MSH3 promotes back-up DNA end-joining pathways that sustain the survival of BRCA2-deficient cells

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Abstract

DNA repair pathways that maintain genomic stability are essential in avoiding carcinogenesis. However, inactivation of these repair pathways frequently occurs during tumorigenesis and cancer cells become dependent on alternative mechanisms for DNA repair. Cancer cells with heterozygous germline mutations in *BRCA1* and *BRCA2* have abrogated HR function, hence they rely on alternative DSB repair pathways known to promote genomic instability, one of which is alternative non-homologous end-joining (A-NHEJ). This pathway requires the activity of error-prone DNA polymerase θ (Polθ) to anneal sequences with microhomology and utilise them as primers for DNA synthesis. Previous studies have shown that Polθ activity is critical for the survival of BRCA2-deficient cells and A-NHEJ has also been implicated in the resistance of BRCA2-deficient tumours to chemotherapy. Hence, factors of A-NHEJ represent potential targets for the elimination of BRCA-deficient tumours. The MSH2/MSH3 heterodimer has been canonically characterised in mismatch repair (MMR) in recognising long distortions in DNA structure. Notably, yeast and hamster MSH2/MSH3 displayed MMR-independent activity in repairing DNA lesions by joining DNA breaks with microhomologies, similar to A-NHEJ. The aim of this thesis was to investigate the potential role of MSH3 in the A-NHEJ pathway in BRCA2-deficient cells. We discovered that concurrent loss of MSH3 and BRCA2 reduced cell viability, thus indicating a role for MSH3 in the survival of BRCA2-deficient cells. Subsequently, we performed epistatic experiments with MSH3 and Polθ which indicated that MSH3 likely has a role in A-NHEJ. PARP-inhibitors are known to eliminate BRCA2-deficient cells. Interestingly, we observed that MSH3 loss in BRCA2-deficient cells led to their increased sensitivity to PARP inhibitor (PARPi) treatment. Thus, we have identified novel functions of MSH3 in the survival and response to treatment of human BRCA2-deficient cells.

List of Abbreviations

DNA	deoxyribonucleic acid
IR	ionising radiation
DSB	double-stranded breaks
DDR	DNA damage repair
indels	insertions-deletions
BRCA1	breast cancer susceptibility 1
BRCA2	breast cancer susceptibility 2
HR	homologous recombination
ssDNA	single stranded DNA
NHEJ	non-homologous end-joining
Ku	Ku70-Ku80
DNA-PKCs	DNA-dependent protein kinase catalytic subunit
DNA-PK	DNA-dependent protein kinase
XRCC4	x-ray repair cross-complementing protein 4
LIG4	DNA ligase IV
53BP1	p53-binding protein
CtIP	CtBP-interacting protein
NBS1	Nijmegen breakage syndrome protein 1
MRN	MRE11-RAD50-NBS1
RPA	replication protein A
BARD1	BRCA1-associated ring domain
PALB2	partner and localiser of BRCA2
D-loop	displacement loop
PCNA	proliferating cell nuclear antigen
SDSA	synthesis dependent strand annealing

dHj	double Holliday Junction
BIR	break-induced DNA replication
A-NHEJ	alternative non-homologous end-joining
MMEJ	microhomology-mediated end-joining
Polθ	DNA polymerase θ
TMEJ	DNA polymerase θ-mediated end-joining
PARP	poly(ADP-ribose) polymerase
PARP1	poly(ADP-ribose) polymerase 1
XPF	xeroderma pigmentosum group F
XRCC1	X-ray cross complimenting 1
LIG3	DNA ligase III
LIG1	DNA ligase I
PARPi	PARP inhibitors
MMR	mismatch repair
MutSα	MSH2/MSH6
MSH2	MutS homolog 2
MSH6	MutS homolog 6
MutSβ	MSH2/MSH3
MSH3	MutS homolog 3
MutLα	MLH1/PMS2
MLH1	MutL Protein homolog 1
PMS2	PMS1 homolog 2
EXO1	exonuclease 1
Polδ	DNA polymerase δ
ATR	ataxia telangiectasia and RAD3-related
DOX	doxycycline

RNA	ribonucleic acid
shRNA	short hairpin ribonucleic acid
DMEM	Dulbecco's Modified Eagle's Medium
FBS	foetal bovine serum
siRNA	small interfering RNA
RT	room temperature
DMSO	dimethyl sulfoxide
PBS	phosphate-buffered saline
DTT	dithiothreitol
PBST	PBS and 0.05% Tween 20
BSA	bovine serum albumin
HRP	horseradish peroxidase
ANOVA	analysis of variance
RPE	retinal pigment epithelial
KAP1	KRAB domain-associated protein 1
ATM	ataxia-telangiectasia mutated

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1. Introduction

1.1. Genome Instability

The deoxyribonucleic acid (DNA) within our cells is reactive and susceptible to modifications that result from exposure to endogenous and exogenous agents. Endogenous agents, such as replication-associated errors and reactive oxygen species produced by cellular metabolism, and exogenous agents, such as ionising radiation (IR), ultraviolet light, and certain chemotherapeutic drugs, can trigger accumulation of DNA lesions, the most deleterious of which are DNA double-strand breaks (DSBs) (Trenner & Sartori, 2019). Consequently, DNA damage repair pathways (DDR) are required to ensure genome integrity and cell survival. Aberrant repair of DSBs can lead to chromosomal translocations and insertions or deletions (indels), which in turn leads to genomic instability (Khanna & Jackson, 2001), a hallmark of human tumours (Hanahan & Weinberg, 2011). Tumour progression is largely dependent on the acquisition of an advantageous mutant genotype through either activation of a proto-oncogene, or loss of tumour suppressor genes. Mutations and chromosomal rearrangements often lead to tumour cells having a selective advantage, hence they are responsible for the aberrant proliferation and metastatic behaviour of cancer cells, as well as their dominance in a local tissue environment.

1.2. *BRCA1* and *BRCA2*

Breast cancer susceptibility 1 (*BRCA1*) and breast cancer susceptibility 2 (*BRCA2*) genes encode tumour suppressors that have two key physiological functions; firstly, they play critical roles in the homologous recombination repair pathway (HR; Figure 1.1A) and secondly, they act during DNA replication to protect stalled forks from nucleolytic degradation (Figure 1.1B). Cells with mutations in *BRCA1* and *BRCA2* display spontaneous aberrations in chromosome structure, which accumulate with increasing

cell divisions (Gretarsdottir et al., 1998; Tirkkonen et al., 1997; Venkitaraman, 2002a). Consequently, inactivation of either one of these two proteins often leads to genomic instability and carcinogenesis. Heterozygous germline mutations of *BRCA1* and *BRCA2* greatly predispose carriers to breast and ovarian cancers (Zhao et al., 2019; Venkitaraman, 2002). Individuals carrying *BRCA1* or *BRCA2* mutations have a 60-85% lifetime risk of developing breast cancer and a 10-40% lifetime risk of ovarian cancer (Nathanson et al., 2001). *BRCA1* mutation carriers have a higher risk of developing early-onset breast cancer, that is almost 20 years earlier than individuals not carrying the mutation (D. F. Easton et al., 1994). Notably, increased cancer risk is not limited to women who carry mutations in these genes, because men with *BRCA2* germline mutations also have an approximately 6% increase in breast cancer risk (D. Easton, 1999). *BRCA1* and *BRCA2* mutations also predisposes individuals to other cancer types, such as prostate, colon, pancreatic, and stomach cancer, amongst others (D. Easton, 1999; Ford et al., 1994). As loss of *BRCA1* and *BRCA2* leads to genomic instability and greatly predisposes to cancer development, understanding the physiological roles of these proteins is critical in developing novel therapies to improve patient survival.

1.2.1. Roles of BRCA1 and BRCA2 in replication

DNA replication is an essential process in cell division where genomic information is copied to produce two complementary DNA molecules (E. Y. C. Chang & Stirling, 2017). During DNA replication, the replication fork may encounter obstacles that leads to the stalling of the replication fork (Cortez, 2015). These obstacles include sites which are difficult to replicate (e.g. G-quadruplexes), DNA-protein crosslinks, and replisome collisions with transcription complexes sites (Zeman & Cimprich, 2013). Stalled forks may lead to fork collapse if not repaired (Hamperl & Cimprich, 2016; Tarsounas & Sung, 2020). In addition to fork restart, BRCA1 and BRCA2 are also able to protect stalled

replication forks from MRE11-mediated nucleolytic degradation (Schlacher et al., 2011). Although mechanisms are not fully understood, it is thought that the loading of RAD51 by BRCA1 and BRCA2 onto the single stranded DNA (ssDNA) at stalled replication forks facilitates their protection. In the absence of BRCA1 and BRCA2, increased replication stress leads to fork collapse and nucleolytic fork degradation, ultimately leading to accumulation of toxic DNA lesions (Feng & Jasin, 2017; Tarsounas & Sung, 2020).

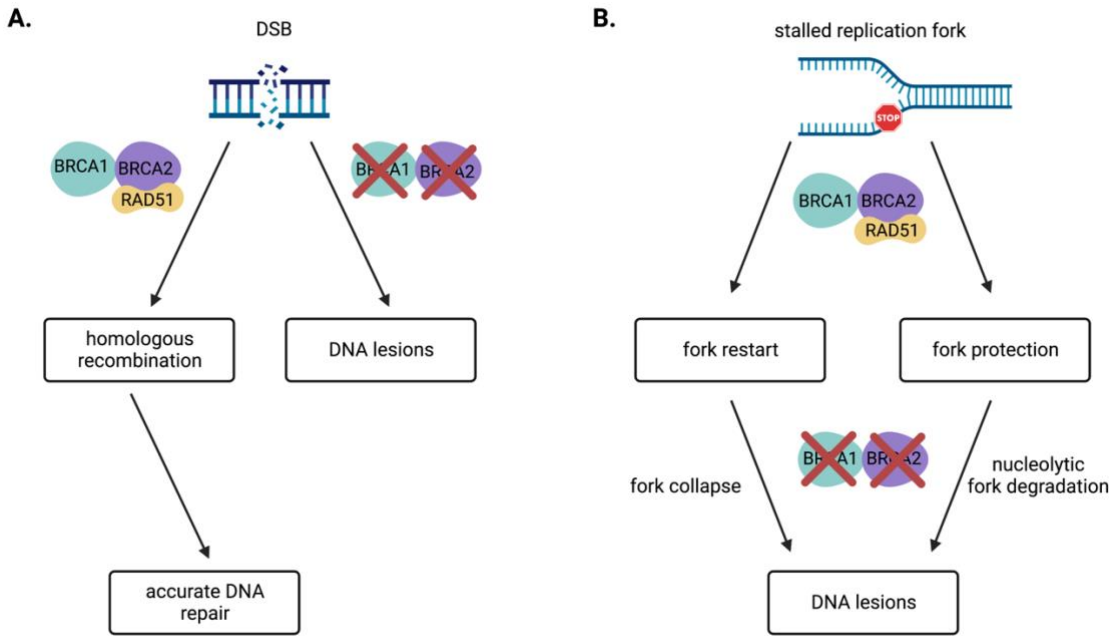


Figure 1.1 | Roles of BRCA1 and BRCA2 in Replication and Repair A. BRCA1 and BRCA2 promote DSB repair through loading of the RAD51 recombinase, which initiates the HR pathway (discussed in following sections). HR is an error-free repair pathway leading to accurate DNA repair. In the absence of BRCA1 or BRCA2, DSB cannot be accurately repaired, hence leading to an accumulation of chromosome aberrations and DNA lesions. **B.** BRCA1 and BRCA2 also facilitate the restart and protection of stalled replication fork through the loading of RAD51. Abrogation of BRCA1 or BRCA2 function leads to fork collapse and/or nucleolytic fork degradation, finally promoting accumulation of DNA lesions. Figure adapted from Tarsounas & Sung, 2020. Created with Biorender.com.

