Investigating the effects of cGAS-STING-dependent innate immune responses in BRCA2-deficient cells

by

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Tumours with *BRCA2* gene deficiency, retain the ability to proliferate, despite the genomic instability resulting from DNA damage accumulation. These tumours can be targeted with Poly-ADP ribose polymerase inhibitors (PARPi), which increase the susceptibility of *BRCA2*-mutated tumours to DNA damaging agents. However, in spite of the therapeutic benefits and low toxicity in patients, PARPi resistance remains a clinical challenge. Therefore, improved approaches for targeting *BRCA2*-mutated tumours are being sought to improve patient outcomes. Previously, BRCA2 depletion was shown to activate the cGAS-STING-dependent innate immune responses which have the potential to promote antitumour immunity. In this project, we set out to investigate the effects of stimulating the cGAS-STING-dependent innate immune responses using the STING agonist DMXAA in two BRCA2-deficient cell models. DMXAA treatment activated the cGAS-STING signalling pathway in only one of the BRCA2-deficient cell models, characterised by an increase in IRF3 phosphorylation, but this did not lead to an increase in DNA damage. PARPi treatment is known to activate the cGAS-STING signalling pathway and induce DNA damage in BRCA2-deficient cells. Therefore, we co-treated BRCA2-deficient cells with the PARPi Talazoparib and DMXAA in order to activate STING mediated innate immune responses and increase DNA damage. The combination treatment led to activation of cGAS-STING signalling, upregulation of interferon-stimulated genes and an increase in DNA damage in both cell models. Finally, we investigated the effect of combining DMXXA and Talazoparib on the viability of BRCA2-deficient cells. However, DMXAA did not lead to increased sensitivity of the BRCA2-deficient cells to Talazoparib. Whilst these are preliminary results, they suggest that the effect of STING agonists may vary depending on cell type and they may not have the predicted efficacy in BRCA2-deficient cells.

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List of abbreviations

%	Percent
ºC	Degree Celsius
53BP1	p53 binding protein 1
alt-NHEJ	alternative non-homologous end joining
ATM	ataxia telangiectasia mutated
BARD1	BRCA1-associated RING domain protein 1
BER	Base-excision repair
BRCA	breast cancer gene
BSA	bovine serum albumin
cDNA	complementary DNA
cGAMP	cyclic 2'3'-GMP-AMP
cGAS	cGAMP synthase
CtIP	C-terminal binding protein 1-interacting protein
D-loop	displacement loop
Da	Dalton(s)
DDR	DNA damage response
DMEM	Dulbecco's Modified Eagle's Medium
DMXAA	5,6-dimethyllxanthenone-4-acetic acid
DMSO	Dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNA-PKcs	DNA-dependent protein kinase catalytic subunit DOX doxycycline
DOX	Doxycycline
DSB	double-strand break

DSS1	deleted in split hand/split foot syndrome 1
DTT	Dithiothreitol
HR	homologous recombination
IFN	interferon
IFNAR	IFNα and β receptor subunit
IRF	IFN regulatory factor
ISG	IFN-stimulated gene
KAP1	Krüppel-associated box zinc finger protein-associated protein 1
MCM	minichromosome maintenance
PALB2	partner and localizer of BRCA2
PARP	PAR polymerase
PBS	phosphate-buffered saline
PBST	0.05% Tween 20 in PBS
PCNA	proliferating cell nuclear antigen
qPCR	quantitative polymerase chain reaction
RT	room temperature
shRNA	short hairpin RNA
siRNA	small interfering RNA
SSA	single-strand annealing
SSB	single-strand break
ssDNA	single-stranded DNA
TBK1	TANK-binding kinase 1

1. Introduction

Cancer has emerged worldwide as one of the leading causes of mortality. Heterozygous germline mutations in *BRCA1* and *BRCA2* tumour suppressor genes account for approximately 20% of ovarian and 5% of breast familial cancers (Daly, Axilbund et al. 2010). Importantly, *BRCA*1/2 mutations have also been reported in sporadic cancers (Nik-Zainal, Davies et al. 2016). Therefore, uncovering novel vulnerabilities for therapeutic targeting of *BRCA2*-mutated tumours will provide novel therapies for different familial and sporadic cancer types carrying mutations in the *BRCA2* gene.

1.1. BRCA1/2-deficiency in tumourigenesis

BRCA1 and BRCA2 are key players in the maintenance of genome integrity (Tarsounas and Sung 2020). They are large proteins encoded by *BRCA1* and *BRCA2* genes which represent classical tumour suppressor genes because their loss of function, due to acquired mutations, drives tumourigenesis. Heterozygous germline *BRCA1* and *BRCA2* mutations are often harboured in patients with familial ovarian, breast, pancreatic, prostate, stomach and other cancer types (Friedenson 2005, Cavanagh and Rogers 2015). *BRCA1* and *BRCA2* germline mutations account for approximately 25% of familial cases of breast and ovarian cancers (Miki, Swensen et al. 1994). Moreover, somatic mutations in *BRCA1* and *BRCA2* genes have been reported in different sporadic cancer types (Lal, Ramazzotti et al. 2019).

1.2. Genomic Instability – a Hallmark of Cancer

1.2.1. DNA Damage

DNA is inherently unstable and a highly reactive molecule that is constantly being damaged and repaired, accumulating damage over a lifetime (Chatterjee and Walker 2017). Hence, DNA damage repair is essential to prevent the accumulation of lethal DNA lesions and ensure normal cellular functions are maintained (Ciccia and Elledge 2010). DNA damage arises from endogenous as well as exogenous sources. The endogenous type of DNA damage can arise during DNA replication and repair, DNA expected rearrangements, or from alterations in the chemistry of DNA bases (such as depurination, depyrimidation, and deamination of bases) (Torgovnick and Schumacher 2015, Chatterjee and Walker 2017). Endogenous DNA damage can arise due to inactivation of DNA repair pathways, during replication and transcription (Tubbs and Nussenzweig 2017). Furthermore, additional endogenous sources can also cause DNA damage including hydrolysis, mismatch of DNA bases, alkylation and oxidation (Hakem 2008). Endogenous DNA damage is prevalent and can lead to as many as 10^5 DNA lesions per cell per day (Hoeijmakers 2009). Exogenous DNA damage results from chemical and physical DNA damaging agents (Chatterjee and Walker 2017). Chemical DNA damaging agents include alkylating agents, aflatoxins, and polycystic aromatic hydrocarbons (Hwa Yun, Guo et al. 2020). Physical DNA damaging agents include ionising radiation (IR) and ultraviolet light (UV) (Hakem 2008). Cells exposed to IR can acquire chromosomal aberrations (Durante and Formenti 2018). Whilst less frequent, exogenous DNA damage sources can also lead to deleterious DNA lesions such as double strand breaks (DSBs), which if not repaired correctly can introduce

genomic instability and result in cancer formation (Jackson and Loeb 2001, Cannan and Pederson 2016).

1.2.2. Maintaining Genomic Stability

Tens of thousands of DNA lesions are formed in each of the 10^{13} human cells every day (Lindahl and Barnes 2000, Bianconi, Piovesan et al. 2013). The presence of DNA lesions within the genome increases the risk of genomic instability, and these lesions can halt fundamental cellular processes including the replication and transcription of DNA (Jeggo, Pearl et al. 2016). Before cell division ensues, DNA has to be accurately copied and delivered to the two daughter cells (Lindahl 1993) in the process called DNA replication. During DNA replication, loss of tumour suppressor genes and gain of oncogenes or chromosomal rearrangements can all lead to DNA lesions. If the lesions are not repaired or are repaired by an inaccurate DNA repair pathway, they can lead to genomic instability. Genomic instability is a well-characterised hallmark of cancer driving tumorigenesis and is defined as the increased tendency of genetic alterations (Yao and Dai 2014). Maintaining genomic stability is therefore essential for the survival of the organism. To safely deliver the genetic material to the next generation, the cells evolved mechanisms of DNA repair, which detect DNA lesions and promote their repair. Consequently, the maintenance of genome stability relies on the fine balance between DNA damage and repair. Therefore, understanding the ways by which genomic stability is maintained in response to DNA lesions is important in order to devise novel targets for the treatment of cancer in the clinic.

1.2.3. DNA Repair

Genome integrity is constantly being maintained by robust DNA damage repair mechanisms (Chatterjee and Walker 2017). For the repair of the DNA damage, cells rely on complex repair mechanisms that detect and signal the presence of DNA lesions, promote the repair of these lesions. These complex repair mechanisms are known as the DNA-damage response (DDR) (Jackson and Bartek 2009, Chatterjee and Walker 2017). There are different DNA damage types including DNA base lesions, intra- and interstrand crosslinks, DNA-protein crosslinks, single strand breaks (SSB), and DSBs (Lindahl 1993). The different types of DNA damage are repaired via different DNA repair pathways. SSBs are among the most common DNA lesions in cells), and if the break is “clean” the DNA SSBs can be religated by DNA ligase (Caldecott 2008, Cannan and Pederson 2016). Despite being rare, DSBs are among the most deleterious DNA lesions. When DSBs are detected, they can lead to cell cycle arrest or repair (Scott and Pandita 2006). DNA DSBs occur 10-50 times per cell per day (Vilenchik and Knudson 2003). The main pathways of repair of DSBs are homologous recombination (HR) and classical non-homologous end joining (c-NHEJ) (Chakraborty, Tapryal et al. 2016). Less frequently, the repair of DSBs relies on other DNA repair pathways including alternative-NHEJ (alt-NHEJ), and single strand annealing (SSA).

1.3. DSB Repair

When DSBs occur, Mre11-Rad50-Nbs1 (MRN) end recognition complex assembles at the break site. This complex stimulates ataxia-telangiectasia mutated (ATM) which is involved in the initiation of DDR (Blackford and Jackson 2017). ATM activation triggers the repair of DSB via HR or NHEJ. The predominant pathway of DSB repair during G1 is NHEJ but can also occur in all stages of the cell cycle whilst HR is restricted to the S and G2 phases.

On a molecular level, the choice of DSB repair pathway has been depicted to be dependent on whether the DNA ends have undergone 5′-to-3′ extensive nucleolytic resection with ends bearing long 3′ DNA tails being repaired via HR (Symington and Gautier 2011, Chapman, Taylor et al. 2012, Daley and Sung 2014). Moreover, since the discovery of the opposing functions between BRCA1 and 53-binding protein (53BP1), BRCA1 was discovered to influence the choice of HR over NHEJ. BRCA1 and 53BP1 compete at the broken ends of DNA and perform opposing functions by engaging with HR as a choice of repair or NHEJ respectively. In particular, 53BP1 prevents resection therefore 53BP1 at the break directs the repair towards NHEJ whilst BRCA1 promotes resection and therefore leads the repair towards HR.

