted by PAXX (21-23). NHEJ is most often error-free, however, it can lead to small deletions and insertions as minimal end-processing is sometimes required for the repair (24).

Homologous recombination

HR is the most faithful DSB repair pathway since the cell uses an unbroken sister chromatid as a template to repair the DSB, leading to error free repair of the break. HR is inhibited during the G1-phase of the cell cycle to ensure recombination is only performed using an available sister chromatid in the S and the G2 phase. HR requires end-resection by the MRN complex (MRE11, RAD50 and NBS) and CTIP to expose 3' single-stranded DNA (ssDNA) which is subsequently bound by the RPA complex (RPA1, RPA2, RPA3) to protect the DNA against nucleases (14). The BRCA2-PALB2-BRCA1 complex displaces RPA and loads RAD51 onto the single stranded 3' overhang. RAD51 and RAD51 paralogues facilitate the search for homology and the RAD51 coated 3' overhang, called a presynaptic filament, will invade the homologous template to faithfully repair the break. Both BRCA1 and BRCA2 are key players in HR as BRCA1 stimulates end-resection (14,25-28) and both BRCA1 and BRCA2 are essential for the loading of RAD51 (14,29,30).

Short-range end-resection by MRN and CTIP can remove up to 300 nucleotides of ssDNA and is, as described above, essential for HR (31). Potential subsequent long-range end-resection by either EXO1 or DNA2-BLM can process thousands of nucleotides. The importance of this process for HR is still enigmatic, which I will elude to in more details in chapter 3. Initially, long-range end-resection has been shown to stimulate both HR and checkpoint activation (32-34). In contrast, loss of long-range end-resection factors does not affect HR between identical sequences, indicating their redundancy for correct HR (35-39).

As explained above, the sister chromatid is often used as a homologous template to repair the break in an error-free manner. However, other identical DNA sequences in the nucleus, either present on the homologous chromosome or an endogenous/exogenous homologous DNA template, can be used as well. Once the 3' overhang invading the homologous sequence has annealed to the complementary DNA template, forming a so called displacement-loop (D-loop), polymerases extend the broken 3' end. Subsequent resolution of the D-loop can occur via synthesis-dependent-strand annealing (SDSA) during which the extended 3' end detaches from the used template and binds to the original break end (40). When the D-loop structure evolves into a double-Holliday junction, this structure is resolved either through dissolution or resolution (41).

Both SDSA and NHEJ are used by the cell to resolve two-ended DNA breaks. Problems during DNA replication can result specifically in one-ended DSBs. Collapse of the replication fork leads to one-ended DSBs which cannot be solved by SDSA. Instead, literature has suggested that such DSB can be repaired by a unique HR mechanism, namely break-induced replication (BIR). In BIR, the break is resolved by performing DNA synthesis all the way to the end of the chromosome arm (42). However, recently it has been shown that collapsed forks are often converted into two-ended DSBs and can also use classical RAD51-dependent HR for their repair (43-46).

Alternative end-joining

Alt-EJ is suggested to act as a back-up mechanism to NHEJ and HR as well as repair specific types of DSBs (47-51). For Alt-EJ, limited end-resection is required to expose microhomology flanking the break, 2-20 bps of perfect homology with an optimum of >2 and <8 bps (52). These nucleotides are subsequently annealed to repair the break and the intervening sequence is lost. Different DNA repair proteins, such as XRCC1, LIG3, PARP1 and POLθ, have been described to play a role in Alt-EJ (53-56). Alt-EJ is also known as micro-homology mediated end-joining (MMEJ) or theta-mediated end-joining (TMEJ). TMEJ is the specific term when POLθ, both a polymerase and a helicase, is used by the cell to repair the break.

Single-strand annealing

Similar to Alt-EJ, SSA is suggested to act as a back-up mechanism to HR as both Alt-EJ and SSA are essential for the survival of HR-deficient cells (47,48,57,58). Long-range end-resection

by EXO1 and DNA2-BLM is important for SSA to reveal tandem repeats flanking the DSB. Subsequently, RAD52 binds to the ssDNA to promote the annealing of the hom(e)ologous repeat sequences downstream and upstream of the break (59). Non-homologous 3' DNA is removed by the ERCC1-XPF complex (60). SSA results in large deletions as the DNA sequence between the tandem repeats is deleted (61,62). Despite the mutagenic nature of SSA, the pathways is conserved, indicating a possible role for SSA in the maintenance of the genome (61).