1.3. DSB repair

There are two main pathways of DSB repair in mammalian cells: non-homologous end-joining (NHEJ) and HR. NHEJ involves the joining of blunt ends in order to repair DNA, while HR requires resection with the formation of 3' single-stranded overhangs and homologous DNA to serve as a template for repair (Chang et al., 2017; Zhao et al., 2019).

1.3.1. NHEJ

NHEJ is an error-prone repair pathway, which can act throughout all phases of the cell cycle (Figure 1.2). The joining of blunt ends (Figure 1.2.A) in NHEJ is initiated by the heterodimer Ku70-Ku80 (Ku). Ku recognises DSBs and binds with high affinity to free DNA ends, thereby preventing end-resection and acting as a scaffold for other NHEJ factors to be recruited to the DSB site (H. H. Y. Chang et al., 2017). DNA-dependent protein kinase catalytic subunit (DNA-PKcs) is then recruited to Ku-bound DNA ends to form the DNA-dependent protein kinase (DNA-PK) complex. The autophosphorylation of DNA-PK is required to signal the presence of a break and to activate other DNA repair proteins (Burgess et al., 2014). Finally, X-ray repair cross-complementing protein 4 (XRCC4) and DNA Ligase IV (LIG4) are loaded onto DNA ends and facilitate the ligation of the two ends (Chen et al., 2000).

Blunt DNA ends are preferentially repaired via this pathway with relatively high efficiency (H. H. Y. Chang et al., 2017). However, DNA ends frequently show overhangs and require the action of other enzymes for further processing prior to ligation (Figure 1.2.B). When DNA ends have incompatible overhangs, the DNA-PK complex facilitates recruitment of other nucleases, such as the endonuclease Artemis. Artemis, in complex with DNA-PKCs, is able to remove 5' and 3' DNA overhangs, which allows DNA ends to be ligated by the XRCC4-LIG4 complex (Goodarzi et al., 2006). As this pathway does

not rely on sequence homology it is error prone and can result in small indels at the DNA lesion site (H. H. Y. Chang et al., 2017).

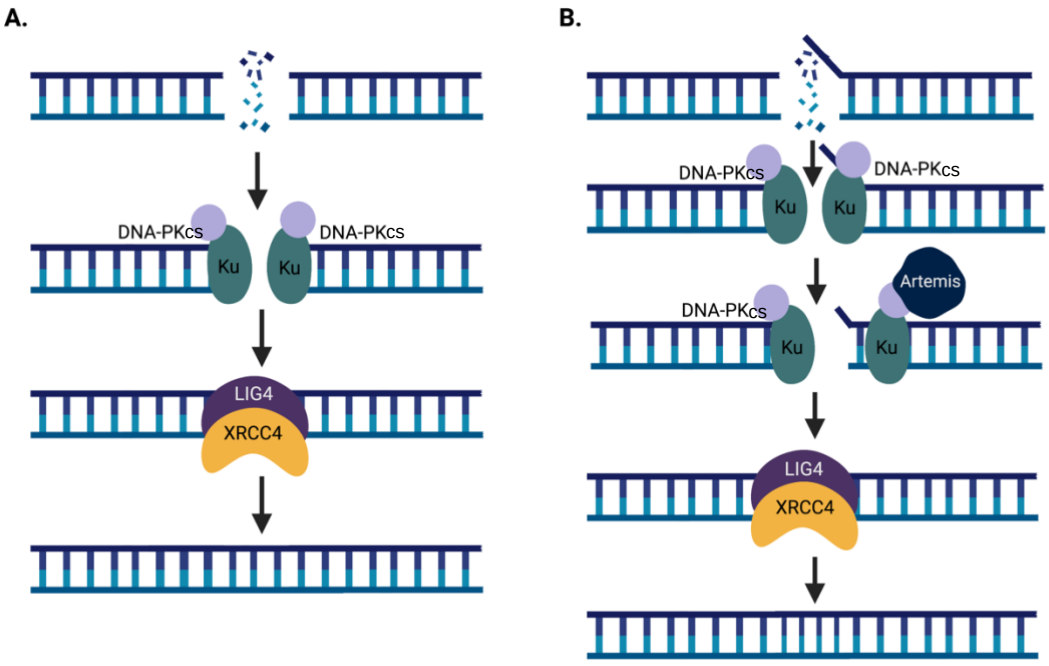


Figure 1.2 | Schematic representation of the NHEJ pathway. **A**. In the case of DSBs with blunt ends the heterodimer Ku recognises and binds to DNA ends. DNA-PKcs is recruited to DNA ends and form a complex with Ku. The DNA-PK and Ku complex is auto phosphorylated to signal the presence of the break and recruit the relevant repair proteins. LIG4 in a complex with XRCC4 then ligates the two ends. **B**. When DNA ends require further processing, DNA-PKcs recruits Artemis. Artemis removes the 5' and 3' DNA overhangs, which allows ends to be ligated by LIG4-XRCC4. Figure adapted from Chang et al., 2017. Created with Biorender.com.

1.3.2. HR

HR is a DSB repair pathway that is essential for maintaining genomic integrity (Figure 1.3). Unlike NHEJ, HR occurs primarily in the G2 and S phases of the cell cycle as it requires a homologous template for accurate repair (W. Zhao et al., 2019). The choice of repair pathway depends on the competitive binding of p53-binding protein 1 (53BP1) or BRCA1 to the break site (Daley & Sung, 2014). During S and G2 phases of the cell cycle, BRCA1 promotes the removal of 53BP1, an inhibitor of end-resection, from DSB

ends to allow access to the resection and HR machineries (Bunting et al., 2010). In the absence of BRCA1, 53BP1 accumulates at DSBs thereby causing cells to rely on alternate pathways of repair such as NHEJ (Tarsounas & Sung, 2020).

Once DSBs are detected, the MRE11-RAD50-Nijmegen breakage syndrome protein 1 (NBS1; MRN) complex is recruited to the damage site where it initiates end-resection. HR requires the end resection of ssDNA in the 5' to 3' direction to create long 3' ssDNA overhangs. The ubiquitination of CtBP-interacting protein (CtIP), a co-factor of the MRN complex, by BRCA1-BRCA1 associated ring domain (BARD1) complex also aids in the initiation of DNA end-resection (F. Zhao et al., 2020). The 3' ssDNA overhangs produced by end-resection are bound by replication protein A (RPA) (X. Li & Heyer, 2008). Subsequently, the BRCA1-BARD1 complex recruits BRCA2 in association with partner and localiser of BRCA2 (PALB2), to displace RPA and load RAD51 recombinase onto the ssDNA, thus creating the pre-synaptic complex (Prakash et al., 2015). RAD51 is essential in initiating the strand-invasion process of HR, by searching for a sequence on the DNA duplex that is homologous to the 3' overhang (X. Li & Heyer, 2008). When homology is found the pre-synaptic complex then invades the template DNA to create a displacement-loop (D-loop) intermediate between the invading 3' strand and the homologous DNA molecule (San Filippo et al., 2008). Following strand-invasion and subsequent D-loop formation, DNA polymerase δ in association with proliferating cell nuclear antigen (PCNA) commences the synthesis of DNA from the 3' ends of the invading strand to copy the sequence from the homologous template (Zhao et al., 2019). HR is completed through processing of the extended D-loop structures by four different pathways: (1) synthesis-dependent single strand annealing (SDSA), (2) double Holliday junction (dHJ) resolution, (3) dHJ dissolution or (4) break-induced DNA replication (BIR) (Tarsounas & Sung, 2020). There is a greater tendency for cells to resolve D-loop structures via SDSA or dHJ dissolution and subsequently form non-crossover recombinants (W. Zhao et al., 2019). BIR is frequently used at DSB proximal to telomeres

(San Filippo et al., 2008). From these four distinct pathways, only dHJ resolution results in the generation of recombinants that has undergone a genetic crossover event (W. Zhao et al., 2019b).

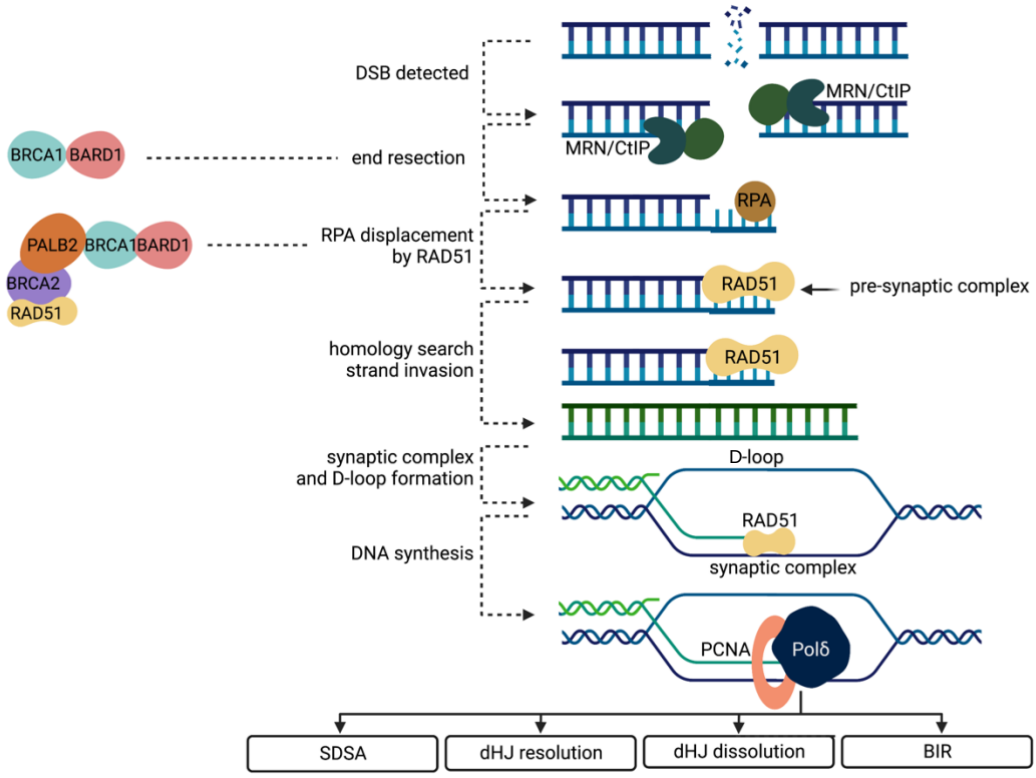


Figure 1.3 | Schematic representation of HR. Once a DSB is detected, BRCA1-BARD1 and MRN/CtIP initiate the resection of DNA from 5' to 3'. The 3' ssDNA overhang generated from this process is protected by RPA, prior to being displaced by RAD51 leading to the formation of the pre-synaptic complex. The loading of RAD51 onto ssDNA is facilitated by BRCA2-PALB2 and BRCA1-BARD1. RAD51 then initiates homology search which allows for the capture of dsDNA that is homologous to the 3' overhang. The presynaptic complex then invades this template strand, creating the synaptic complex and subsequently a D-loop. Following this, Polδ (in complex with PCNA) synthesises DNA from the 3' ends of the invading strand using the homologous template. HR can be resolved in four different pathways: SDSA, dHJ resolution, dHJ dissolution, and BIR. Figure adapted from Tarsounas & Sung, 2021. Created with Biorender.com.