1.3.1. Homologous recombination (HR)

Found in all life forms, HR provides a high-fidelity, template-dependent DNA repair pathway (Li and Heyer 2008). HR has been described as error-free pathway of DSB repair (Rodgers and McVey 2016). Error-free repair by HR depends on utilising the sister chromatid as a template for repair. In addition to repairing DSBs, HR is also

involved in the repair of DNA gaps and interstrand crosslinks (ICLs) contained within the DNA (Li and Heyer 2008). HR is not only involved in DNA repair, but also plays a key role in DNA replication by protecting and restarting stalled replication forks.

During HR, the formation of single-stranded DNA (ssDNA) overhangs at the site of DSBs is required (Chang, Pannunzio et al. 2017, Scully, Panday et al. 2019). End resection is initiated by the nucleolytic activity of the MRN complex and C-terminal binding protein 1-interacting (CtIP) protein (Sartori, Lukas et al. 2007). The resection is further promoted by other factors such as BLM and WRN helicases and EXO1 and DNA2 nucleases (Sturzenegger, Burdova et al. 2014). Upon end resection, a ssDNA tail is generated (Figure 1.1). Next, replication protein A (RPA) binds and protects the ssDNA tail from degradation or annealing to another strand. Protein complexes consisting of BRCA1-BARD1, BRCA2-DSS1 and PALB2 act at this stage (Tarsounas and Sung 2020). PALB2 acts as a bridge for the interaction between BRCA1-BARD1 and BRCA2, while BRCA2 has been depicted to have a key role in regulating the DNA binding of RAD51 and its intracellular localization (Gorodetska, Kozeretska et al. 2019). In order for the downstream steps to occur, RPA coating the ssDNA tail needs to be replaced by RAD51 (Scully, Panday et al. 2019). The concerted action of PALB2, BRCA2 and BRCA1-BARD1 facilitate the loading of RAD51 onto the ssDNA tail. RAD51 mediates strand invasion into the sister chromatid, which is used as a template for repair. Within the sister chromatid strand, the annealing of RAD51-ssDNA nucleoprotein complex to the complementary sequence leads to displacement of non-complementary strand. A displacement loop (D-loop) is formed after strand invasion (Fasching, Cejka et al. 2016). DNA synthesis is mediated by polymerase-δ (Pol) and proliferating cell nuclear antigen (PCNA).

D-loops can be processed via 4 pathways including break-induced DNA replication (BIR), synthesis-dependent SSA (SDSA), canonical DSB repair (DSBR) and double Holliday junction (dHJ) dissolution (Tarsounas and Sung 2020). SDSA is a non-crossover and a conservative pathway, and a predominant pathway of repair in somatic cells (Scully, Panday et al. 2019). SDSA involves the dissociation of the D-loop by cellular helicases (Daley, Gaines et al. 2014). This pathway promotes re-annealing of invading with non-invading strand, followed up with gap filling and ligation to complete repair of DNA. Moreover, as a result of a D loop, holiday junction (HJs) or dHJs can be formed and will subsequently need to be resolved. A dHJ can form if the second end of DNA becomes captured. DNA resolvases mediate dHJ resolution generating crossover or non-crossover events (Daley, Gaines et al. 2014). Alternatively, the dHJ may be dissolved by migrating the two DNA junctions towards each other which results in hemicatenated intermediate formation. The decatenation of the intermediate regenerates the original DNA and complete HR reaction (Bizard and Hickson 2014). BIR is error prone pathway (Scully, Panday et al. 2019). In BIR, the invading strand replicates the entire distal chromosome part by using the sister chromatid (Anand, Lovett et al. 2013).

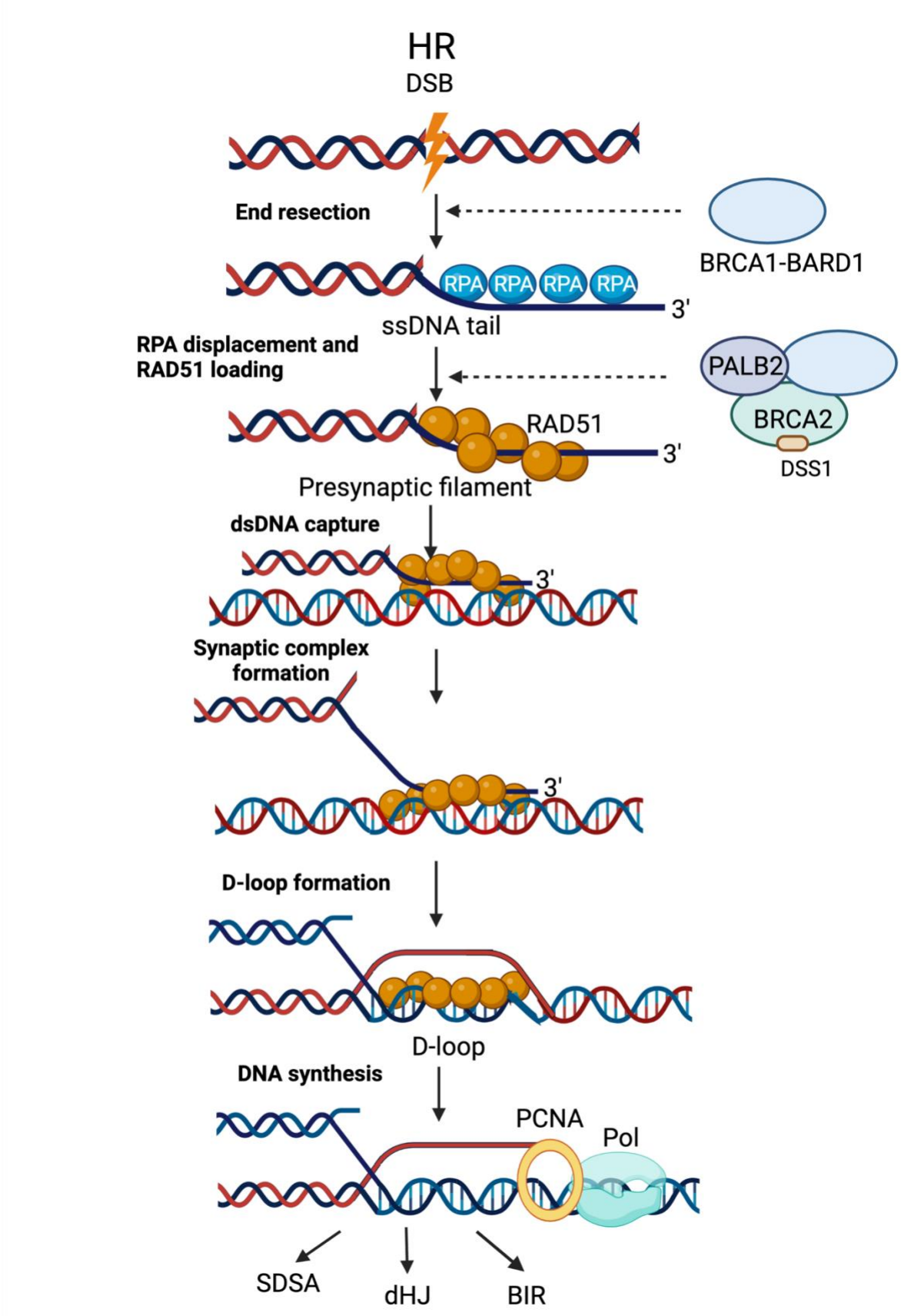


Figure 1.1. Schematic representation of DNA repair via HR.

End resection is initiated by MRN complex and C-terminal binding protein 1-interacting (CtIP) protein and further promoted by BLM and WRN helicases and EXO1 and DNA2 nucleases. End resection generates a ssDNA tail. Replication Protein A (RPA) binds ssDNA tail and is replaced by RAD51 in order for the steps downstream in the pathway to occur. The replacement of RPA by RAD51 is facilitated by concerted actions of BRCA1-BARD1, BRCA2-DSS1, and PALB2. Sister chromatid strand invasion is mediated by RAD51. RAD51 uses the sister chromatid strand as a template for repair. Upon annealing of RAD51-ssDNA nucleoprotein complex to the complementary sequence displacement of non-complementary strand occurs resulting in the formation of displacement loop (D-loop). Following DNA synthesis by polymerase-δ (Pol) and proliferating cell nuclear antigen (PCNA), the D-loop can be resolved by different pathways including synthesis-dependent single strand annealing (SDSA), double Holiday Junction (dHJ), and break-induced DNA replication (BIR).

1.3.2. Canonical non-homologous recombination (c-NHEJ)

c-NHEJ plays a prominent role in the repair of DSBs. c-NHEJ is initiated through the DNA ends recognition and protection by the Ku70/Ku80 heterodimer, which forms a ring that encircles the DNA Duplex (Figure 1.2). This protects the DNA ends against resection and provides a platform for the recruitment of the DNA-PK catalytic subunit (Deriano and Roth 2013). Artemis nuclease trims the broken ends, which can then be ligated by different factors including X-ray repair cross complementing protein 4 complex (XRCC4), XRCC4- like factor, Ligase 4 (Lig 4), and others (Meek, Dang et al. 2008, Mahaney, Meek et al. 2009, Lieber 2010). Another role of c-NHEJ is in V(D)J recombination during the development of T and B cells (Malu, Malshetty et al. 2012). c-NHEJ repairs DSBs quickly (10-30 mins) however, compared to HR, it is more error prone (Dueva and Iliakis 2013), as it re-ligates two DNA ends irrespective of homology, thereby increasing the risk of introducing mutations, insertions, and deletions. Importantly, loss of tumour suppressor genes such as *BRCA1/2* can cause HR to become abrogated, and therefore c-NHEJ becomes the pathway of choice for DSBs repair.