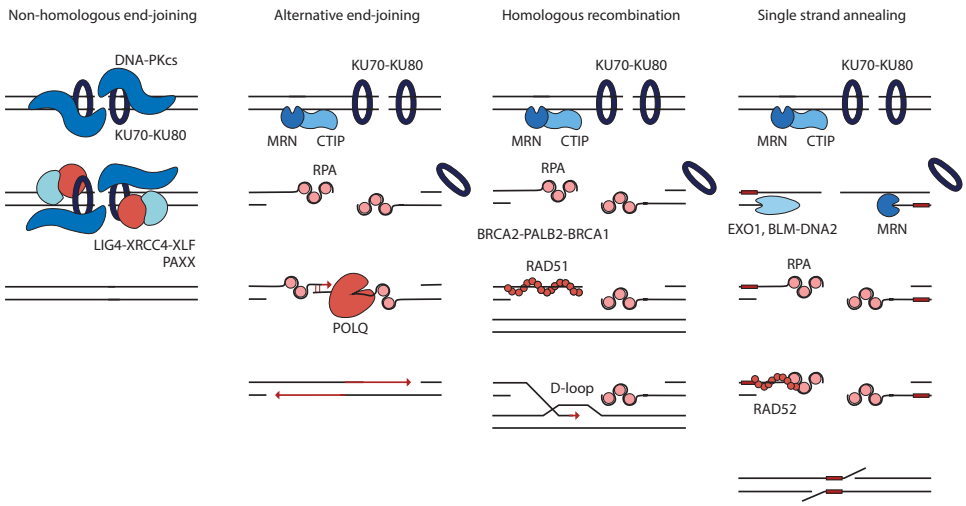

Figure 2. The different DSB repair pathways. DSBs in the cell can be repaired via multiple repair mechanisms. During NHEJ the broken DNA ends are ligated back together. Alt-EJ depends on microhomology flanking the break for repair. HR uses the sister chromatid as an error-free template for faithful repair. During SSA, extensive end-resection is required to unveil regions of hom(e)ology flaking the break.

REPAIR PATHWAY CHOICE

Before the cell can repair a DSB using one of the above explained DSB repair pathways, the cell needs to choose between the different repair methods, a tightly regulated process to ensure genomic stability (63). The DSB signalling cascade starts with the phosphorylation of histone variant H2AX by the kinase ATM. Phosphorylated histone variant H2AX (γH2AX) assists in the loading of MDC1 on the broken DNA (64). This is followed by the recruitment of RNF8, an E3 ubiquitin ligase which ubiquitinates histone variant H1 and recruits another E3 ubiquitin ligase, RNF168. RNF168 ubiquitinates histone variant H2A on Lys 13 and 15 (H2AK13Ub and H2AK15Ub) (65,66). The methylation of yet another histone variant, H4K20, depends on the cell cycle phase of the cell. H4K20me2 in G1-phase in combination with H2AK15Ub leads to the recruitment of 53BP1 and stimulation of NHEJ (67,68).

Cell cycle phase is an important regulator of pathway choice. In S-phase, H4K20 is unmethylated and thereby favours the recruitment of BARD1 (69-71), which forms an obligatory heterodimeric protein complex with BRCA1, subsequently stimulating HR. More recently it was described that phosphorylation of H2AK15 at Thr12 blocks the binding of 53BP1 and not BARD1/BRCA1 to H2AK15Ub (72), indicating additional pathway regulation. Another post-translational modification (PTM) important for pathway choice are the phosphorylation of CTIP by CDK in S/G2-phase to drive end-resection (73).

Both alt-EJ and SSA are suggested to act as back-up mechanism to HR (47,48,57,58), however the specific regulation of these pathways remains unknown. The type of break (50), presence of tandem repeat sequences flanking the break and the amount of end-resection are presumably influencing this regulation. In addition, chromatin context (e.g. heterochromatin vs. euchromatin) has been described to regulate pathway choice. Euchromatin is more likely to be repaired by NHEJ, whereas lesions in heterochromatin favour repair by alt-EJ (49).