1.3.3. Alternative non-homologous end-joining (A-NHEJ)

HR abrogation, often due to loss of BRCA1 or BRCA2, forces cells to rely on alternate pathways of DSB repair. The alternative non-homologous end-joining (A-NHEJ) pathway is a critical compensatory pathway when HR is abrogated (H. H. Y. Chang et al., 2017). A-NHEJ, also often called the microhomology-mediated end-joining (MMEJ) or DNA

Polymerase θ (Polθ)-mediated end-joining (TMEJ), is an end-joining pathway that relies on short length of sequence homology in order to facilitate repair (Figure 1.4.). In the initial step of A-NHEJ poly (ADP-ribose) polymerase 1 (PARP1) recognises the DNA damage and recruits MRN and CtIP to the broken end (Audebert et al., 2004). DNA end-resection then occurs, which involves the endonuclease complex MRN and its co-factor CtIP, and generates 15-100 nucleotide 3' overhangs (Xie et al., 2009). Polθ, encoded by the gene *POLQ*, binds to the overhangs generated by the end resection, and anneals sequences which share 2-6 base pairs of sequence homology, using them as primers for DNA synthesis (Mateos-Gomez et al., 2015). Occasionally, resection creates non-homologous 3' ssDNA which requires removal by the xeroderma pigmentosum group F (XPF) in complex with DNA excision repair protein ERCC1, prior to gap filling by Polθ (Wyatt et al., 2016). The DNA ends are then ligated by X-Ray cross complementing 1 (XRCC1) in a complex with DNA ligase 3 (LIG3) or DNA ligase 1 (LIG1; Schrempf et al., 2021).

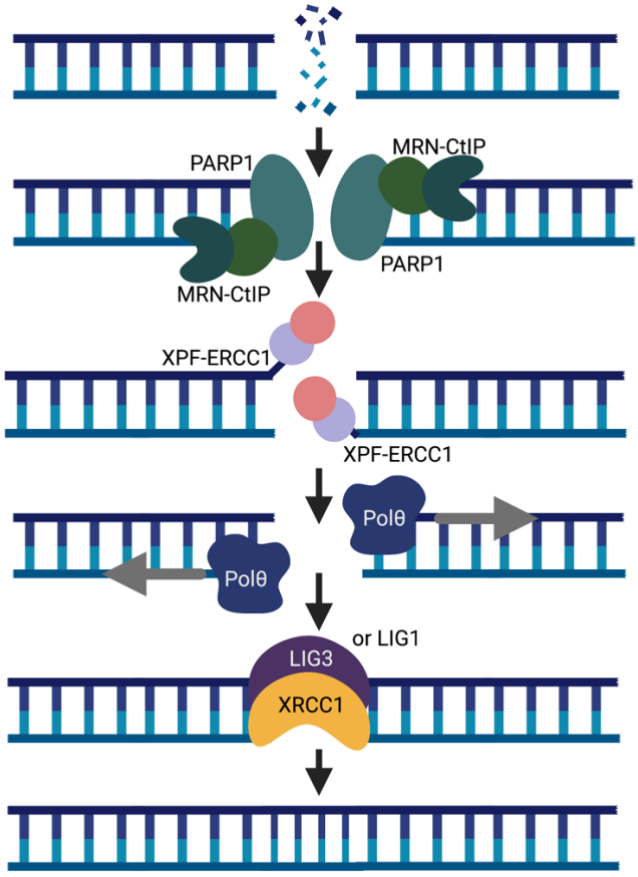


Figure 1.4 | Schematic representation of A-NHEJ. PARP1 recognises DNA damage and recruits the MRN-CtIP complex to initiate DNA end-resection. Resection may lead to non-homologous 3' ssDNA overhangs which are removed by XPF-ERCC1 prior to filling by Polθ. Polθ binds to the overhangs, anneals sequences which share small amounts of homology and uses them as primers for DNA synthesis. DNA ends are then ligated by XRCC1 in complex with DNA ligase I or III. Figure adapted from Schrempf et al., 2021. Created with Biorender.com.

Repair by A-NHEJ is mutagenic and often leads to indels and chromosomal translocations (Zhang & Jasin, 2011), as well as microhomology-flanked chromosomal aberrations (Sallmyr & Tomkinson, 2018). Notably, A-NHEJ activity is upregulated in cancer cells in which HR has been abrogated (Seol et al., 2018). Polθ expression is upregulated in *BRCA1*- and *BRCA2*-mutated tumours and Polθ polymerase activity is essential for the survival of BRCA2-deficient cells (Ceccaldi et al., 2015). These results indicate the importance of A-NHEJ as a survival compensatory pathway in cancer cells deficient for BRCA1 or BRCA2. Due to this, factors of the A-NHEJ pathway represent potential targets for the elimination of BRCA-deficient tumours. In addition, A-NHEJ has

been implicated in the resistance of BRCA2-deficient tumours to chemotherapy by mediating mutations that restore *BRCA2* activity (Edwards et al., 2008; Sakai et al., 2008; Tobalina et al., 2021).

1.4. Targeting tumours with *BRCA1/2* mutations in the clinic

BRCA1- and BRCA2-deficient tumours are routinely treated with chemotherapeutic drugs which exploit the genomic instability caused by the loss of these tumour suppressor proteins. Small molecule inhibitors of PARP (PARP inhibitors; PARPi), such as Olaparib and Talazoparib, have been approved for the treatment of BRCA1/2-deficient cancers (Ashworth & Lord, 2018). PARP represents a family of 17 proteins which are able to catalyse the transfer of ADP-ribose onto target proteins (Rose et al., 2020). The best characterised protein of the family is PARP1, an enzyme which is an important sensor in DNA repair (Ray Chaudhuri & Nussenzweig, 2017). PARPi acts by trapping PARP on chromatin, thereby interfering with DNA replication and repair, and ultimately leading to apoptosis (H. Li et al., 2020).

Despite initial clinical successes, BRCA1- and BRCA2-deficient tumours commonly acquire resistance to PARPi, either through the restoration of HR function or by stabilising their replication forks (Chaudhuri et al., 2016; D'Andrea, 2018; H. Li et al., 2020). Approximately 40% of BRCA1- and BRCA2-deficient ovarian cancer patients fail to respond to PARPi treatment (Fong et al., 2010). Due to this, it has become imperative to discover alternative methods of targeting HR-deficient tumours and overcoming PARPi resistance.

A-NHEJ factors represent attractive therapeutic targets for the elimination of HR-deficient tumours as BRCA-deficient cells has been shown to rely on A-NHEJ for DSB repair (Ceccaldi et al., 2015). Abrogating this compensatory repair pathway results in accumulation of DNA damage and/or replication stress, and, subsequently, apoptosis.

Two Polθ inhibitors are currently in pre-clinical development or undergoing clinical trials (Gourley et al., 2019; Zatreanu et al., 2021; Zhou et al., 2021). The antibiotic novobiocin was shown to be able to bind to Polθ and inhibit its recruitment to DSB ends, thereby halting repair via A-NHEJ and selectively killing HR-deficient tumours (Zhou et al., 2021). Similarly, ART558 is a new drug shown to have a synthetic lethal effect in both *BRCA1* and *BRCA2* mutant cells (Zatreanu et al., 2021). Both these small molecule inhibitors were also able to overcome PARP inhibitor resistance (Zatreanu et al., 2021; Zhou et al., 2021). Hence, they could be independently or concomitantly administered with PARP inhibitors to treat HR-deficient tumours.

Clinical studies have shown that the clinically relevant serum concentrations of novobiocin can kill HR-deficient tumours and caused no adverse side-effects (Drusano et al., 1986; Eder et al., 1991). However, an early phase I clinical trial showed that although novobiocin was well tolerated, it only slowed breast cancer progression in a small number of patients (Kennedy et al., 1995). Currently, clinical trials are in preparation to evaluate the ability of novobiocin to treat patients with *BRCA1*- and *BRCA2*-mutated cancers. Likewise, although ART588 was shown to be efficient in HR-deficient tumours in mice, it has not proceeded to clinical trials (Zatreanu et al., 2021). Currently, a different formulation called ART4125 is undergoing a Phase I/IIa study (NCT04991480) to determine its safety, pharmacokinetics, and efficiency as single treatment or in combination with PARP inhibitors in treating advanced and metastatic breast cancer patients.

1.5. MutS Homolog 3 (MSH3)

1.5.1. MSH3 in mismatch-repair (MMR)

The MMR pathway recognises and repairs spontaneous base-base mismatches and small loops that are generated during DNA replication and genetic recombination

(Jiricny, 2006). The MMR reaction can be divided into three main steps: recognition, excision and resynthesis, and ligation (Figure 1.5). MMR is initiated with the recognition of DNA distortions by two complexes, MutSα and MutSβ, which recognise different types of lesions and promote repair. The most abundant mismatch-binding factor, MutSα is comprised of the heterodimer MutS homolog 2 (MSH2)/MutS homolog 6 (MSH6). MutSα recognises base-base mismatches and indel loops of 1-2 nucleotides (Li, 2007). On the other hand, MutSβ, a heterodimer of MSH2/MutS homolog 3 (MSH3), recognises longer indel loops, up to 14 nucleotides (Gupta et al., 2012). Once a DNA lesion is recognised, MutSα or MutSβ recruit MutLα, a heterodimer of MutL protein homolog 1 (MLH1)/ PMS1 homolog 2 (PMS2). PCNA then interacts with MutLα and stimulates its endonuclease function to generate nicks in the DNA strands (Kadyrov et al., 2006). Subsequently, MSH2 and MLH1 recruit Exonuclease 1 (EXO1) which performs a 5' to 3' DNA degradation at the MuLα incision site (Tran et al., 2004). DNA is then re-synthesised by DNA polymerase δ (Polδ) and ligated by DNA ligase 1 (Liu et al., 2017). Loss of MMR factors has been implicated in carcinogenesis. Mutations in *MSH2* and *MSH6* have been shown to predispose individuals to hereditary non-polyposis colorectal cancer/Lynch syndrome (Steinke et al., 2013). Furthermore, *MSH3* loss has been reported to drive the progression of a variety of tumours in mice (van Oers et al., 2014).