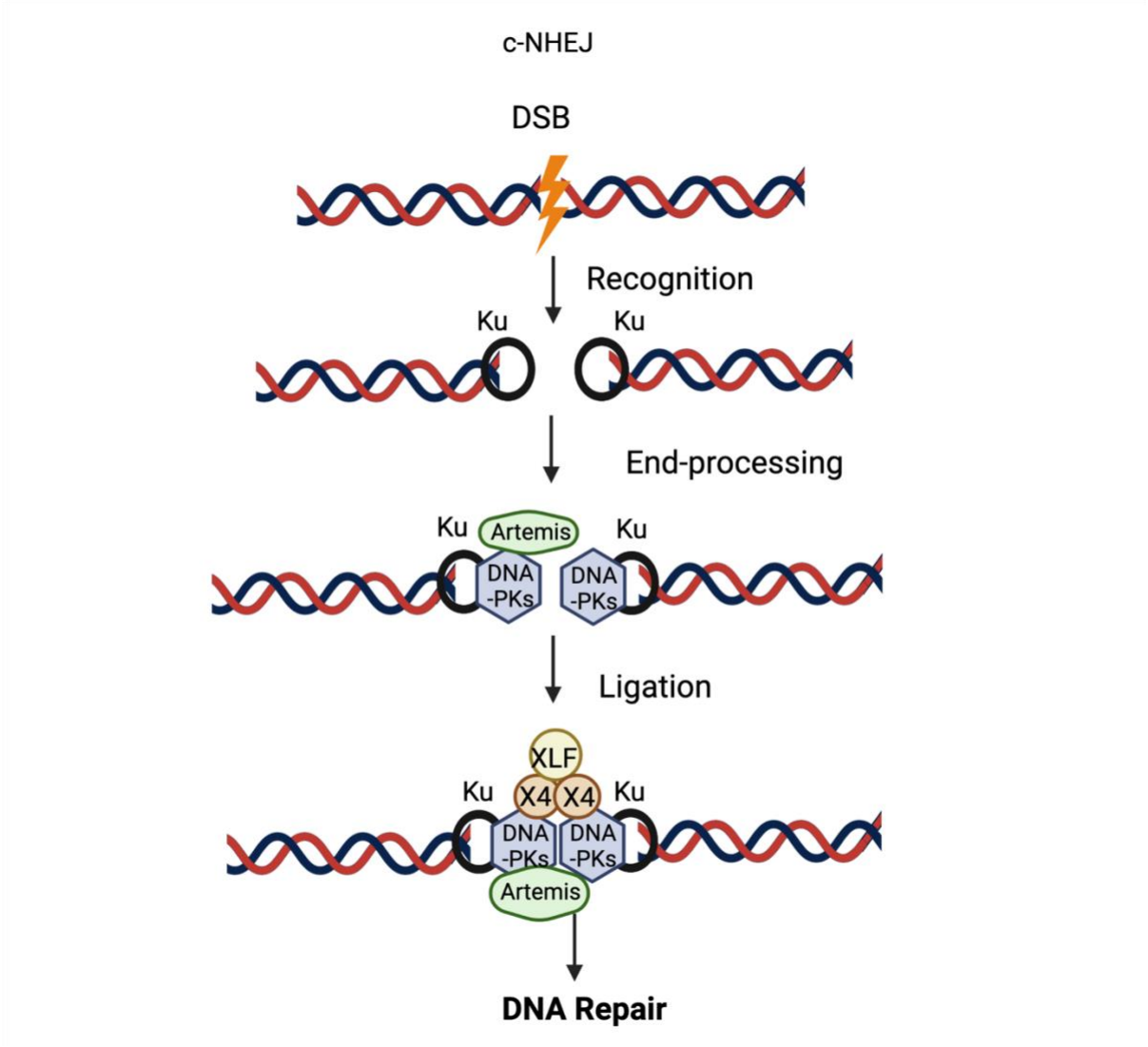


Figure 1.2. Schematic representation of c-NHEJ DNA repair pathway.

The key steps of the c-NHEJ DSB repair pathway are DSB recognition, end-processing and ligation. The Ku70/Ku80 recognises the DSB and binds. The Ku70/Ku80 complex has a ring structure and encircles the DNA duplex. The binding of the Ku70/Ku80 complex allows the recruitment of DNA-PKcs, Artemis complex, polymerases λ and μ, and ligation complex composed of XRCC4 (X4), XRRC4-like factor (XLF), and DNA ligase IV. DNA-PKCs act as a platform for repair. The DNA-PK is activated by DNA ends which results in autophosphorylation of DNA-PK and downstream effects including the phosphorylation of Artemis. The recruitment of the ligation complex allows direct ligation of the ends.

1.4. BRCA1/2 roles in DNA replication

DNA replication is the duplication of the genomic DNA necessary for mitosis to ensue. Commonly, during this process, the replication fork stops progressing in response to a barrier (Tarsounas and Sung 2020). Replication barriers include G-quadruplexes, RNA-DNA hybrids and inter-strand crosslinks and protein-DNA complexes that prevent replication. Consequently, the replicative helicase minichromosome maintenance protein complex (MCM) and the polymerases become uncoupled from each other because the helicase moves on, but the polymerase is blocked by the barrier (Byun, Pacek et al. 2005). MCM is a DNA helicase essential for genomic DNA replication (Forsburg 2008). Stalled replication forks can cause uncoupling of polymerase and replicative helicase (Figure 1.3; Alexander and Orr-Weaver 2016).

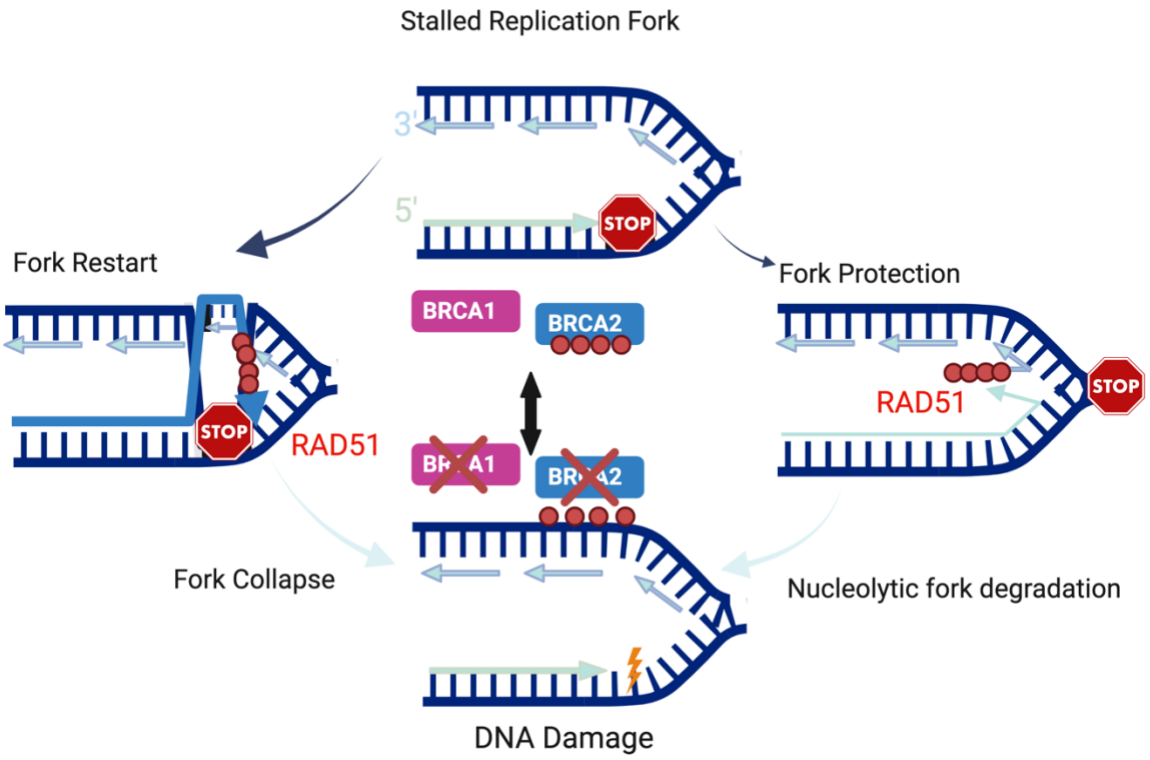


Figure 1.3. Stalled Replication Restart and Protection

Replication forks stop progressing in response to replication barriers such as RNA-DNA hybrids. BRCA1 and BRCA2 are involved in fork protection by preventing the degradation of the fork by nucleases. These tumour suppressors also play a key role in fork restart by promoting HR-like reactions, thereby, enabling the fork to bypass the block. To promote fork restart, BRCA2 binds RAD51 which protects the stalled replication fork from nucleases. However, upon loss of BRCA1 or BRCA2 abrogation, the forks become permanently stalled which results in fork collapse or fork degradation by nucleases. Both of these events can lead to DNA damage.

BRCA1 and BRCA2 can promote fork restart by promoting a DNA reaction that enables the fork to bypass this block (Quinet, Lemaçon et al. 2017). This is dependent on the loading of RAD51 protein which is not only essential for HR repair but also for the restart of stalled replication forks. BRCA2 is also involved in protecting a stalled replication fork from degradation by nucleases (Schlacher, Christ et al. 2011, Rickman, Noonan et al. 2020). RAD51 binds to ssDNA that forms at the side of the stalled fork and in doing so it prevents access of nucleases that could otherwise degrade the ss DNA at site of the stalled fork (Bhat and Cortez 2018).

Consequently, upon inactivation of *BRCA1* or *BRCA2* due to mutations, for example in cancer, both functions of BRCA1/2 protein in replication are abrogated. Fork restart cannot occur in a BRCA-dependent manner and hence the forks frequently collapse. When the forks collapse, DNA damage is introduced via DSB formation. Additionally, without fork protection, nucleases start degrading the fork which leads to DNA damage accumulation.

1.5. Targeting *BRCA1/2* inactivated tumours in the clinic

Understanding BRCA1 and BRCA2 roles in tumorigenesis is crucial for providing improved treatment modalities for the treatment of *BRCA1/2*-mutated tumours in the clinic. BRCA2 loss in normal cells leads to apoptosis or cell cycle arrest whilst in tumour cells BRCA2 loss leads to HR deficiency which disrupts the genomic stability maintenance (Roy, Chun et al. 2011, Feng and Jasin 2017). Thus, the hallmark of BRCA2-deficient tumours is genomic instability. Genomic instability is a vulnerability of *BRCA*1/2-mutated tumours that has been exploited therapeutically by pharmacological agents, with poly (ADP- ribose) polymerase inhibitors (PARPi) being a prominent example.

1.5.1. PARPi

Small molecule inhibitors of PARP are at the forefront in the treatment of *BRCA1/2*-mutated ovarian and breast tumours and have therefore been at the forefront of investigations in clinical research (Mirza et al., 2016, Robson et al., 2017). These inhibitors act by trapping PARP enzymes on the chromatin. This interferes with DNA replication and repair and leads to cancer cell death (Kedar, Stefanick et al. 2012, Bignold 2020). PARPi have been a promising treatment for BRCA1/2-deficient cancers (Montemorano et al., 2019) since they are selectively toxic to HR-deficient cells (Verma et al., 2021). Olaparib and Talazoparib, are FDA- and NICE-approved PARPi, that have been shown to have high specificity for BRCA1/2-deficient tumours. PARPi initially elicit a good response in patients, however, most patients acquire resistance to these small molecule inhibitors. Therefore, the key clinical challenge

remains the resistance developed upon treatment with PARPi. Consequently, efforts have been made to improve the treatment of *BRCA1/2*-mutated tumours in order to prevent or overcome PARPi resistance. For example, by exploiting innate immune responses in *BRCA1/2*-mutated tumours as a novel therapeutic targeting approach (Reisländer, Lombardi et al. 2019).

1.6. Innate immune responses

Immunosurveillance is the ability of the immune system to protect the host from cancer development, by constantly clearing newly formed tumour cells. This concept was initially proposed fifty years ago (Burnet, 1957). The literature on the effects of BRCA2 inactivation and subsequent activation of innate immune responses remains scarce. However, Reislander et al. (2019) have shown that abrogation of the *BRCA2* gene activates innate immune responses, which are potentiated by PARPi. Chronic BRCA2 inactivation, depleted for 28 days by DOX-inducible shRNA against BRCA2, was shown to upregulate genes involved in the innate immune responses. Moreover, DNA damage has been depicted to elicit innate immune responses via the cGAS-STING signalling pathway (Shen, Liu et al. 2021). Activation of the cGAS-STING signalling pathway and subsequent innate immunity activation has been suggested as a novel target for treatment of cancer (Gan, Li et al. 2021).