FUNCTIONS OF BRCA1 OUTSIDE DNA REPAIR

Besides the important role of BRCA1 in HR and DNA repair pathway choice as described in the previous paragraphs, BRCA1 also plays a role in cell cycle check-point activation, replication fork protection, ssDNA gap suppression, R-loop dissolution and mitosis.

BRCA1 in cell cycle checkpoint activation

BRCA1 is implied to play a role in G1 arrest as BRCA1 binds to hypophosphorylated RB, a protein that forms a transcription inhibitory complex (74). Additionally, BRCA1 interacts with many cell cycle related proteins: e.g. Cyclin D1, CDC2, CDK2, CDK4, γ-TUBULIN, p21, p27 and p53 (75). Furthermore, ATM, ATR and CHK2 have been shown to phosphorylate BRCA1 upon genotoxic stress at different cell cycle checkpoints (76). However, the direct role for BRCA1 in the cell cycle regulation is not well understood.

BRCA1 in replication fork protection

Problems that arise during replication can lead to replication stress. As organisms are highly dependent on replication for their survival, proliferation and differentiation, multiple processes are in place to deal with replication stress. During such a stress response mechanism- fork reversal- the replication fork is remodelled by the formation of a reversed arm containing the nascent DNA. BRCA1, together with BRCA2 and RAD51, is important for the protection of this nascent DNA from nucleolytic degradation by nucleases MRE11, EXO1 or DNA2 (77-82). Uncontrolled resection of the nascent DNA in the absence of BRCA1 will lead to degradation of the replication fork and eventually genomic instability (77,83).

BRCA1 in ssDNA gap suppression

Many recent papers describe the role of BRCA1 in the prevention of ssDNA gap formation as well as resolving these gaps. BRCA1-deficient cells have increased levels of ssDNA gaps, which is further enhanced after treatment with damaging agents (84-86). Additionally, the chemosensitivity of BRCA1-deficient cells has been linked to the increase of ssDNA gaps in BRCA1-deficient cells. The implications of these findings are discussed in chapter 2.

BRCA1 in R-loop dissolution

RNA:DNA hybrids, including so-called R-loops, are naturally occurring structures in the cell. For example, replication requires RNA primers to start replication on both the leading and the lagging strand, forming small R-loops. The loss of both BRCA1 and BRCA2 has been described to induce R-loop accumulation (87,88). Subsequently, BRCA1 has been found to be recruited to naturally formed R-loops and acts as an anchor for Senataxin, which is a protein involved in R-loop dissolution (89). Additionally, it has been suggested that BRCA1 prevents R-loop accumulation by binding to COBRA1 (88,90). Unscheduled R-loop formation

and accumulation can lead to genomic instability. In Ewing sarcoma cells, increased R-loop accumulation has been shown to sequester BRCA1 away from DSBs, thereby further threatening genomic stability (91).

BRCA1 in mitosis

It has been reported that BRCA1 localizes to centromeres during mitosis and that the phosphorylation of BRCA1 by CHK2 is important for the correct assembly of the mitotic spindle (92-94). Additionally, BRCA1 has been described to associate with the mitotic kinase, the Aurora-A kinase, during mitosis (92). Loss of CHK2-mediated phosphorylation of BRCA1 leads to an increase of Aurora-A bound to BRCA1, subsequently inducing mitotic abnormalities. These findings suggest phosphoylation of BRCA1 is important to keep the oncogene Aurora-A under control (95). This role of BRCA1 is less well established and additional research is required.

BRCA1-COMPLEXES

BRCA1 is a large protein that consists of 22 coding exons which code for different domains: a RING-domain on the N-terminus, two nuclear localisation signals (NLS), a coiled coil (CC) domain and two BRCT-domains on the C-terminus of the protein. BRCA1 binds to a plethora of proteins with its different domains, forming multiple multiprotein complexes. Well known and studied proteins that BRCA1 forms complexes with are BARD1, PALB2, ABRAXAS, BRIP1, and CTIP (Figure 3).