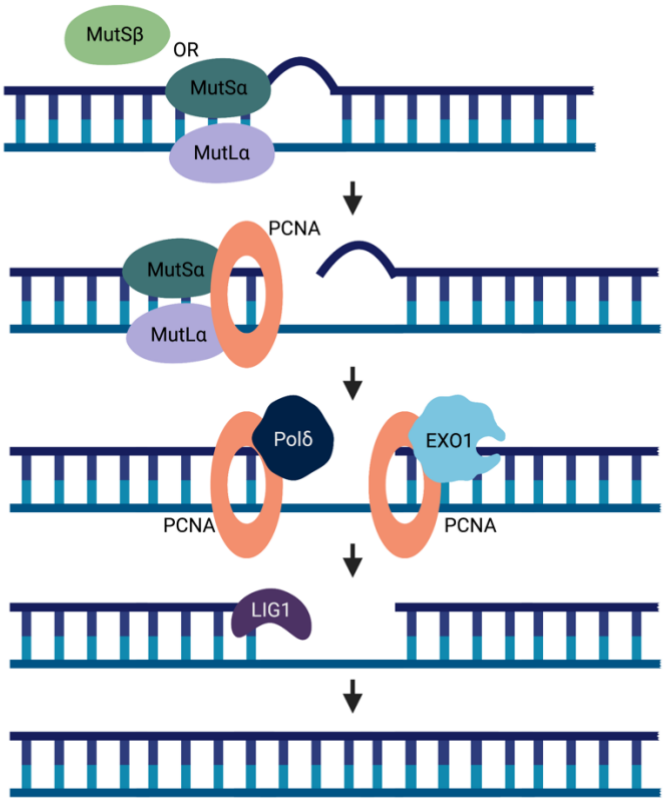


Figure 1.5 | Schematic representation of the MMR pathway. DNA lesions are detected by MutSα (single base mismatches) or MutSβ (longer insertion/deletion loops). These heterodimers are able to bind and recruit MuLα. MuLα interacts with PCNA which activates the endonuclease activity of MuLα to generate nicks in the DNA strand and to recruit EXO1, which excises the DNA lesion. DNA is re-synthesised in the excision gap by Polδ and ligated by DNA ligase 1. Figure adapted from Pećina-Šlaus et al., 2020. Created with BioRender.com.

1.5.2. MMR-independent roles of MSH3

Although initially characterised in the MMR pathway, MutSβ consisting of MSH2 and MSH3, also has MMR-independent activities and has been implicated in DSB repair (Kunkel & Erie, 2005). In *Saccharomyces cerevisae,* this heterodimer is essential in recognising and stabilising non-homologous DNA ends during DSB break repair by gene conversion and single strand annealing (SSA), leading to the removal of unpaired tails by the endonuclease RAD1-RAD10 (Sugawara et al., 1997). The MSH2-MSH3 heterodimer also plays a role in repairing DNA lesions by joining DNA breaks with

microhomologies, similar to A-NHEJ, in both budding yeast and Chinese hamster ovary cells (Kumar et al., 2013).

MutSβ has been implicated in the HR repair of IR-induced DSBs, in addition to its role in replication-associated MMR. HR repair of IR-induced DSBs was impaired in MSH2-deficient mouse cells (Franchitto et al., 2003). Furthermore, MSH3-deficient mouse cells had increased chromosomal abnormalities, even in the absence of a DNA damaging agent. Moreover, they showed impaired repair of IR-induced DNA lesions, suggesting that loss of MutSβ function compromised DSB repair (van Oers et al., 2014). Recently, MutSβ was implicated in ataxia telangiectasia and RAD3-related (ATR) protein kinase activation in response to DSBs. Mutations in the binding domain of MSH3 resulted in failure to binding DNA, thus abrogating its ability to promote ATR activation and ATR-mediated DSB repair (Burdova et al., 2015). However, the role of MSH3 in DSB repair remains poorly understood.

1.6. Aim

Previous work in our laboratory has demonstrated that MSH3 is required for the proliferation of BRCA2-deficient cells. As MSH2/MSH3 has MMR-independent functions, some of which are similar to A-NHEJ, we postulated that the MSH2/MSH3 heterodimer could be a novel A-NHEJ factor in human cells. As cells lacking HR rely on compensatory DNA repair pathways such as A-NHEJ, this works aims to provide further insight into the mechanisms required for the survival of HR deficient cells. In this project, we sought to investigate the role of MSH3 in the survival of BRCA2-deficient cells. Firstly, we investigated whether MSH3 functions in the A-NHEJ pathway in BRCA2-deficient cells. To address this, we used epistasis analyses between Polθ and MSH3 to determine whether the two proteins act in the same repair pathway. To characterise the role of MSH3 in BRCA2-deficient cells we also examined DNA damage signalling, markers of replication stress and the contribution of MSH3 to PARP inhibitor sensitivity. This work could help elucidate the role of MSH3 in BRCA2-deficient cell survival, which is a critical first step in identifying novel clinical targets for the elimination of BRCA2-deficient tumour cells.

2. Materials and Methods

2.1. Cell lines

Human non-small-cell lung carcinoma H1299 cells that carry a doxycycline (DOX)-inducible short hairpin ribonucleic acid (shRNA) against BRCA2 (GGG AAA CAC UCA GAU UAA A; H1299+shBRCA2^{DOX}; Lau et al., 2017) were cultivated in Dulbecco's Modified Eagle's Medium (DMEM; Sigma Aldrich, D5796-500ML) supplemented with filter-sterilised, tetracycline-free 10% foetal bovine serum (FBS; Takara Bio UK Ltd, 631106). Expression of shRNA against *BRCA2* was induced by the addition of 2 µg/mL DOX (Sigma-Aldrich, D9891) to the growth medium 4 days prior to commencing experiments. *BRCA2*$^{+/+}$ (RPE1 hTERT TP53$^{-/-}$ WT) and *BRCA2*$^{-/-}$ (RPE1 hTERT *TP53*$^{-/-}$ *BRCA2*$^{-/-}$) human retinal pigment epithelial RPE1 cells expressing Cas9, transduced with hTERT and *TP53*-deleted (Dagg et al., 2021; Groelly et al., 2022) were cultivated in DMEM supplemented with 10% FBS (Merck Life Science UK, F7524-500ML), and 2 µg/ml blasticidin (Life Technologies, A1113903). Cell lines were grown at 37˚C with 5% CO_2 atmosphere unless otherwise indicated and were routinely tested for mycoplasma contamination.

2.2. Small interfering RNA (siRNA) transfection

2.2.1. siLentFect siRNA transfection

siRNAs utilised in transfections were re-suspended in siRNA buffer (Horizon Discovery, B-002000-UB-100) to 40 µM. Reverse transfections were conducted using the siLentFect transfection solution (Bio-Rad Laboratories Ltd, 1703360) diluted 1:25 with Opti-MEM media (Life Technologies, 31985062). This mixture was then incubated at room temperature (RT) for 5 minutes. siRNA was diluted with Opti-MEM to a final concentration of 10 nM. Both solutions were mixed at a 1:1 ratio and incubated for another 20 minutes at RT. The final mixture of the transfection solution was then added dropwise to 10–30X10^4 cells in 6-well plates or 6 cm dishes and incubated at 37ºC, 5%

CO_2 for 24 hours. The growth medium was replaced with fresh medium the following day. Two days post-initial transfection, following the same procedure, a re-transfection was conducted. The siRNAs utilised were siMSH3-02, siMSH3-01, siPOLθ, siGENOME BRCA2 siRNA SMARTpool (Horizon Discovery Biosciences Ltd, M-003462-01-0020, and AllStars siRNA (Qiagen, 1027281) which was used as a negative control (Table 1). Cell pellets were collected for immunoblotting to confirm depletion of the protein upon seeding of every experiment.

RPE1 hTERT *TP53*$^{-/-}$ *BRCA2*$^{-/-}$ cells were generated by CRISPR with a guide RNA (gRNA) that targeted a region in the middle of the BRC repeats. This may result in a truncated BRCA2 protein that retains some ability to bind RAD51. Consequently, RPE1 hTERT *TP53*$^{-/-}$ *BRCA2*$^{-/-}$ cells were transfected with siRNA against BRCA2 to ensure BRCA2 function was abrogated in all subsequent experiments.

Table 2.1. siRNA sequences

siRNA	**Sequence**
siMSH3-02 (Horizon Discovery Biosciences Ltd, custom siRNA sequence)	GCA AGG AGU UAU GGA UUA A
siMSH3-01 (Horizon Discovery Biosciences Ltd, custom siRNA sequence)	AUA CGC CGC UAG AAU UAC A
siPOLθ (Horizon Discovery Biosciences Ltd, custom siRNA sequence)	UUU CAU AUA AAC AUU CUG G
siGENOME BRCA2 siRNA SMARTpool (Horizon Discovery Biosciences Ltd, M-003462-01-0020)	GAA ACG GAC UUG CUA UUU A, GUA AAG AAA UGC AGA AUU C, GGU AUC AGA UGC UUC AUU A, GAA GAA UGC AGG UUU AAU A

2.2.2. Dharmafect siRNA transfection

For the proliferation assay using H1299 cells, cells were reverse transfected using the DharmaFECT-1 (Horizon Discovery Biosciences Ltd, T-2001-03) siRNA transfection

reagent. The respective siRNAs were diluted to 40 nM. The reverse transfection is carried out following the protocol outlined in 2.2.1.

2.3. Population doubling (Proliferation) assay

H1299+shBRCA2^{DOX} cell cultures were supplemented with 2 µg/mL DOX for 3 days prior to seeding the experiment. Moreover, a siRNA transfection using siMSH3-01 and siMSH3-02 was conducted two days prior to the seeding of the assay to deplete MSH3. Cells (1.5-2 X10^3 cells per well) were seeded in triplicate in a 96-well plate so that growth reached 70-80% confluency on the final day of measurement (day 8 post-seeding). To measure the population doublings, cells were incubated with 10 µg/mL resazurin (Sigma, R7017), a fluorogenic oxidation-reduction indicator, in medium at 37˚C for 2 hours. After incubation, fluorescence was then measured (544 nm excitation and 590 nm emission) using POLARstar Omega plate reader (BMG Labtech). Measurements were conducted 5 hours after seeding (day 0) and subsequently on day 2, 4, 6, and 8 post-seeding. The population doubling on each of these days was calculated relative to the cells seeded on day 0.

2.4. Survival (Clonogenics) assay

2.4.1. Survival assay after siRNA transfection

Four days prior to seeding RPE1 hTERT *TP53*$^{-/-}$ cells were transfected twice with siMSH3-02, siPOLθ, and siBRCA2 to deplete the respective target proteins. Cells (300-600 cells per well) were then seeded in technical triplicates in a 6-well plate and incubated at 37˚C, 5% CO_2 and 2% O_2 for 10-14 days until colonies of >50 cells/colony were observed. Colonies were stained with 5 mg/mL crystal violet (Merck, 1.15940.0025) in 50% methanol and 20% ethanol. Colonies were then counted, and cell survival expressed relative to control cells.

2.4.2. Drug dose-response survival assay

H1299+shBRCA2^{DOX} cells, grown in the presence or absence of DOX and transfected with control or MSH3-02 siRNA, were seeded (200-400 cells per well) in triplicate in 6-well-plates. After 24 hours the cells were treated with a serial dilution of Talazoparib (Cambridge Bioscience Ltd, HY-108413; 0.625 nM, 0.313 nM, 0.156 nM, 0.078 nM, and 0.039 nM) or vehicle dimethyl sulfoxide (DMSO; Sigma Aldrich, D450-500ML; volume equivalent to the highest Talazoparib concentration used) and grown at 37˚C and 5% CO_2 for 24 hours. The following day, the drug was removed and replaced with fresh media. Cells were incubated at 37˚C and 5% CO_2 for 5-10 days prior to staining with crystal violet. Cell survival was expressed relative to vehicle control cells.