1.7. cGAS-STING signalling pathway

Cyclic GMP-AMP synthase (cGAS) is activated by dsDNA. It senses accumulated DNA in the cytoplasm, subsequently cGAS can binds the micronuclei-encapsulated DNA, and catalyses the synthesis of cGAMP by utilising GTP and ATP (Figure 1.4) (Zhao, Wang et al. 2021). The cGAMP synthesis activates ER-bound stimulator-of-interferon-genes (STING) which from the endoplasmic reticulum translocated to Golgi apparatus where it promotes phosphorylation of TBK1 and IKK α/β. Next TBK1 and IKK α/β phosphorylate IRF3 and Ikβα respectively (Reisländer, Groelly et al. 2020). Ikβα releases NF-kβ. Phosphorylated IRF3 and NF-kβ translocate into the nucleus and induce transcriptional changes such as production of type I IFNs including IFN-α and IFN-β. Subsequently, IFN-α and IFN-β bind IFNAR1/2, which is their heterodimeric receptor. This leads to phosphorylation of STAT1 and STAT2 via JAK activation. The STAT1/2 heterodimer triggers transcription of Interferon-stimulated genes (ISGs). ISGs play a key role in bridging the innate and adaptive immunity (Wong and Chen 2016). For example, IFN type I signalling triggers the activation of effector immune cells via mediating the (neo)antigen presentation on dendritic antigen-presenting cells at the major histocompatibility complex (MHC) (Boukhaled, Harding et al. 2021). This process leads to the activation of T cells by binding and activating the T cell receptor (TCR). This process is dependent on two activation signals including stimulatory signals (B7-CD28) and inhibitory signals (B7-CTLA4). T cells, upon their activation, infiltrate tumours and recognise the antigen presented on the tumour cell surface. The interaction between CTLA-4 on T cell and B7 on tumour cells, and the interaction between PD-1-on T cell and PDL-1 lead to inhibition of the anti-tumourigenic immune activity. As a result, immune checkpoint inhibitors have been developed in the clinic to prevent the inhibition of anti-tumourigenic responses. The

cGAS/STING signalling pathway therefore has an important role in the immune system (Jiang et al., 2020). Pharmacological activation of this signalling pathway, for example by employing STING agonists, may elicit antitumour immunity and cancer clearance.

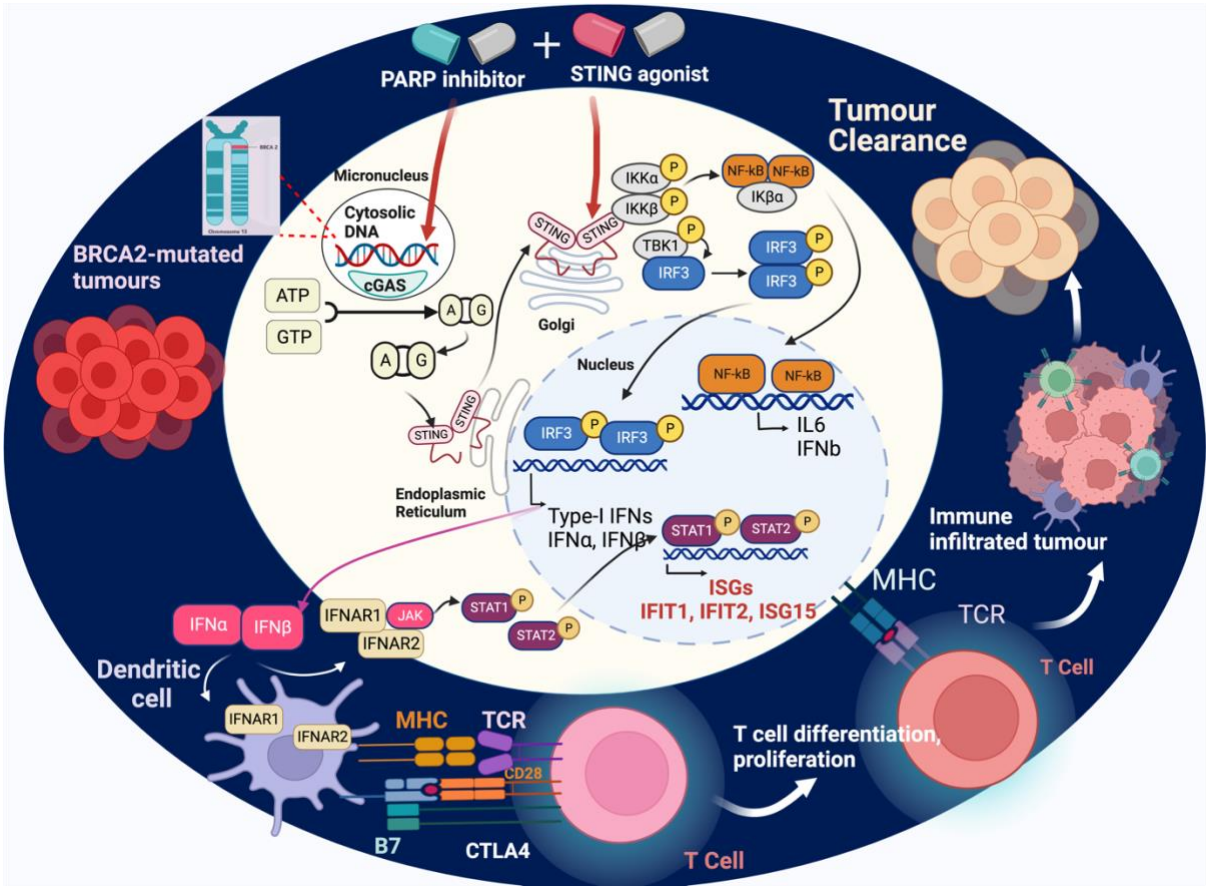


Figure 1.4. cGAS-STING-STAT pathway

Loss of key genes such as *BRCA2* tumour suppressor *gene* can lead to the accumulation of DNA damage in the cytosol. The cytosolic sensor cGAS detects and binds to the DNA encapsulated into micronuclei found in the cytosol. Subsequently, cGAS generates cGAMP from ATP and GTP. STING is activated by cGAMP, and translocates from the endoplasmic reticulum to Golgi apparatus. Next, STING stimulates phosphorylation of TBK1 and IKK α/β, which phosphorylate IRF3 and release NF-Kβ respectively. Phosphorylated IRF3 and NF-Kβ translocate to the nucleus and elicits transcriptional changes such as the synthesis of type I interferons. IFNα and IFNβ bind dendritic cells and enhance antigen presentation and act on the JAK-STAT pathway which leads to the synthesis of interferon stimulated genes (ISGs). The pathway can be stimulated pharmacologically by employing STING agonists, PARPi or combination treatment with both.

1.8. STING agonists

The cGAS-STING pathway is an appealing target for the development of new drugs that will induce anti-tumourigenic immune responses. Immune checkpoint inhibitors have been suggested for this purpose in the clinic. However, the efficacy of these checkpoint inhibitors is limited to patients with a tumour microenvironment permissive to T cell infiltration and IFN secretion (Reislander Groelly et al., 2020). An alternative approach is to induce pharmacological activation of cGAS-STING signalling through the use of STING agonists which act to activate the cGAS-STING signalling pathway and therefore can lead to antitumour immunity. One prominent example of STING agonists is 5,6-dimethylxanthenone-4-acetic acid (DMXAA) also referred to as Vadimezan. It has proceeded as far as phase III clinical trials, for example in non-small cell lung cancer, although DMXAA employed in combination with chemotherapy (platinum-based) did not show improved efficacy in this tumour type (Lara, Douillard et al. 2011). In macrophages, DMXAA was shown to elicit robust cytokine responses, thereby reducing the cytoplasmic presence of viral nucleocapsids (Guo, Han et al. 2015). DMXAA has also been shown to induce apoptosis in T cells but not in bone marrow-derived dendritic cells (BMDCs), mouse embryonic fibroblasts (MEFs) or bone marrow-derived macrophages (BMDM) (Gulen, Koch et al. 2017). This finding suggested that the apoptosis induced by STING activation using DMXAA is cell type specific (Gulen, Koch et al. 2017). This STING agonist has been shown to induce interferon release (Perera, Barber et al. 1994, Roberts, Goutagny et al. 2007), and robust cytokine responses in macrophages (Guo, Han et al. 2015). This is important because certain cytokines including interferons have been shown to promote anti-tumour immune responses by directly activating key immune cells including dendritic cells which are involved in antigen presentation (Fenton, Saleiro et al. 2021).

Furthermore, DMXAA has been shown to induce antitumour immunity by inducing type I interferon responses and by activating cytotoxic CD8+ T cells (Corrales et al., 2015; Sivick et al., 2018). One major impediment of DMXAA is that this agonist has been shown to induce limited activation of human STING and has been shown to have better efficacy in activating mouse STING (Conlon, Burdette et al. 2013). The difference in sensitivity to DMXAA between mouse and human STING has been attributed to dynamic structural differences between the two types of STING (Shih, Damm-Ganamet et al. 2018). Newer cyclic compounds such as ADU-S100 have been developed which may be more specific for human STING and could be used to progress this work.

STING agonists have also been shown to synergise with PARPi in order to suppress tumour growth and breach immunosuppression (Wang, Bergholz et al. 2022). The combination therapy was depicted to elicit type I IFN response and cytotoxic T cell activation. Another study combining STING agonists with PARPi in BRCA1-deficient human and mouse cell lines showed that the combination treatment led to greater STING activation and cytokine synthesis (Pantelidou, Jadhav et al. 2021). Therefore, combining STING agonists with PARPi in BRCA2-deficient cells may lead to greater cGAS-STING pathway activation and therefore improved antitumour immunity compared to treatment with STING agonists alone.

2. Aims of the study

Genomic instability, arising from the accumulation of DNA lesions, has been exploited as a therapeutic vulnerability in BRCA-mutated tumours, which can be targeted with highly specific therapies such as PARPi. However, resistance to PARPi remains a major clinical challenge. Therefore, novel therapeutic approaches are required to improve patient outcomes. Previously, we have observed that loss of BRCA2 expression led to the activation of the cGAS-STING signalling pathway and the downstream induction of type I interferon (IFN) responses (Reisländer, Lombardi et al. 2019). These responses have the potential to promote tumour eradication by activating the innate and adaptive immune responses (Burnette, Liang et al. 2011, Fuertes, Woo et al. 2013). Notably, cGAS-STING pathway activation was shown to be potentiated by treatment with PARPi (Pantelidou, Jadhav et al. 2022). In this project, we aimed to examine STING agonist mediated activation of the cGAS-STING pathway and its impact on DNA damage levels and survival of human BRCA2-deficient cells, either as a single agent or in combination with PARPi.