The BRCA1/BARD1 heterodimer

BRCA1 binds to BARD1 through its RING-domain, forming a heterodimer with E3 ligase activity (96). Binding of BRCA1 to BARD1 is essential for the stability of BRCA1, highlighted by the pathogenicity of the $BRCA1^{C61G}$ mutation, which reduces the binding to BARD1 (97). BARD1 is important for the recruitment of BRCA1 to DNA damage in S phase through recognition of both H4K20me0 and H2AK15ub (69,98,99). Additionally, both BRCA1 and BARD1 contribute to the DNA binding capacity of the heterodimer, recombinase acitivity of RAD51 and their isomerization promotes replication fork protection (27,100).

BRCA1-PALB2-BRCA2

With its coiled coil domain, BRCA1 binds to PALB2, subsequently forming a complex with RAD51 and BRCA2 (101). As discussed before, this complex plays a pivotal role during HR in the loading of RAD51 into the ssDNA (27,102). Pathogenic mutations in either RAD51 and PALB2 can result in BRCAness, increasing breast and ovarian cancer susceptibilty.

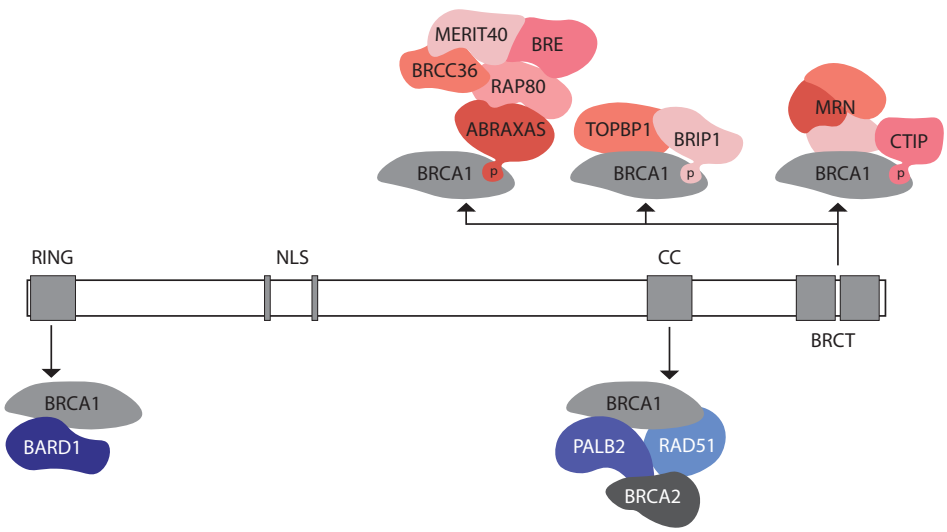

Figure 3. Schematic representation of BRCA1 and BRCA1-complexes. The structure of BRCA1 consists of a RING-domain on the N-terminus, two NLS domains, a coiled coil domain and two BRCT-domains on the C-terminus. Well known and studied proteins that BRCA1 forms complexes with are BARD1, PALB2, ABRAXAS, BRIP1, and CTIP.

The BRCA1-A/B/C complex

The tandem BRCT domains of BRCA1 bind to ABRAXAS1, BRIP1 and CTIP, forming respectively the BRCA1-A,-B and-C complex. This binding is dependent on the phosphorylation of the serine in the SXXF motif in the binding proteins (103-105). The interaction between BRCA1 and either ABRAXAS1, BRIP1 or CTIP is mutually exclusive and how this is precisely regulated has not yet been elucidated. The binding affinities of each protein are comparable, therefore it is either regulated by their abundance or an alternative mechanism.

The BRCA-A complex consists of BRCA1, and two of each of the following proteins: ABRAXAS1, ubiuitin binding protein RAP80, deubiquitinase BRCC36, BRE and scaffold protein MERIT40 (106). Phosphorylation on serine 406 in ABRAXAS1 is essential for the binding of ABRAXAS1 to BRCA1 and thereby the formation of the BRCA1-A complex. Auxiliary to the recruitment of BRCA1 by BARD1, RAP80 in the BRCA1-A complex facilitates the recruitment of BRCA1 through recognition of K63-linked ubiquitin chains on the DNA (107-109). The BRCA1-A complex is thought to inhibit HR and limit excessive end-resection (110-112). Chapter 5 further describes the importance of the BRCA1-A complex.