2.5. Resazurin-based viability assay

A resazurin-based viability assay was conducted to evaluate the effects of Talazoparib on the viability of RPE1 hTERT *TP53*$^{-/-}$ cells. Cells were transfected twice with AllStars Control siRNA, siMSH3-02, and siBRCA2 prior to seeding. Cells were seeded (500-700 cells per well) in triplicate in a 96-well-plate to reach 70-80% confluency after 6 days. Once cells had adhered, 4-5 hours post-seeding, Talazoparib was added to the cells at the indicated doses (1:2 serial dilution for 8 doses beginning at 10 nM). Control cells were treated with 100% v/v with a volume of DMSO equivalent to the Talazoparib concentration of 10 nM. Cells were then incubated for 6 days at 37˚C and 5% CO_2. To determine viability, cells were incubated at 37˚C for 2 hours with 10 µg/mL of resazurin (Sigma, R7017) in media. After reading the fluorescence the cell viability was calculated relative to the vehicle control.

2.6. Immunoblotting

For siRNA transfection timecourses, 10-15 X10^4 cells were seeded in a 6-well plate (Greiner Bio-One, 657160) and harvested 2- and 6-days post transfection. To examine DNA damage and replication stress, a 6 cm dish (Greiner Bio-One, 628160) was seeded

with 15-25X10^4 cells. Once the cells had adhered (4-5 hours later), 5 nM of Talazoparib was added to the growth medium for 3 days; the equivalent volume of the vehicle DMSO was used to treat control cells.

To prepare whole cell extracts, cells were washed with 1X phosphate-buffered saline (PBS; Fisher Scientific, BR014G) and detached from the plates by trypsin-EDTA (Sigma Aldrich, T3924). Cells were collected, washed with 1X PBS and pelleted by centrifugation at 250 x *g* for 5 minutes. Cell pellets were then lysed in SDS-PAGE loading buffer (0.16M Tris.HCl pH 6.8, 4% SDS, 25% glycerol, 0.01% bromophenol blue) supplemented with 100 mM dithiothreitol (DTT; Sigma Aldrich, D9779-5G) and cocktails of protease (Roche, 04693159001) and phosphatase (Roche, 04906845) inhibitors. Samples were sonicated for 6-10 seconds using Ultrasonic Homogeniser 300 V/T (Biologics Inc.) to fragment the DNA, then heated at 80˚C for 5 minutes to denature the proteins and centrifuged at 13,000 x *g* for 7 minutes to remove any cellular debris. The NanoDrop-1000 spectrophotometer was used to quantify the protein concentration of samples.

An equal amount of protein (100-120 µg) was then analysed by gel electrophoresis followed by immunoblotting. NuPAGE Novex 3-8% Tris-Acetate gels (Life Technologies, EA03752BOX) or NuPAGE Novex 4-12% Bis-Tris Protein gels (Life Technologies, NP0322BOX), with the HiMark prestained protein standard marker (Life Technologies, LC5699) and NuPAGE Tris-Acetate SDS Running Buffer (Life Technologies, LA0041) or Fermentas PageRuler Prestained Protein Ladder (Fisher Scientific, 11812124) and NuPAGE MOPS SDS Running Buffer (Life Technologies, NP0001), respectively, were run at 180 V until the desired separation of bands was achieved. Proteins were transferred onto a nitrocellulose membrane (VWR International Ltd, 10600001) by semi-dry transfer at 30 V with NuPAGE Transfer buffer (Life Technologies, NP00061) supplemented with 10% methanol. The subsequent membrane was blocked with 5% Marvel non-fat-milk in 0.05% Tween 20 (Merck Life Science UK

Ltd, P7949-500ML) in 1X PBS (PBST) for 1 hour at RT and then incubated overnight at 4˚C with primary antibodies (Table 2.2) diluted in 2% bovine serum albumin (BSA; Sigma Aldrich, A7906-100g) and 0.05% azide in PBST or 5% Marvel non-fat-milk in PBST.

Table 2.2. Primary antibodies used for Western Blot

Target	Host	Provider	Catalogue Number	Dilution	Target size (kDa)
BRAC2	Mouse	Calbiochem	OP95	1:500	384
GAPDH	Mouse	Novus Biologicals	NB600-502	1:30,000	36
KAP1	Rabbit	Bethyl Laboratories	A300-273A	1:1,000	89
Phosphorylated Ser824 KAP1	Rabbit	Bethyl Laboratories	A300-767A	1:1,000	89
MSH3	Mouse	BD Biosciences	611390	1:1,000	127
POLQ	Mouse	A gift from Jean-Sebastien Hoffman's group	N/A	1:5,000	289
RPA32	Mouse	Abcam	AB2175	1:1,000	32
Phosphorylated Ser33 RPA32	Rabbit	Bethyl Laboratories	A300-246A	1:1,000	32
SMC1	Rabbit	Bethyl Laboratories	A300-055A	1:10,000	143

The following day, the membrane was washed 3 times for 5 minutes with PBST and then incubated for 1 hour at RT in the relevant horseradish peroxidase (HRP)-conjugated secondary antibodies (Table 2.3.) diluted 1:5,000 in 5% Marvel non-fat-milk in PBST. Next, membranes were washed as before with PBST. Protein detection was achieved using enhanced chemiluminescence (Thermo Scientific, 32106; Fisher

Scientific, WBKLS0500; GE Healthcare, RPN2235) detected by CL-XPosure films (Fisher Scientific, 34089) using the ECOMAX X-ray film processor (PROTEC, 1186-3-4000).

Table 2.3. Secondary Antibodies Used in Western Blot

Target	Host	Provider	Catalogue Number	Dilution
Mouse	Goat	Dako	P0447	1:5,000
Rabbit	Goat	Dako	P0448	1:5,000

2.7. Statistical Analyses

Statistical analyses for experiments were performed using GraphPad Prism. For comparison of means in the survival (clonogenics) assay a one-way analysis of variance (ANOVA) test was applied. To test for statistical significance of means in the dose-response clonogenics assays, an unpaired T test was applied. *P*-value of $P \leq 0.05$ was considered as statistically significant.

3. Results

MSH3 depletion reduces the proliferation of BRCA2-deficient cells

The A-NHEJ pathway is a critical compensatory pathway in BRCA2-deficient cells, which lack HR repair (Ceccaldi et al., 2015). Polθ is the essential DNA polymerase responsible for microhomology-primed DNA synthesis and repair (Luedeman et al., 2022). The overexpression of Polθ is commonly observed in HR-deficient cancers and is associated with poor prognosis (Wang et al., 2019). The MSH2/MSH3 heterodimer, also known as MutSβ, initially characterised in the MMR pathway, recognises long distortions in DNA structure (Gupta et al., 2012). Notably, in hamster cells and yeast, it has been shown that MutSβ has MMR-independent roles in repairing DNA lesions by joining DNA breaks with microhomologies, similarly to A-NHEJ (Kunkel & Erie, 2005). Consequently, we sought to investigate the hypothesis that BRCA2 abrogated cells may require MSH3 for their survival, and that MSH3 possibly promotes A-NHEJ in BRCA2-deficient cells.

First, we needed to establish that siRNAs were effective in depleting MSH3 and Polθ protein in the cell line models used in our subsequent experiments. Two cellular models for BRCA2 inactivation were used: human non-small cell lung carcinoma H1299 cells that carry a DOX inducible shRNA against BRCA2 (H1299+shBRCA2^{DOX}), which were grown in the presence or absence of DOX for four days in order to deplete BRCA2, and retinal pigment epithelial (RPE) cells, hTERT-immortalised and *TP53* deleted (RPE1 hTERT *TP53*$^{-/-}$ cells) in which *BRCA2* was deleted using the CRISPR/Cas9 system (Groelly et al., 2022). Both H1299 and RPE1 cells were transfected twice with siRNAs against MSH3, Polθ or non-targeting control siRNA and protein expression was monitored by immunoblotting 2 or 6 days after the initial transfection (Figure 3.1A). Immunoblotting of BRCA2-proficient and BRCA2-deficient H1299 cells indicated that MSH3 and Polθ were depleted 2 days after siRNA transfection and that this persisted for up to 6 days (Figure 3.1B). Similar results were observed in RPE1 hTERT *TP53*$^{-/-}$

BRCA2$^{+/+}$ and *BRCA2*$^{-/-}$ cells (Figure 3.1C). Expression of MSH3 or Polθ was also inhibited with siRNA and this was still observed 6 days after the initial siRNA transfection. Hence, we concluded that MSH3 and Polθ were effectively depleted using our siRNAs and these could therefore be used in subsequent experiments.

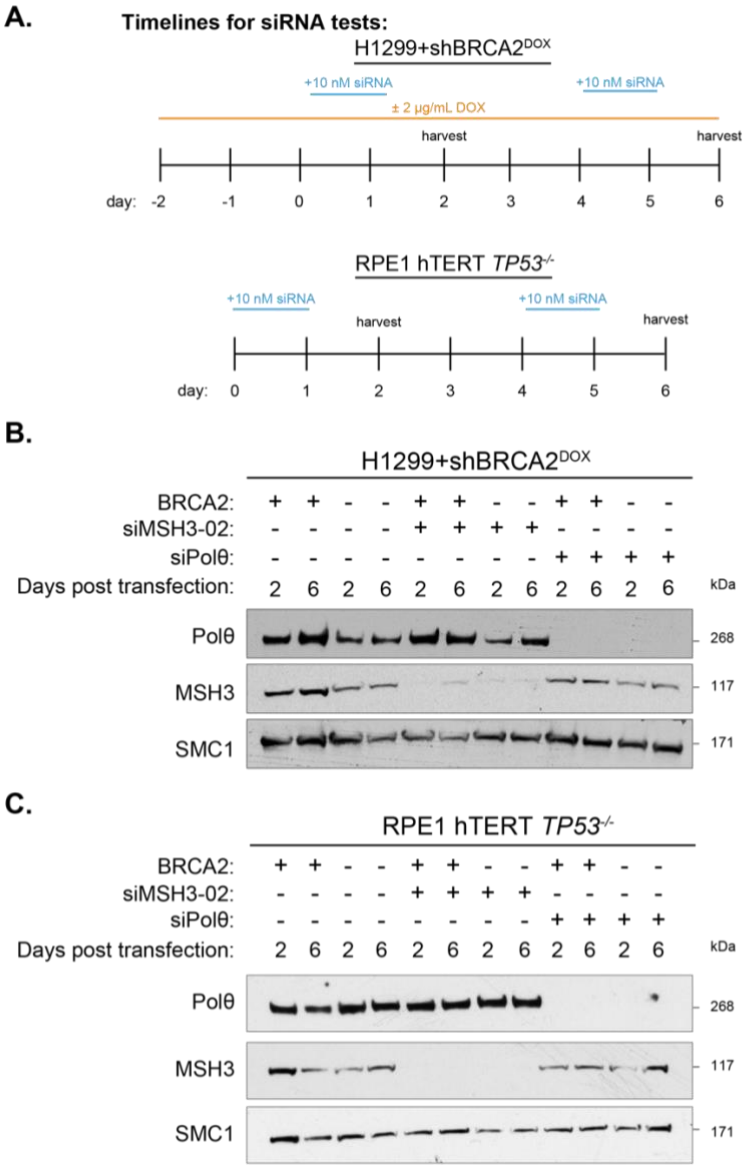


Figure 3.1 | Optimisation of siRNA transfection. A. Timeline of human H1299+shBRCA2^{DOX} and RPE1 hTERT *TP53*$^{-/-}$ cells transfected with siRNA targeting MSH3, Polθ or with a non-targeting control siRNA. H1299 cells were grown in the presence or absence of 2 ug/ml DOX. Cells were transfected on two separate days, as indicated in blue, and samples were collected for immunoblotting on the indicated days. **B.** Immunoblotting of H1299 cells was performed for the indicated proteins on samples prepared according to (**A**). SMC1 was used as a loading control. **C.** Samples of RPE1 cells were prepared as described in (**A**). Protein expression of MSH3 and Polθ were examined by immunoblotting, with SMC1 used as a loading control.