3. Materials and methods

3.1. Cell lines

Human retinal pigment epithelial RPE1 cells expressing Cas9, transduced with hTERT and *TP53*-deleted (*BRCA2*$^{+/+}$ and *BRCA2*$^{-/-}$) were grown in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich, D5796), supplemented with 2 µg/ml blasticidin (Life Technologies, A1113903) and 10% foetal bovine serum (FBS; Merck Life Science UK, F7524-500ML) (Zimmermann, Murina et al. 2018, Groelly, Porru et al. 2022). Human non-small cell lung carcinoma H1299 cells with doxycycline (DOX)-inducible BRCA2 shRNA (GGG AAA CAC UCA GAU UAA A; H1299+shBRCA2^{DOX}) were grown in DMEM supplemented with 10% tetracycline-free FBS (Takara Bio, 631106) (Lau, Zhu et al. 2017). Expression of the shRNA against BRCA2 was induced by addition of 2 µg/mL DOX (Sigma-Aldrich, D9891) to the medium for 4 days. The cell lines were cultivated as monolayers at 37°C in 5% CO_2 containing atmosphere. Cells were tested routinely for contamination with mycoplasma.

3.2. Drug Treatments

Cells were seeded in 60 mm ($15x10^4$ – $25x10^4$ cells) or 100 mm ($55x10^4$ – $99x10^4$ cells) dishes to reach 70-80% confluency after 72 hours growth. The STING agonist DMXAA (Sigma Aldrich, D5817) was added for 72 hours at concentration of 5 or 10 µM for RPE1 cells and 10 or 50 µM for H1299 cells. Control cells were treated with the equivalent amounts of the vehicle, dimethyl sulfoxide (DMSO; Sigma Aldrich, D2438). Cells were treated for 72 hours with 10 nM of the PARPi Talazoparib (Selleckchem, S7048) or the equivalent amount of the vehicle DMSO.

3.3. Viability Assays

H1299+shBRCA2^{DOX} cells (cultured in the presence or absence of 2 µg/ml DOX for 4 days) and RPE1 cells were seeded in triplicate in 96-well plates and treated with the indicated doses of DMXAA. Five hours later, the cells were treated with the indicated doses of Talazoparib. The cells were cultured in the presence of drugs for 6 days at 37°C, 5% CO_2. Viability was determined by a resazurin-based method. Cells were incubated with resazurin (Sigma-Aldrich, R7017), diluted to 10 µg/mL in culture media, for 2 hours at 37°C, 5% CO_2. The fluorescence was measured using POLARstar OMEGA plate reader. Cell viability was expressed relative to control cells.

3.4. Immunoblotting

We washed the cells in 1X PBS and trypsinised the cells in order to harvest them. The cell pellets were lysed in sodium dodecyl sulphate (SDS) lysis buffer (0.16 M Tris-HCl pH 6.8, 4% SDS, 25% glycerol, 0.01% bromophenol blue, 100 mM DTT) containing phosphatase inhibitor cocktail (Roche, 04906845001) and protease inhibitor cocktail (Roche, 04693159001). Samples were sonicated for 6-12 seconds to fragment the DNA. The samples were then heated for 5 minutes at 80°C to denature the proteins. The samples were subsequently centrifuged for 7 minutes at 13,000 x *g* to remove cellular debris. The Spectrophotometer ND-100 (Nanodrop Technologies Inc.) was used for protein quantification. Equal protein amounts (80 – 120 µg) were evaluated using gel electrophoresis using 3-8% 15-well Tris-acetate gels (Thermo Fisher Scientific, EA03785) or 10% 15-well Bis-Tris gels (Thermo Fisher Scientific,

EA03752). MOPS (Thermo Fisher Scientific, N0001) was used as the running buffer for Bis-Tris gel and Tris-Acetate buffer (Thermo Fisher Scientific, LA0041) for the Tris-Acetate gel. The gels were run for 50-55 mins at 180 V using XCell SureLock Mini-Cell (Thermo Fisher Scientific, EI0001). The proteins were then transferred to 0.2 µM nitrocellulose membranes (GE Healthcare Life Sciences, 10600001) using semi-dry transfer (Thermo Fisher Scientific, NP0006-1) with 10% methanol. The membranes were blocked for 1 hour in 5% skimmed milk in PBS-Tween (PBST; 1X phosphate-buffered saline [Thermo Fisher Scientific, BR0014G] and 0.05% Tween 20 [Sigma-Aldrich, P7949]) and incubated overnight in primary antibody at 4°C. The primary antibody (Table 1) was diluted in 2% bovine serum albumin (BSA; Sigma-Aldrich, A7906) and 0.05% azide in PBST. The next day the membrane was washed 3 times for 5-10 mins with PBST and incubated with secondary antibody (Table 2) for 1 hour at room temperature. The membrane was then washed with PBST 3 times for 5-10 mins. Proteins were detected by enhanced chemiluminescence (ECL; VWR International Ltd and Millipore) and X-ray films (Thermo Fisher Scientific, 34089) developed using the Ecomax (Protec) developer.

Table 1 Primary antibodies used for immunoblotting

Target	**Host**	**Supplier**	**Catalogue Number**	**Dilution**	**Size (kDa)**
BRCA2	Mouse	Calbiochem	OP95	1:1,000	384
cleaved PARP (Asp214)	Rabbit	Cell Signalling	D214	1:1,000	89
PARP	Rabbit	Cell Signalling	9532S	1:1,000	89
KAP1	Rabbit	Bethyl Laboratories	A300-274A	1:5,000	117
Phosphorylated KAP1 (S824)	Rabbit	Bethyl Laboratories	A300-767A	1:1,000	117
IRF3	Rabbit	ABCAM	AB76409	1:1,000	47
Phosphorylated IRF3 (S386)	Rabbit	ABCAM	AB76493	1:1,000	47
SMC1	Rabbit	Bethyl Laboratories	A300-055A	1:10,000	143
GAPDH	Mouse	Novus	NB600-502	1:30,000	37

Table 2 Secondary antibodies used for immunoblotting

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

3.5. Reverse transcription–quantitative Polymerase Chain Reaction (RT-qPCR)

Cells grown in 60 mm dishes were washed in PBS and harvested by trypsinisation. RNA extraction and reverse transcription to complementary DNA (cDNA) were performed using the SYBR Green Cells-to-CT Kit (Ambion, 4402954) according to instructions from manufacturer. The resulting cDNA was diluted 1:10 with DEPC-treated water (Thermo Fisher Scientific, AM9906) and analysed using SYBR Green technology. For each sample, technical triplicate qPCR reactions were prepared. Each 8 µl reaction contained 2 µl of diluted cDNA, 1X Power SYBR Green PCR (Applied Biosystems, 2111618), 0.2 µM forward primer (Table 3), 0.2 µM reverse primer (Table 3) and DEPC-treated water. We performed the reactions using the StepOnePlus Real-Time PCR System (Thermo Fisher Scientific) and the PCR program in Table 4. For the analysis of RT-qPCR results, gene expression was first normalised to *GAPDH* expression and then calculated relative to control untreated cells as $2^{-\Delta\Delta CT}$.

Table 3 Pairs of primers used for RT-qPCR

Target	Sense	Sequence
GAPDH	Forward	GACAGTCAGCCGCATCTTCT
	Reverse	ACCAAATCCGTTGACTCCGA
STING	Forward	GAGTGTGTGGAGTCCTGCTC
	Reverse	CTGGAGTGGGGCATCTTCTG
ISG15	Forward	GCGAACTCATCTTTGCCAGTA
	Reverse	CCAGCATCTTCACCGTCAG
IFIT3	Forward	CTTCAGTATTTACTTGAGGCAGAC

	Reverse	CTTGGTGACCTCACTCATGAC

Table 4 Running Programme of RT-qPCR reaction

Stage	Cycles	Ramp Rate (℃/s)	Temperature (℃)	Time (s)
Enzyme activation	1x	1.6	95	600
PCR cycles	40x	1.6	95	15
		1.6	60	60
Dissociation curve	1x	1.6	95	15
		1.6	60	60
		0.075	95	15

3.6. Statistical analyses

The data was processed in Excel and subsequently analysed using GraphPad Prism 9. The statistical significance of the results was evaluated by an unpaired two-tailed Student's *t*-test. A value of $p \leq 0.05$ was considered statistically significant. The results were represented as mean values ± standard error of mean (S.E.M.).

4. Results

4.1. Activation of the cGAS-STING signaling pathway by DMXAA is cell line dependent

Previous studies have shown that *BRCA2* gene inactivation leads to cGAS-STING dependent induction of type I IFN responses, which lead to the upregulation of immune response genes including ISGs such as *ISG15*, *IFIT3*, and *IFI6* (Reisländer, Lombardi et al. 2019). Type I IFN responses have been shown to elicit anti-cancer immune responses (Fuertes, Woo et al. 2013). Consequently, we set out to stimulate STING-mediated IFN responses using a STING agonist in human BRCA2-deficient cells. Several STING agonists have been developed to stimulate STING-mediated responses. The STING agonist DMXAA has been shown to induce STING-dependent innate immune pathways (Prantner, Perkins et al. 2012). Moreover, DMXAA was shown to lead to tumour regression by triggering T cell cooperation (Weiss, Guérin et al. 2017). Here, we characterise the ability of DMXAA to activate innate immune responses in human BRCA2-proficient and BRCA2-deficient cells, either alone or in combination with a PARPi. Human non-small cell lung carcinoma H1299 cells carrying doxycycline (DOX)-inducible short hairpin RNA (shRNA) against *BRCA2* (H1299+shBRCA2^{DOX}) were used as a cellular model for *BRCA2* gene inactivation. To monitor cellular responses to DMXXA, H1299 cells were cultured with or without DOX for 4 days and treated with DMXAA for 3 days before being harvested for immunoblotting (Figure 4.1A). The addition of DOX effectively suppressed *BRCA2* expression, detected by immunoblotting of whole-cell extracts (Figure 4.1B). We next evaluated cGAS-STING pathway activation upon DMXAA treatment by monitoring

phosphorylation of IRF3 (Figure 4.1C). IRF3 phosphorylation at residue Ser386 is a marker of STING activation since it indicates IRF3 nuclear translocation (Abraham, Botto et al. 2020). IRF3 phosphorylation is the downstream effect of STING activation. IRF3 nuclear translocation is required for IRF3 to induces transcription of ISGs (Majumdar, Chattopadhyay et al. 2015). IRF3 phosphorylation was not significantly altered by DMXAA treatment in BRCA2-proficient or -deficient cells compared to vehicle control cells.