The BRCA1-B complex consists of BRCA1, the helicase BRIP1 (also known as BACH1 or FANCJ) and the topoisomerase TOPBP1. This complex plays a role in HR and it has been suggested to play a role in the S-phase checkpoint (113).

The BRCA1-C complex, contains BRCA1, the endonuclease CTIP and the MRN-complex. The interaction between BRCA1 and CTIP depends on the CDK-mediated phosphorylation of serine 327 in CTIP. The complex stimulates end-resection and thereby DSB repair processes that require end-resection (26,114). Chapter 5 further describes the importance of the binding between BRCA1 and CTIP.

BRCA1-DEFICIENCY PROVIDES THERAPEUTIC POTENTIAL

While mutations in BRCA1 increase cancer risk, BRCA1-deficiency also provides vulnerabilities that can be exploited for the treatment of the same cancer (3). BRCA1-deficient tumours are sensitive to DNA damaging drugs such as platinum compounds or PARP inhibitors (PARPi) (115). The platinum compound cisplatin was approved for clinical use in 1978, while the toxicity of PARP inhibitors was first described in 2005 (116,117). Since this discovery, different PARPi have been developed that are currently used in the clinic to treat HR-deficient tumours (118).

PARPi block the activity of PARP1, a protein important for the repair of ssDNA breaks. Besides inhibiting PARP1 activity, PARPi also trap the PARP1 protein to the DNA (119,120). A protein stuck to the DNA creates an obstacle for replication, causing stalled replication forks, which will ultimately lead to an increase of DSBs. Stalled replication forks require BRCA1 for protection and DSBs require BRCA1 for repair via HR during S-phase. It is thought that, without BRCA1, cells are unable to resolve these problems and cell death is induced. Whether this is indeed the precise mechanism behind the synthetic lethality of PARPi is still under investigation.

Therapy resistance

Unfortunately, cancer cells frequently acquire resistance to both platinum-based drugs and PARPi (121-123). The currently known mechanisms of PARPi resistance are: increased efflux of PARPi, decreased PARP trapping, stabilization of stalled replication forks or restored HR (121,124). Cells are able to restore HR either via reactivation of BRCA1/2 function through reversion mutations in the BRCA genes (125-128) or the acquisition of mutations in factors of the 53BP1-mediated NHEJ pathway (15-20,129-134).

As BRCA1-mutated tumours keep finding ways to acquire therapy resistance, additional therapeutic targets and improved understanding of BRCA1 biology are required. With the rise of CRISPR/Cas9-mediated genome-wide synthetic lethality screens, more synthetic lethal interactors (Figure 4) of BRCA1 are unveiled (135). The future of HR-deficient cancer treatment lies in inhibiting multiple synthetic lethal interactors of BRCA1/2, to overcome or prevent resistance.