Cells lacking HR repair rely on A-NHEJ as a compensatory repair pathway, as evidenced by the reduced survival of BRCA2-deficient cells in which Polθ was inactivated

(Ceccaldi et al., 2015). If MSH3 is similarly involved in A-NHEJ, then its depletion would decrease proliferation and survival of BRCA2-deficient cells, due to the abrogation of the backup repair pathway A-NHEJ. To test this hypothesis, the proliferation of BRCA2-proficient and -deficient cells was examined upon MSH3 depletion using two siRNAs that target distinct regions of MSH3 (Figure 3.2A, B). The work shown in Figure 2 was performed by Timo Reïslander (unpublished PhD data). To abrogate BRCA2 expression in H1299 cells, we used an shRNA against BRCA2 induced by DOX treatment for three days (Figure 3.2B). BRCA2 depletion slightly reduced the growth of these cells, compared to H1299 cells that retained BRCA2 expression (Figure 3.2C; solid red line compared to solid black line). MSH3 depletion, using both siRNA, further decreased the viability of BRCA2-deficient cells (Figure 3.2C; dotted red line compared to solid red line). However, the proliferation of BRCA2-proficient cells was only slightly affected when MSH3 was depleted (Figure 3.2C; grey dotted line compared to solid red line). These results suggest that MSH3 is required for the proliferation of BRCA2-deficient cells, and to a lesser extent of BRCA2-proficient cells.

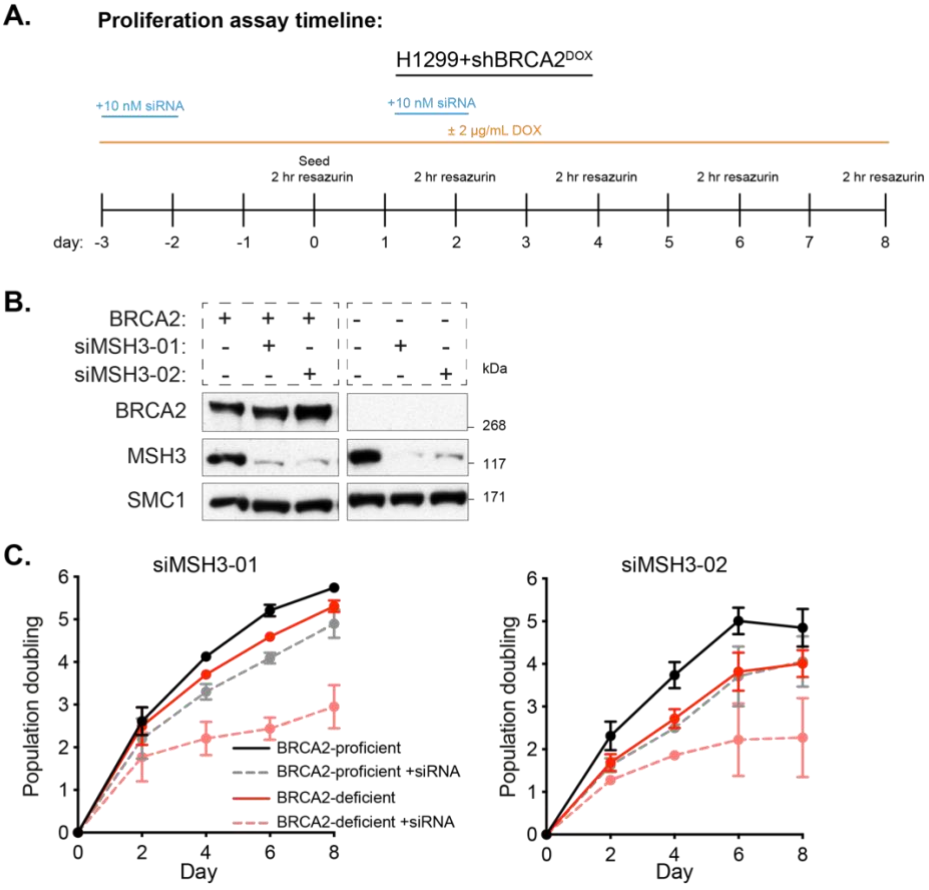


Figure 3.2 | MSH3 depletion decreases the proliferation of BRCA2-deficient cells A. Timeline of human H1299+shBRCA2^{DOX} proliferation assay performed following 3 days of 2 µg/mL DOX treatment as indicated in orange, and siRNA depletion of MSH3 as indicated in blue. Cells were reverse transfected twice, both instances with siRNA targeting MSH3 or non-targeting control siRNA. Samples were harvested for immunoblotting at day 0 to confirm depletion of MSH3 and BRCA2. **B.** Cells were prepared as described in (**A**) and immunoblotted to determine the depletion of indicated proteins. SMC1 was used as a loading control. **C.** Resazurin-based viability assay to quantify population doublings was conducted at indicated days and reported relative to day 0. Error bars represent the standard error mean of $n = 3$ independent experiments, each performed in technical triplicates. All work shown in Figure 3.2 was performed by Timo Reïslander (unpublished PhD data).

MSH3 role in the A-NHEJ pathway

Polθ loss is synthetic lethal with HR loss in cells and tumours, indicating that in the absence of HR cells rely on A-NHEJ for DNA repair (Ceccaldi et al., 2015). Our results described above suggested that MSH3 plays a significant role in the proliferation of BRCA2-deficient cells. Therefore we sought to investigate its potential involvement in the

A-NHEJ pathway through epistasis analyses between the *POLQ* and *MSH3* genes, as an indicator that these two proteins act in the same repair pathway. If depletion of MSH3 in Polθ/BRCA2-double deficient cells caused no change in cell survival relative to single-factor depletion, then it is likely that MSH3 is involved in A-NHEJ.

In order to demonstrate the involvement of MSH3 in the A-NHEJ pathway, we determined the survival of BRCA2-proficient and -deficient RPE1 cells when MSH3 was depleted alone or concomitantly with Polθ (Figure 3.3A). Whole-cell lysates were collected on day 0 and immunoblotted to demonstrate inhibition of MSH3 and Polθ expression (Figure 3.3B). Single depletion of MSH3 or Polθ in BRCA2-proficient cells led to a 11.3 or 19.6% decrease in cell survival, respectively (Figure 3.3C, D; light grey bar compared to grey bar). No further decrease in cell survival was observed when depletion of Polθ was combined with depletion of MSH3 in RPE1 *BRCA2*$^{+/+}$ cells (Figure 3.3C, D; solid grey bar and light grey bar when compared to shaded bar) indicating that A-NHEJ is functional in BRCA2-proficient cells. Consistent with Ceccaldi et al. (2015), single-factor depletion of Polθ resulted in a 58.8% reduction in the cell survival of RPE1 *BRCA2*$^{-/-}$ cells (Figure 3.3C, D; pink bar compared to red bar). Interestingly, MSH3 depletion only decreased the survival of cells lacking BRCA2 by 21.1% (Figure 3.3C, D; red bar compared to light red bar). Notably, our results showed that depletion of MSH3 in Polθ/BRCA2-double deficient cells did not result in a further decrease in cell survival relative to depletion of Polθ alone (Figure 3.3C, D; solid light red bar and pink bar compared to shaded bar). This indicates that MSH3 and Polθ are acting in the same pathway promoting survival of BRCA2-deficient cells, most likely A-NHEJ.

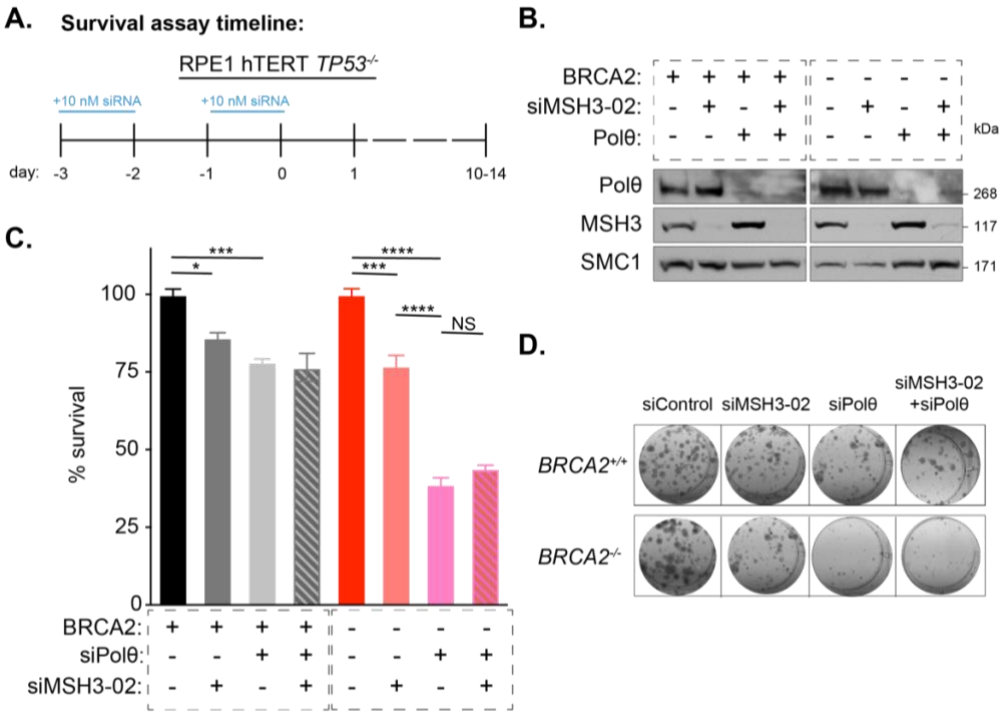


Figure 3.3 | MSH3 and Polθ are epistatic in promoting the survival of BRCA2-deficient cells **A.** Timeline of human RPE1 hTERT *TP53*$^{-/-}$ survival assays reverse transfected on two separate occasions, as indicated in blue, with siRNA against MSH3 and Polθ or a control non-targeting siRNA. Samples were seeded for survival assays on day 0 and immunoblotted to determine protein depletion. **B.** Immunoblotting of samples prepared as described in (**A**). SMC1 was used as a loading control. **C.** Quantification of surviving fraction of cells after a 7-15 day incubation. Error bars represent the standard error of the mean of n = 4 independent experiments each performed in technical triplicates. Statistical significance was determined by a one-way ANOVA test. NS, $P > 0.05$; *, $P \leq 0.05$; **; $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$. **D.** Representative images of colony formation of RPE1 *BRCA2*$^{+/+}$ and *BRCA2*$^{-/-}$ cells prepared as described with (**A**).