We next investigated DMXAA-induced phosphorylation of KRAB-associated protein 1 (KAP1) at Ser824 which is indicative of DSB and ATM activation (White, Rafalska-Metcalf et al. 2012) (Figure 4.1D). Loss of *BRCA2* gene has been shown to induce DNA damage (Reisländer, Lombardi et al. 2019). Consistent with this finding, we observed increased phosphorylation of KAP1 in BRCA2-deficient cells compared to BRCA2-proficient cells. There was no further increase in DSBs, as measured by phosphorylation of KAP1, when BRCA2-deficient cells were treated with DMXAA for 3 days. To examine the effect of prolonged treatment with DMXAA in BRCA2-proficient and BRCA2-deficient cells, cell viability assays were performed (Figure 4.1E). DMXAA treatment did not lead to a significant change in the cell viability of BRCA2-proficient and BRCA2-deficient cells (Figure 4.1F).

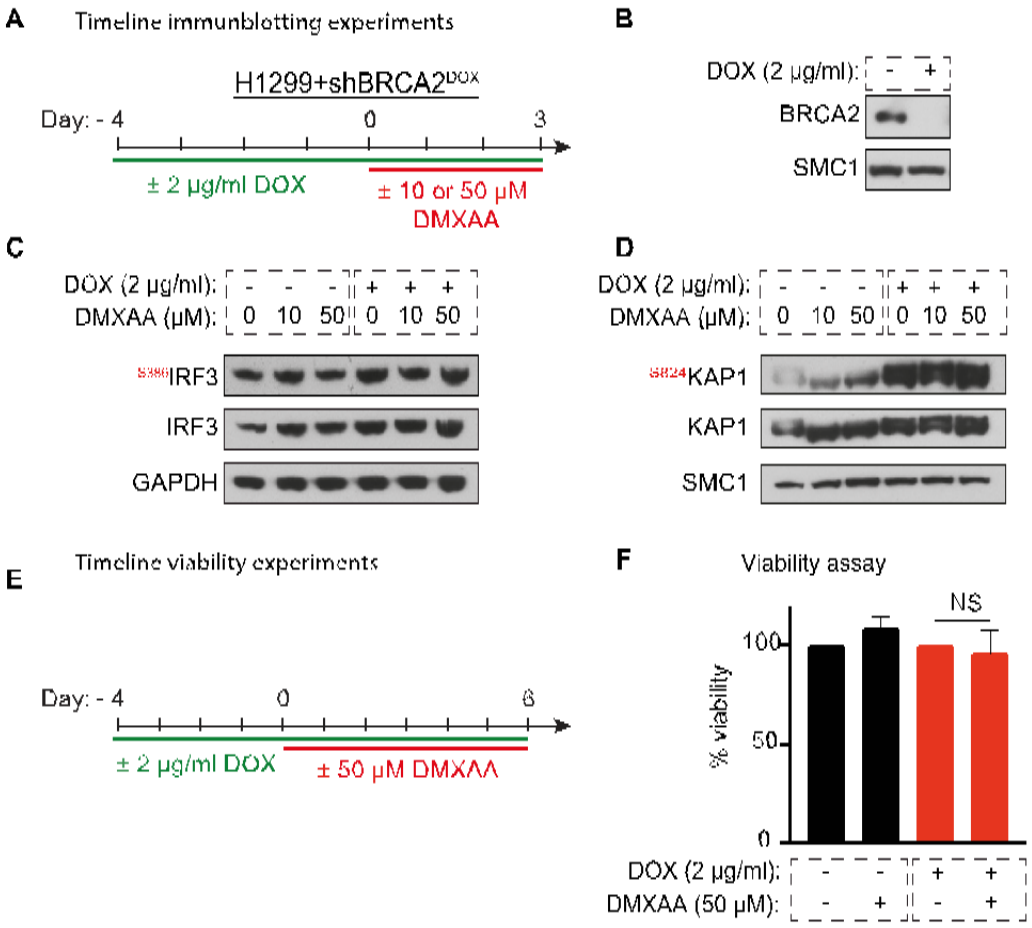


Figure 4.1: DMXAA does not significantly activate the cGAS-STING signalling pathway and does not induce DNA damage in H1299 BRCA2-deficient cells.

(A) Experimental timeline for treatment of human H1299+shBRCA2^{DOX} cells cultured in the presence or absence of doxycycline (DOX; 2 µg/ml) for 4 days and subsequently treated or not with 10 µM or 50 µM DMXAA for 3 days. Cells were collected after 3 days and processed for immunoblotting. (B) Whole cell extracts were immunoblotted to confirm BRCA2 depletion in H1299+shBRCA2^{DOX} cells cultured as in (A). SMC1 was used as a loading control. (C, D) Whole-cell extracts of H1299 cells treated as in (A) were immunoblotted as shown. The sites of phosphorylation are indicated in red. GAPDH or SMC1 were used as a loading control. (E) Experimental timeline for measuring cell viability of H1299 cells grown in the presence or absence of 2 µg/ml DOX. Four days later, cells were treated or not with 50 µM DMXAA for 6 days and processed for cell viability assays. (F) Viability assay of BRCA2-proficient and BRCA2-deficient cells treated as in (E). Data are representative of mean of $n = 3$ independent experiments. Error bars represent standard error of the mean (SEM). Statistical significance was determined by an unpaired two-tailed Student's *t*-test. NS, $P > 0.05$

Next, to rule out any cell model specific effects, we performed the experiment in a second cell line. For this purpose, we employed human RPE1 *BRCA2*$^{+/+}$ and *BRCA2*$^{-/-}$ cells which were generated using CRISPR/Cas9-mediated gene deletion. Although there is a debate as to whether RPE1 cells can activate or not the cGAS-STING pathway (Basit, Cho et al. 2020, Hong, Tijhuis et al., 2019, Li, Zhang et al. 2022, Santaguida, Richardson et al. 2017), we have demonstrated that treatment with DNA damaging agents (e.g. pyridostatin) can effectively achieve this (Groelly, Porru et al. 2022). To investigate DMXAA-dependent cGAS-STING pathway activation in RPE1 cells, *BRCA2*$^{+/+}$ and *BRCA2*$^{-/-}$ cells were treated with DMXAA for 3 days (Figure 4.2A). BRCA2 deletion was confirmed by immunoblotting (Figure 4.2B). Subsequently, we monitored induction of cGAS-STING-dependent immune responses upon treatment with DMXAA by evaluating IRF3 phosphorylation (Figure 4.2C). IRF3 phosphorylation in BRCA2-deficient cells was increased upon DMXAA treatment compared to vehicle control cells indicating that DMXAA treatment is sufficient to activate the cGAS-STING pathway in BRCA2-deficient RPE1 cells. As previously observed in H1299 cells, BRCA2 inactivation led to an increase in phosphorylated KAP1 but DMXAA treatment did not induce further DNA damage compared to vehicle control cells (Figure 4.2D). Subsequently, we investigated the possibility that longer (6 days) DMXAA treatment was required to alter the cell viability of BRCA2-deficient cells (Figure 4.2E). However, DMXAA-induced STING pathway activation did not change the cell viability of BRCA2-proficient or -deficient RPE1 cells (Figure 4.2F).

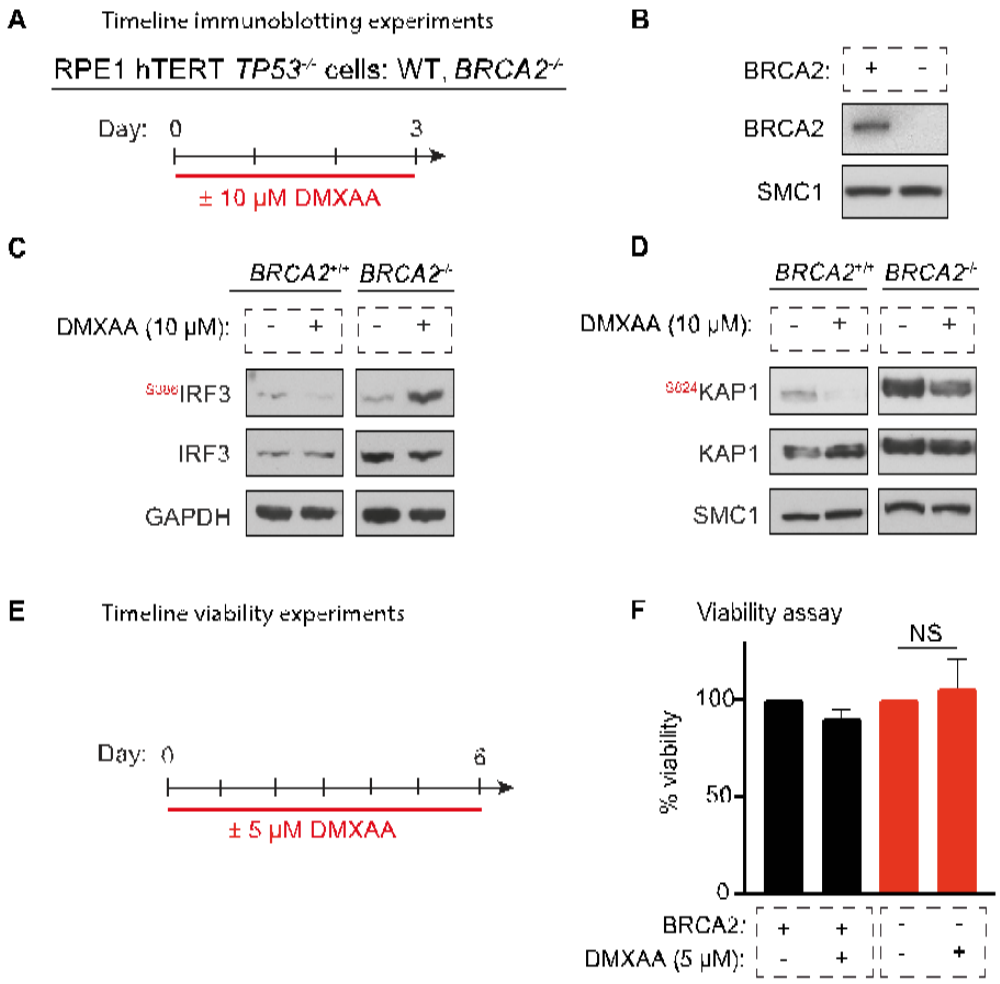


Figure 4.2: DMXAA activates the cGAS-STING signalling pathway but does not induce DNA damage in BRCA2-deficient cells.