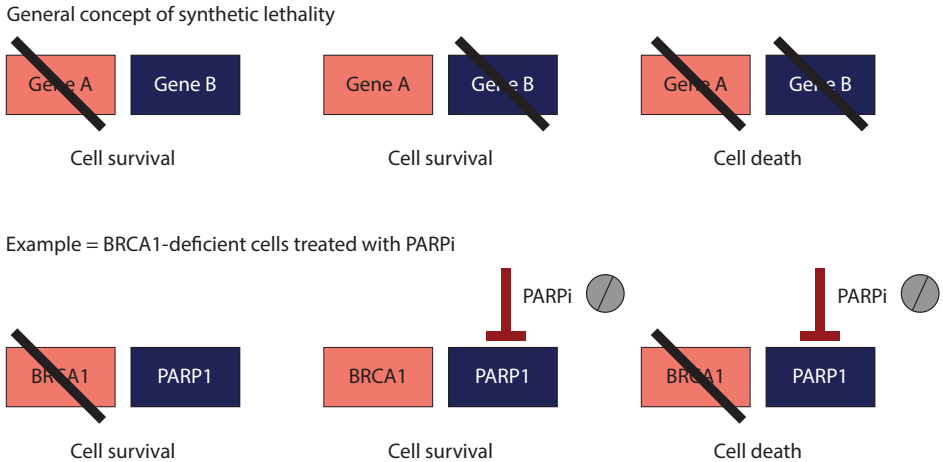

Figure 4. Synthetic lethality. Diagram explaining synthetic lethality, including the example of BRCA1-deficient cells treated with PARP inhibitors. The loss of two specific genes results in cell death, whereas the loss of either one gene does not.

THESIS OUTLINE

During my PhD, I dove into the world of genomic stability and DNA repair, important mechanisms in our body that protect us against cancer and many other diseases (13). The main focus of my PhD was BRCA1, a key protein in the DSB repair pathway HR, important for genomic stability. To complicate matters, BRCA1 is not only involved in HR, but also protects the replication fork during replication stress and supresses ssDNA gap formation. Furthermore, BRCA1 is a large protein that forms different complexes with multiple different proteins. The specific BRCA1-complex formed in turn also influences the specific role BRCA1 can play in the cell (26,110). With this thesis, I aim to improve our understanding of the labyrinthine BRCA1 biology as well as highlight current gaps and contradictions within the field.

Chapter 2
Currently, the importance of each separate BRCA1/2 function (HR, replication fork protection and ssDNA gap suppression) in tumour suppression is under debate. In the review in chapter 2, we discuss the recent papers highlighting the role of BRCA1/2 in ssDNA gap suppression and the importance of this function in tumour suppression. HR, replication fork protection and ssDNA gap suppression are all closely intertwined, complicating the ongoing research. Better separation-of-function-mutations for BRCA1 and BRCA2 are required to solve the ongoing debate. We conclude that, since both ssDNA gaps and unprotected forks lead to an increase of DSBs, the genomic instability is ultimately caused by a DSB repair defect in BRCA1/2-deficient cells.

Chapter 3
This chapter focusses on the search for new synthetic lethal interactors with BRCA1-deficiency. We discovered that the long-range end-resection factor EXO1 is essential for the survival of BRCA1-deficient but not BRCA2-deficient cells. Loss of EXO1 leads to an accumulation of DSBs and increased genomic instability in BRCA1-deficient cells. Our data indicate that BRCA1/EXO1 double deficient cells have impaired SSA on top of their HR-defect. The loss of both SSA and HR is lethal to these cells, indicating EXO1 is a promising therapeutic target for BRCA1-deficient tumours.

Chapter 4
In chapter 4, the search for new synthetic lethal interactors with BRCA1-deficieny continues. We study the genetic vulnerabilities of two hypomorphic BRCA1 missense mutations and compare these to a BRCA1-proficient and-depleted setting. These vulnerabilities were uncovered by performing CRISPR/Cas9-mediated genome-wide synthetic lethality screens. We uncover that NDE1 loss leads to increased genomic instability specifically in BRCA1^{R1669Q} mutated cells, and not BRCA1-proficient or-deficient cells. In general, we conclude that

BRCA1-deficient and BRCA1-mutated cells do not share all their genetic vulnerabilities. This is a relevant finding for the clinic, as many patient-derived mutations do not result in complete loss of the protein but often concern missense mutations with hypomorphic phenotypes .

Chapter 5

BRCA1 forms multiple multiprotein complexes with different functions. The aim in chapter 5 is to further elucidate the role of these multiprotein complexes, focussing mainly on the oxymoronic BRCA1-A complex formed with i.a. ABRAXAS1. We confirm that the loss of ABRAXAS1 leads to excessive end-resection. Contrastingly, we discover that impaired BRCA1-ABRAXAS1 binding drastically decreases end-resection. This decrease in end-resection is combined with an increase in genomic instability in the form of micronuclei. Future work is required to improve our understanding of the mechanism behind these phenotypes.

Chapter 6

In the final chapter, the findings of this thesis are discussed and put into the context of the current field of DSB repair and BRCA1 biology.

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