DNA damage induced by Talazoparib in BRCA2-deficient cells is exacerbated by loss of MSH3

Abrogation of BRCA1 and BRCA2 proteins leads to accumulation of DNA damage, due to their inability to perform accurate DSB repair (Zámborszky et al., 2016). This genomic instability can be clinically exploited for their selective elimination. PARPi have been approved for the treatment of BRCA1- and BRCA2-deficient cancers due to the synthetic lethality observed between *PARP1* and *BRCA1/BRCA2* genes (Ashworth & Lord, 2018). Although PARPi had great initial success, *BRCA1* and *BRCA2* mutated tumours

frequently develop resistance (D'Andrea, 2018). Hence, it became imperative to find alternative methods to eliminate HR-deficient tumours. Proteins involved in A-NHEJ represent potential clinical targets due to the reliance of BRCA1- and BRCA2-deficient cells on A-NHEJ for DSB repair. As previous results indicated that MSH3 has a role in A-NHEJ, we hypothesised that MSH3 depletion in BRCA2-deficient cells may sensitise the cells to PARPi.

Firstly, we examined the effect of MSH3 depletion on markers of replication stress and DNA damage, using immunoblotting of H1299 cells grown in the presence or absence of DOX to deplete BRCA2 (Figure 3.4A). Additionally, these markers were examined in MSH3-depleted cells, as well as in PARPi (Talazoparib)- or vehicle-treated cells. Immunoblotting indicated depletion of BRCA2 and MSH3 in the cells at the start of Talazoparib treatment (Figure 3.4B).

The phosphorylation of KRAB domain-associated protein 1 (KAP1) at serine 824 by Ataxia-Telangiectasia Mutated (ATM) protein occurs in response to DNA damage (Podhoreckaet al., 2010; White et al., 2006). Hence, an accumulation of phosphorylated KAP1 (pKAP1) equates to an increase in DNA damage (White et al., 2006). Consistent with previous reports (Reisländer et al., 2019), loss of BRCA2 triggers an increase in pKAP1 compared to BRCA2-proficient cells (Figure 3.4C). MSH3 depletion and Talazoparib treatment, alone or in combination, did not induce phosphorylation of KAP1 in BRCA2-proficient cells. Similarly, depletion of MSH3 in vehicle control treated BRCA2-deficient cells did not increase the phosphorylation of KAP1 compared to control BRCA2-deficient cells. In contrast, MSH3 depletion in BRCA2-deficient cells treated with Talazoparib led to increased DNA damage relative to control or Talazoparib-treated cells that retained MSH3 expression.

In response to stalled replication forks, ATR protein mediates the phosphorylation of RPA32 —a subunit of RPA— at serine 33 (Vassin et al., 2009). Hence, we examined RPA phosphorylation by immunoblotting as a marker of replication stress. Consistent

with retention of HR activity, the BRCA2-proficient cells did not accumulate phosphorylated RPA, even when treated with PARPi (Figure 3.4C). However, we observed that Talazoparib treatment led to an increase in phosphorylated RPA in BRCA2-deficient cells. Unlike phosphorylation of KAP1, MSH3 depletion in Talazoparib treated BRCA2-deficient cells did not result in a further increase in RPA phosphorylation relative to Talazoparib treatment alone (Figure 3.4C). This indicates that MSH3 depletion exacerbates DNA damage accumulation, but not replication stress in BRCA2-deficient cells in response to Talazoparib treatment.

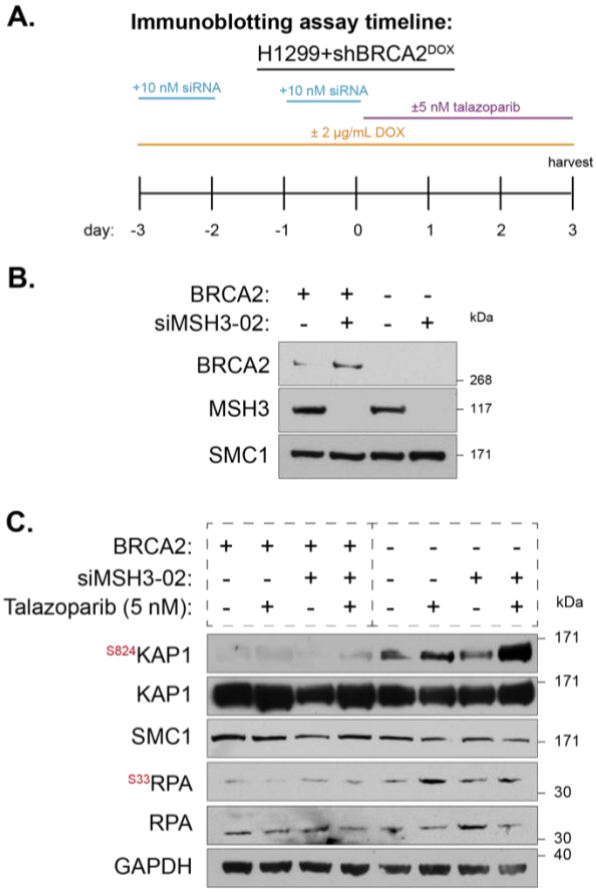


Figure 3.4 | MSH3 depletion increases DNA damage in BRCA2-deficient cells treated with Talazoparib A. Timeline of treatments for immunoblotting of human H1299shBRCA2^{DOX} cells grown with or without 2 µg/mL of DOX (indicated in orange), and depleted of MSH3 through 2 siRNA transfections against MSH3 (indicated in blue). Following this, H1299 cells were treated with 5 nM of Talazoparib or an equivalent amount of the vehicle control DMSO. **B.** H1299 cells were treated as described in (**A**) and immunoblotted at day 0 to confirm depletion of indicated proteins. SMC1 was used as a loading control. **C.** Whole cell lysates of samples treated with Talazoparib or vehicle control (**A**) were collected and immunoblotted for the presence of DNA damage and replication stress markers as indicated. Phosphorylation sites are indicated in red. SMC1 and GAPDH were used as loading controls.

MSH3 depletion sensitises BRCA2-deficient cells to Talazoparib treatment

As MSH3 loss led to an increase in the accumulation of DNA damage in BRCA2-deficient cells treated with Talazoparib (Figure 3.4), we hypothesised that MSH3 loss would further sensitise BRCA2-deficient cells to Talazoparib. To address this, we conducted survival assays of H1299 cells treated with five increasing doses of Talazoparib (Figure 3.5A). Immunoblotting showed that cells cultured in the presence of DOX were indeed BRCA2-deficient and that siRNA transfections depleted MHS3 (Figure 3.5B).

Consistent with existing literature (de Bono et al., 2017; Litton et al., 2018), BRCA2-deficient cells were highly sensitive to Talazoparib treatment (Figure 3.5C, D). Even at low concentrations of Talazoparib, we observed a decrease in survival of cells lacking BRCA2 expression, relative to BRCA2-proficient cells (Figure 3.5C; red line compared to black line). Interestingly, we observed that MSH3 depletion resulted in a slight sensitisation of BRCA2-proficient cells to Talazoparib (Figure 3.5C; grey line compared to black line). Importantly, loss of MSH3 in Talazoparib treated BRCA2-deficient cells caused a significant decrease in cell survival at all doses examined (Figure 3.5C; dashed light-red line compared to solid red line). A dose of 0.039 nM of Talazoparib caused a 26.9% decrease in survival of siMSH3-treated BRCA2-deficient cells relative to siControl-treated BRCA2-deficient cells (Figure 3.5C, D). This suggested that BRCA2-deficient cells rely on MSH3 for the repair of Talazoparib-inflicted DNA damage and that MSH3 may promote A-NHEJ repair in these cells (Ceccaldi et al., 2015; de Bono et al., 2017).

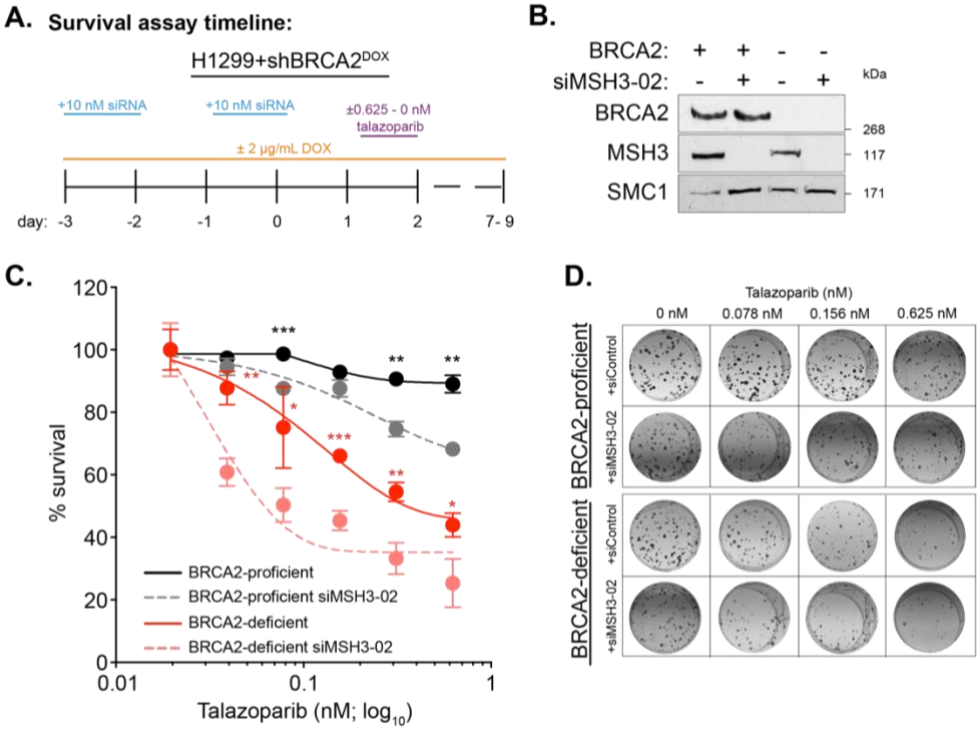


Figure 3.5 | MSH3 depletion sensitises BRCA2-deficient cells to Talazoparib treatment. **A.** Timeline of a Talazoparib dose-response clonogenic survival assay of H1299+shBRCA2^{DOX} cells following DOX treatment for the period of time indicated in orange, and double siRNA transfections to deplete MSH3, as indicated in blue. Length and timing of Talazoparib treatment is indicated in purple. **B.** Human H1299 cells were treated as outlined in (a) and whole-cell lysates were collected and immunoblotted to confirm depletion of relevant proteins upon seeding of the experiment. **C.** Percentage of surviving fraction of colony formation of H1299 cells 5-10 days after treatment with a serial dilution of Talazoparib (0.625 – 0.039 nM) for 24 hours. Error bars represent the standard error of the mean of n = 3 independent experiments, each performed in technical triplicates. Statistical significance was determined by an unpaired two-tailed Student's t-test. NS, $P > 0.05$; *, $P \leq 0.05$; **; $P \leq 0.01$; ***, $P \leq 0.001$. **D.** Representative images of colony formation stained with crystal violet in BRCA2-proficient and BRCA2-deficient H1299 cells treated with a serial dilution of 0.625 – 0.039 nM of Talazoparib.