(A) Experimental timeline for treatment of RPE1 hTERT *TP53*$^{-/-}$ *BRCA2*$^{+/+}$ and *BRCA2*$^{-/-}$ cells treated or not with 10 µM DMXAA for 3 days. Cells were collected after 3 days and subsequently processed for immunoblotting. (B) Whole cell extracts were immunoblotted to confirm BRCA2 deletion in hRPE1 hTERT *TP53*$^{-/-}$ *BRCA2*$^{-/-}$ cells cultured as in (A). SMC1 was employed as loading control. (C, D) Whole-cell extracts of RPE1 hTERT *TP53*$^{-/-}$ *BRCA2*$^{+/+}$ and *BRCA2*$^{-/-}$ cells treated as in (A) were immunoblotted. The sites of phosphorylation are indicated in red. SMC1 and GAPDH were employed as loading controls. (E) Experimental timeline for measuring cell viability of RPE1 hTERT *TP53*$^{-/-}$ *BRCA2*$^{+/+}$ and *BRCA2*$^{-/-}$ cells, treated or not with 5 µM DMXAA for 6 days and subsequently processed for cell viability assays. (F) Viability assay of BRCA2-proficient and BRCA2-deficient cells treated as indicated in (E). Data are representative of mean of $n = 3$ independent experiments. Error bars represent SEM. Statistical significance was determined by employing unpaired two-tailed Student's t test. NS, $P > 0.05$.

4.2. DMXAA in combination with Talazoparib treatment elicits cGAS-STING-dependent innate immune responses

PARPi upregulate innate immune response genes in BRCA2-deficient cells (Reisländer, Lombardi et al. 2019) and can also enhance the efficacy of immunotherapy by inducing IFN-mediated antitumour responses (Shen, Zhao et al. 2019). Moreover, combining PARPi with a STING agonist in BRCA1-deficient cells led to a synergistic effect and tumour growth suppression (Wang, Bergholz et al. 2022). We therefore assessed if combining DMXAA with PARPi in BRCA2-deficient cells would lead to activation of the cGAS-STING pathway and subsequent ISG expression. We cultured H1299 cells in the presence or absence of DOX for 4 days and then treated the cells for 3 days with 50 µM DMXAA alone or in combination with 10 nM Talazoparib (Figure 4.3A). Firstly, expression of *STING*, detected by quantitative RT-PCR, was examined in BRCA2-proficient and BRCA2-deficient H1299 cells. The *STING* expression was modestly increased upon Talazoparib treatment compared to control cells in both BRCA2-proficient and BRCA2-deficient cells (Figure 4.3B). Surprisingly, a decrease in *STING* expression was observed upon DMXAA treatment and when DMXAA was combined with Talazoparib in BRCA2-deficient cells. As *STING* expression can be regulated by a negative feedback loop (Li, Wilson et al. 2017), other markers were required to confirm cGAS-STING pathway activation. STING activation leads to TBK1 kinase phosphorylation which in turn phosphorylates IRF3 (Tanaka and Chen 2012). Hence, we examined phosphorylation of IRF3 (Figure 4.3C). The combination of DMXAA and Talazoparib upregulated IRF3 phosphorylation irrespective of BRCA2 status. In contrast, DMXAA alone was not sufficient to lead to increased phosphorylation of IRF3 in H1299 cells.

IRF3 phosphorylation leads to ISG induction (DeFilippis, Robinson et al. 2006). To confirm that phosphorylation of IRF3 led to the downstream activation of its targets we examined the expression of ISGs including *ISG15* (Figure 4.3D) and *IFIT3* (Figure 4.3E). Expression of *ISG15* was significantly increased upon DMXAA treatment alone ($p < 0.0001$) and in combination with Talazoparib compared to control cells in both BRCA2-deficient ($p < 0.0001$) and BRCA2-proficient cells. Similarly, *IFIT3* expression was increased upon DMXAA treatment alone and combined with Talazoparib in BRCA2-deficient cells, although this was not statistically significant. The same trend of *IFIT3* expression was also observed in BRCA2-proficient cells.

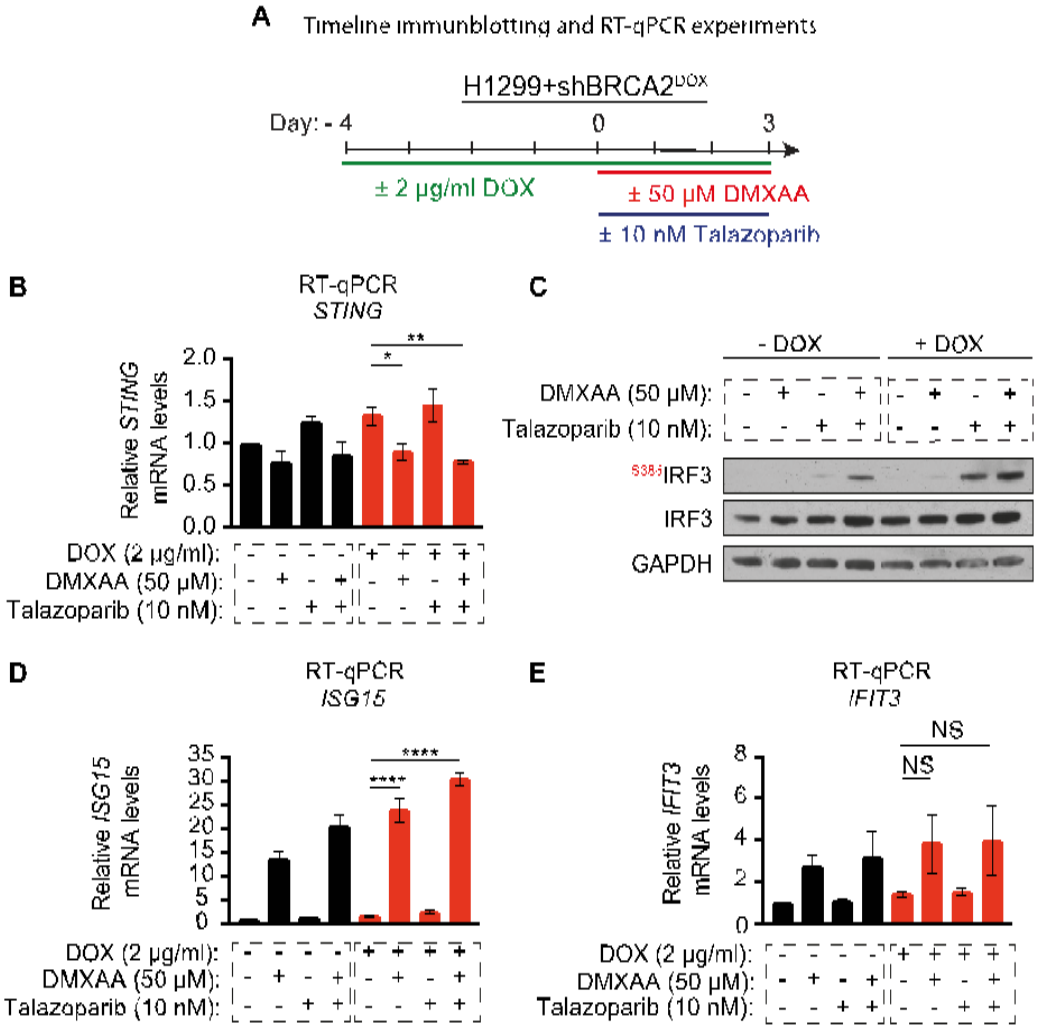


Figure 4.3: Co-treating BRCA2-deficient cells with DMXAA and PARPi Talazoparib activates the cGAS-STING signalling pathway.

(A) Experimental timeline for treatment of H1299+shBRCA2^{DOX} cells cultured in the presence or absence of DOX (2 µg/ml) for 4 days and treated or not with 50 µM DMXAA and 10 nM Talazoparib for 3 days. Cells were collected after 3 days. (B) RT-qPCR was performed to measure *STING* mRNA expression in H1299+shBRCA2^{DOX} cells cultured and treated as indicated in (A). Error bars represent SEM of n = 3 independent experiments. GAPDH was used as the reference gene. Statistical significance was determined by an unpaired two-tailed Student's t-test. (C) Immunoblot of whole-cell extracts of H1299 cells treated as in (A). The site of phosphorylation is indicated in red. GAPDH was used as a loading control. (D) RT-qPCR was performed to measure *ISG15* (D) mRNA levels in H1299+shBRCA2^{DOX} cells cultured and treated as indicated in (A). Error bars represent SEM of n = 2 independent experiments. (E) *IFIT3* mRNA levels were measured using RT-qPCR in H1299+shBRCA2^{DOX} cells cultured and treated as indicated in (A). Error bars represent SEM of n = 3 independent experiments. Statistical significance was determined by an unpaired two-tailed Student's t-test. NS, $P > 0.05$; *, $P \leq 0.05$; **; $P \leq 0.01$; ****, $P \leq 0.0001$.

Next, we investigated the effects of combining PARPi and STING agonist treatment in a second cell line, human RPE1 *BRCA2*$^{+/+}$ and *BRCA2*$^{-/-}$ cells. The cells were treated with DMXAA and Talazoparib at indicated concentrations and harvested for immunoblotting and quantitative RT-PCR (Figure 4.4A). Unlike H1299 cells, in RPE1 BRCA2 abrogated cells the relative mRNA expression of *STING* was slightly elevated upon Talazoparib treatment alone or in combination with DMXAA compared to vehicle control cells (Figure 4.4B). To examine the downstream activation of the cGAS-STING signalling pathway, phosphorylation of Ser386 of IRF3 was examined by immunoblotting (Figure 4.4C). IRF3 phosphorylation was observed upon DMXAA treatment alone and in combination with Talazoparib in BRCA2-deficient cells indicating activation of the cGAS-STING signalling pathway. Similarly, treatment with the combination of DMXAA and Talazoparib induced IRF3 phosphorylation in BRCA2-proficient cells.

Subsequently, expression of *ISG15* (Figure 4.4D) and *IFIT3* (Figure 4.4E) were examined to confirm activation of downstream targets of the cGAS-STING signalling pathway. As previously observed in H1299 cells, the mRNA expression of *ISG15* was increased in *BRCA2*$^{-/-}$ cells when DMXAA and Talazoparib were treated concurrently compared with vehicle control cells. Although this increase was not statistically significant. In contrast to H1299 cells, DMXAA alone did not lead to increased expression of *ISG15* in BRCA2 inactivated cells. Expression of *IFIT3* increased upon DMXAA treatment alone and in combination with Talazoparib relative to control BRCA2-deficient cells consistent with the changes in expression observed in H1299 cells. Taken together these results indicate that combining DMXAA with Talazoparib leads to cGAS-STING pathway activation which ultimately induces upregulation of ISG expression in two independent models of BRCA2 inactivation.