To ensure that the observed sensitisation of BRCA2-deficicient cells to Talazoparib treatment when MSH3 was depleted was not a cell line specific effect, we conducted a dose-response viability assay using RPE1 hTERT *TP53*$^{-/-}$ *BRCA2*$^{+/+}$ and *BRCA2*$^{-/-}$ cells treated with siRNA against MSH3 and control non-targeting siRNA (Figure 3.6A). Depletion of MSH3 was confirmed by immunoblotting (Figure 3.6B). As expected, Talazoparib was specifically lethal to BRCA2-deficient, but not BRCA2-proficient cells

(Figure 6C; red line compared to black line). Similarly to the H1299 cells, we saw that RPE1 *BRCA2*$^{-/-}$ cells became more sensitive to Talazoparib treatment when MSH3 protein expression was inhibited (Figure 3.6C; dashed light-red line when compared to solid red line). On the other hand, we did not observe Talazoparib sensitivity in BRCA2-proficient MSH3-depleted RPE1 cells, compared to cells that retained MSH3 expression (Figure 3.6C; dashed grey line when compared to solid black line), which confirmed in an independent cell model, that concurrent MSH3 and BRCA2 abrogation sensitises cells to Talazoparib.

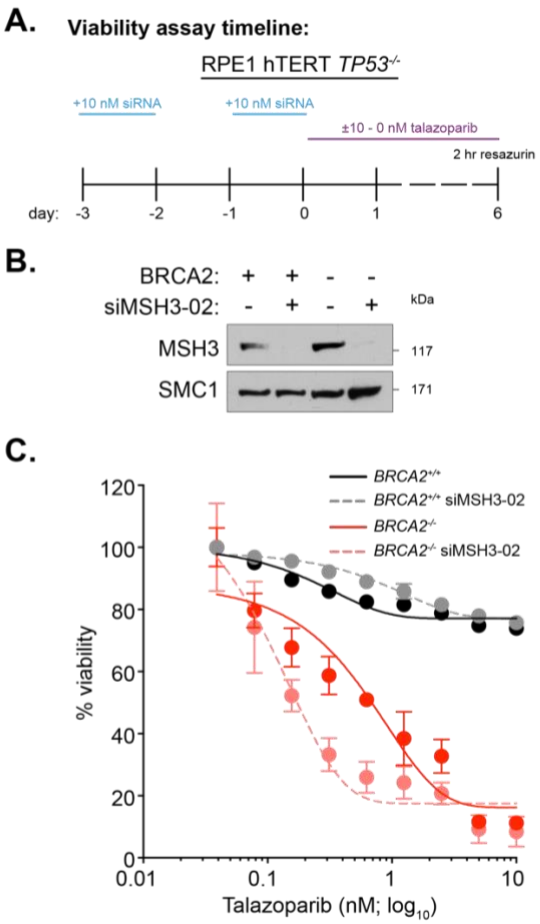


Figure 3.6 | MSH3 depletion decreases the viability of BRCA2-deficient cells treated with Talazoparib. A. Timeline of a viability assay of human RPE1 hTERT *TP53*$^{-/-}$ cells. RPE1 cells had MSH3 depleted through an siRNA-mediated depletion as indicated in blue. Cells were then treated with Talazoparib or DMSO as indicated in purple. **B.** Following treatment described in (**A**) MSH3 expression was confirmed through immunoblotting of cell lysates collected at day 0. SMC1 was used as a loading control. **C.** Dose-dependent viability assay of *BRCA2*$^{+/+}$ and *BRCA2*$^{-/-}$ RPE1 cells treated with a serial dose of Talazoparib at indicated concentrations for 6 days. Graph represents mean values and the standard error of the mean of n = 1 independent experiment performed in technical triplicates, and this trend was observed in n = 2 independent experiments.

4. Discussion

In order to maintain genomic integrity and to avoid the accumulation of DNA lesions, the DDR is responsible for the detection of damaged DNA, the most toxic being DSBs, leading to signalling that promotes DNA repair (Jackson & Bartek, 2009). Tumours that have inactivated HR, an accurate DSB repair pathway, commonly by mutating tumour

suppressor proteins BRCA1 and BRCA2, become reliant on alternative pathways of repair particularly the A-NHEJ pathway (Ceccaldi et al., 2015). Thus, A-NHEJ factors represent promising clinical targets for the treatment of BRCA2-deficient tumours. In this study, we discovered that concurrent loss of MSH3 and BRCA2 led to reduced cell viability, hence indicating a role for MSH3 in the survival of BRCA2-deficient cells. MSH3 is involved in sensing long DNA distortions during MMR repair reactions, but MSH3 has been also shown to have MMR-independent roles, including in microhomology-mediated DNA repair that resembles A-NHEJ (Kumar et al., 2013; Sugawara et al., 1997). Consequently, we performed epistatic analyses of MSH3 and Polθ, the latter being the key polymerase involved in A-NHEJ (Ramsden et al., 2021). We found that MSH3 likely has a role in A-NHEJ. Reporter assays (Grabarz et al., 2013; Mateos-Gomez et al., 2015) could be used to confirm that MSH3 is a factor used by the A-NHEJ pathway to repair DSBs.

Although MSH3 loss was detrimental to cell survival, it was not as toxic as the loss of Polθ. This is contrary to the effect observed with the loss of Polθ, in which its loss is synthetic lethal to BRCA2-deficient cells. This alludes to the possibility that MSH3 is not essential to A-NHEJ, unlike Polθ. In MMR, MSH3 in complex with its binding partner MSH2, acts as a sensor for DNA lesions and binds to indel loops of 1-14 nucleotide base length (Palombo et al., 1996). The DNA binding pocket of the heterodimer MutSβ is able to recognise different branched structures of DNA and bind to mismatches in order to initiate the recruitment of MMR factors and facilitate its repair (Young et al., 2020; Owen et al., 2009). Should MSH3 also detect and signal DNA damage in A-NHEJ, factors such as PARP1 which also senses DNA damage in A-NHEJ (Audebert et al., 2004; Luedeman et al., 2022), could compensate for its loss. To determine the molecular function of MSH3 in A-NHEJ, immunoprecipitation assays could be used to investigate whether MSH3 interacts with other known factors in A-NHEJ. Moreover, abrogating MSH3 DNA binding by mutating the DNA binding domain (Burdova et al., 2015) and repeating the

experiments in this study would indicate a role for MSH3 in binding to and sensing damaged DNA in A-NHEJ.

Although A-NHEJ has been implicated as the essential back-up repair pathway when cells are deficient of HR-related genes (Ceccaldi et al., 2015), we observed that Polθ depletion also led to a decrease in the survival of BRCA2-proficient cells, although to a lesser degree. This indicates that a subset of DSBs are repaired via the A-NHEJ pathway even in a HR-proficient setting. Prior studies have also observed this limited role of Polθ in DSB repair when HR activity is proficient (Feng et al., 2019; Wyatt et al., 2016). This suggests that A-NHEJ repair may play a dual role in both promoting and suppressing genome stability in healthy cells.

Factors of A-NHEJ make attractive therapeutic targets for the elimination of HR-deficient tumours due to their reliance on A-NHEJ for DSB repair (Ceccaldi et al., 2015; Schrempf et al., 2021). Currently novobiocin and ART558 have been shown to be specific and potent Polθ inhibitors which showed promising results *in vitro* and *in vivo* for the treatment of BRCA-deficient cancers (Zatreanu et al., 2021; Zhou et al., 2021). In our study we observed that MSH3 loss in BRCA2-deficient cells led to their increased sensitivity to PARPi treatment. A study has also previously observed that MSH3 loss sensitises colorectal cancer cells to PARPi treatment (Takahashi et al., 2011). This increased sensitivity suggests that MSH3 is working in a compensatory DNA repair mechanism, likely A-NHEJ, that is critical for the repair of PARPi induced DNA damage. This is consistent with results observed in Zhou et al. (2021) and Zatreanu et al. (2021) in which they saw increased sensitivity to PARPi treatment when Polθ function was abrogated. Therefore, we have identified a novel target for the elimination of BRCA2-mutated tumours.

Currently, there are no MSH3 inhibitors available. However, studies of the MutSβ complex have shown that in MMR, the tyrosine 245 and lysine 246 residue of the mismatch-binding domain in MSH3, are essential to its ability to recognise indel loops

(Burdova et al., 2015; Clark et al., 1999; Gupta et al., 2012; Owen et al., 2009). Hence, a potential strategy to inhibit MSH3 activity would be to discover or develop a molecule able to bind to this domain, thereby abrogating the DNA lesion sensing function of MSH3. Crystal structures of human full length MSH2 in complex with a trimmed MSH3 (211-1125 aa) have been characterised (Gupta et al., 2012) and could be utilised for *de novo* design of small molecule inhibitors against MSH3.

In addition to treating patients with *BRCA1* and *BRCA2* mutations, the development of MSH3 inhibitors would be beneficial to treating other conditions. An upregulation in A-NHEJ activity can be seen in a variety of cancer types (Feng et al., 2019; Schrempf et al., 2021). Given the role of MSH3 in A-NHEJ, MSH3 inhibitors, like Polθ inhibitors, could be used to treat cancers with hyperactive A-NHEJ activity such as neuroblastoma, acute myeloid leukaemia, and multiple myeloma amongst others (Caracciolo et al., 2018; Newman et al., 2015; Pashaiefar et al., 2018; Ramsden et al., 2021). Moreover, over-expression of MSH3 has been correlated with dysregulation of the balance of MutSα and MutSβ ratios, leading to the sequestering of MSH2 and the degradation of MSH6 (Marra et al., 1998). Hence, cells are unable to accurately recognise and repair short 1-2 base mismatches leading to an increased rate of microsatellite instabilities and somatic mutations. MSH3 overexpression has also been implicated in the progression of trinucleotide repeat expansion disorders like Huntington's disease (Flower et al., 2019). In mice models, the knockout of MSH3 led to the reduced somatic expansion, hence MSH3 inhibitors would be beneficial in treating those conditions as well (Moss et al., 2017).

Although MSH3 is a promising therapeutic target, it is important to note that its application is limited to cancers and disorders which do not have an existing deficiency in MSH3. The loss of MSH3 is often observed in colorectal cancer, where mutations in *MSH3* leads to an increase in microsatellite instability hence leading to disease progression (Haugen et al., 2008; Plaschke et al., 2004). MSH3 loss can also be seen in

a small percentage of prostate and lung adenocarcinoma (AACR Project GENIE Consortium, 2017). Future work could investigate the percentage of tumours with an existing mutation in both *MSH3* and *BRCA1/2* to fully gauge the limitations of utilising MSH3 as a therapeutic target.

5. Conclusion

In this study, we have identified novel DNA repair functions for MSH3 in BRCA2-deficient cells, which can provide molecular insights into the mechanisms that promote tumorigenesis in patients that carry *BRCA2* mutations. Furthermore, we have identified MSH3 (possibly in complex with its interacting partner MSH2) as a clinical therapeutic target for the elimination of BRCA2-deficient tumour cells.

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