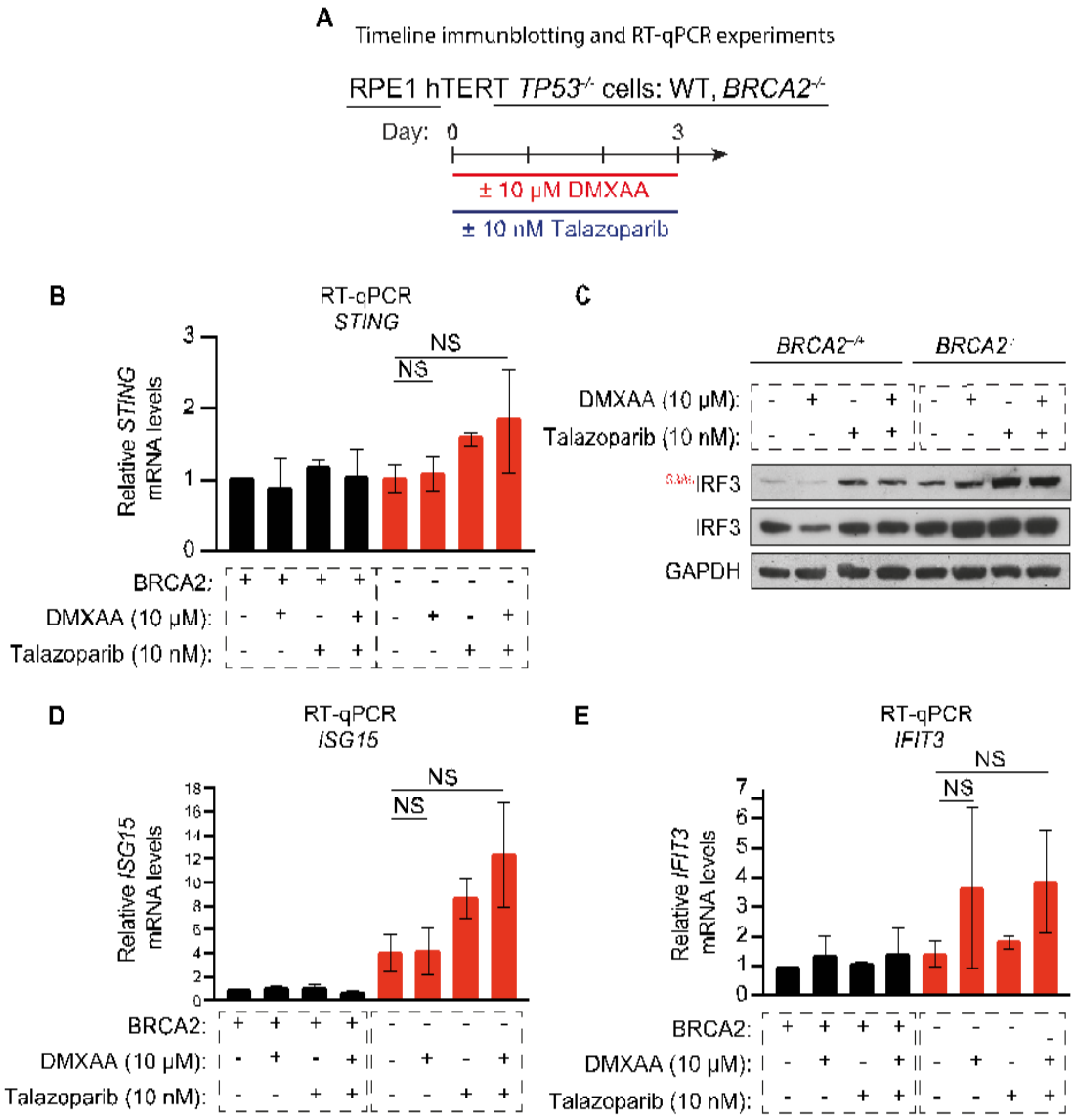


Figure 4.4: Combination treatment with DMXAA and Talazoparib activates the cGAS-STING pathway in BRCA2-deficient cells.

(A) Experimental timeline for treatment of RPE1 hTERT *TP53*$^{-/-}$ *BRCA2*$^{+/+}$ and *BRCA2*$^{-/-}$ cells treated or not with 10 µM DMXAA and 10 nM Talazoparib for 3 days. Cells were collected after 3 days. (B) RT-qPCR was performed to measure *STING* mRNA expression in RPE1 hTERT *TP53*$^{-/-}$ *BRCA2*$^{+/+}$ and *BRCA2*$^{-/-}$ cells treated as indicated in (A). Error bars represent SEM of $n = 3$ independent experiments. GAPDH was used as reference gene. (C) Whole-cell extracts of RPE1 hTERT *TP53*$^{-/-}$ *BRCA2*$^{+/+}$ and *BRCA2*$^{-/-}$ cells treated as in (A) were immunoblotted. The site of phosphorylation is indicated in red. GAPDH was used as a loading control. (D) RT-qPCR was performed to measure *ISG15* mRNA levels in hTERT *TP53*$^{-/-}$ *BRCA2*$^{+/+}$ and *BRCA2*$^{-/-}$ cells treated as indicated in (A). Error bars represent SEM of n=2 independent experiments. (E) *IFIT3* mRNA levels were measured using RT-qPCR in hTERT *TP53*$^{-/-}$ *BRCA2*$^{+/+}$ and *BRCA2*$^{-/-}$ cells treated as indicated in (A). Error bars represent SEM of n=3 independent experiments. Statistical significance was determined by an unpaired two-tailed Student's *t*-test. NS, $P > 0.05$.

4.3. DNA damage is induced when DMXAA is combined with Talazoparib however cell viability is unaltered

We previously showed that DMXAA treatment alone did not potentiate DNA damage in BRCA2-deficient cells. In contrast, PARPi induce cytotoxicity specifically in BRCA2 abrogated cells (Chen 2011). Therefore, we investigated the hypothesis that combining DMXAA and the PARPi Talazoparib could potentiate DNA damage in BRCA2-deficient cells. H1299+shBRCA2^{DOX} cells were cultured in the presence or absence of DOX for 4 days, treated for 3 days with 50 μM DMXAA and 10 nM Talazoparib, and then whole cell extracts were harvested for immunoblotting (Figure 4.5A). To investigate DNA damage, phosphorylation of KAP1 at residue Ser824 was monitored in BRCA2-proficient and -deficient cells (Figure 4.5B). Talazoparib treatment led to increased KAP1 phosphorylation in BRCA2-deficient cells compared to vehicle control or DMXAA treated BRCA2-deficient cells. However, the combination of DMXAA and Talazoparib did not significantly increase phosphorylation of KAP1 compared to Talazoparib treatment alone.

To determine if BRCA2-deficient cells were more susceptible to the DNA damage induced by combined treatment of DMXAA and Talazoparib, apoptosis was examined by immunoblotting for cleaved PARP (Figure 4.5C). Cleaved PARP was observed when H1299 cells were treated with the combination of Talazoparib and DMXAA and was elevated in BRCA2-deficient compared to BRCA2-proficient cells. As BRCA2-deficient cells has increased susceptibility for apoptosis when treated with DMXAA and Talazoparib we went on to evaluate the viability of H1299 cells cultured with or without DOX for 4 days and subsequently treated with DMXAA and Talazoparib for 6 days (Figure 4.5D). However, combining treatment of DMXAA with Talazoparib did not alter the sensitivity of H1299 cells to Talazoparib (Figure 4.5E).

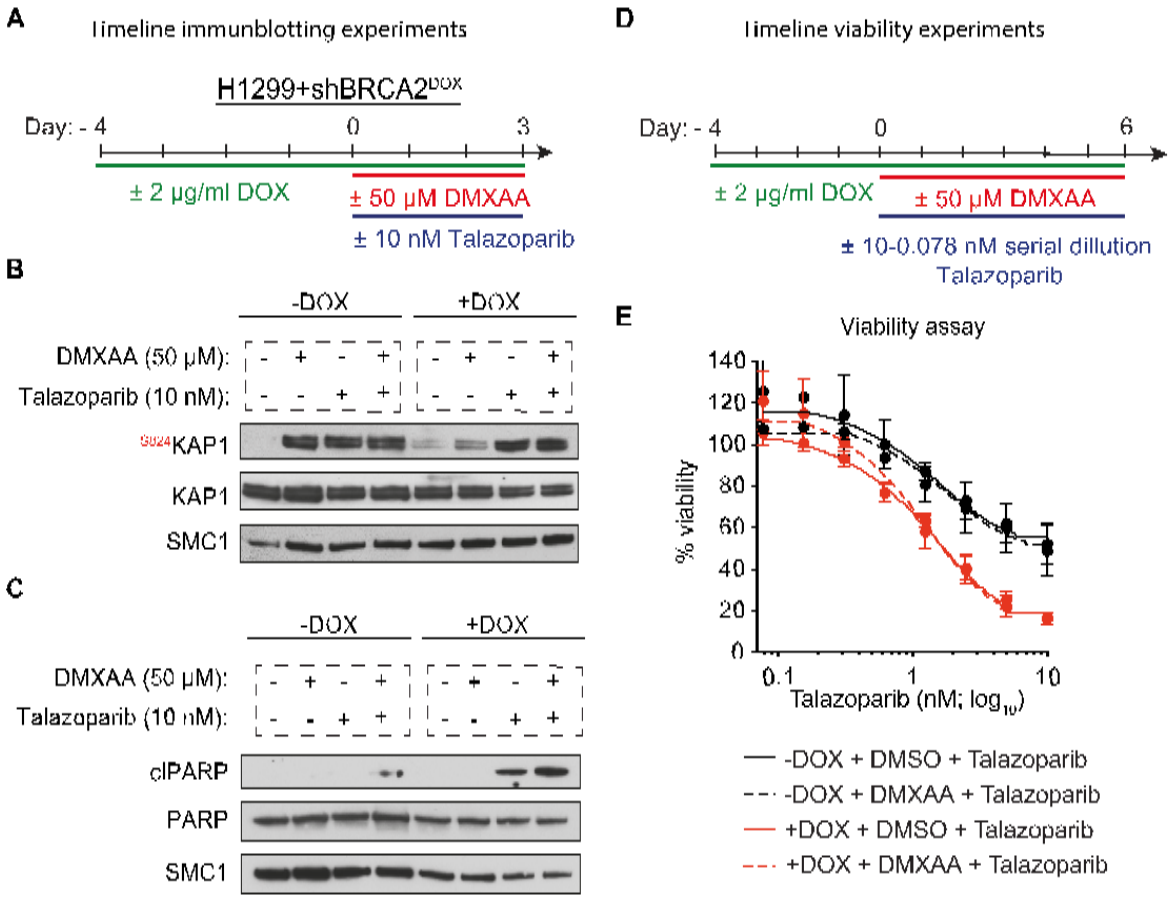


Figure 4.5: Co-treating BRCA2-deficient cells with DMXAA and Talazoparib induces DNA Damage.

(A) Experimental timeline for treatment of H1299+shBRCA2^{DOX} cells cultured for 4 days in the presence or absence of DOX (2 µg/ml) and subsequently treated or not with 10 nM Talazoparib and 50 µM DMXAA for 3 days. Cells were collected after 3 days of treatment and were then processed for immunoblotting. (B, C) Whole-cell extracts of H1299+shBRCA2^{DOX} cells treated as in (A) were immunoblotted. The site of phosphorylation is indicated in red. SMC1 was used as a loading control. (D) Experimental timeline for treatment of H1299+shBRCA2^{DOX} cells cultured in the presence or absence of DOX (2 µg/ml) for 4 days and treated or not with serial dilution of Talazoparib and 50 µM DMXAA for 6 days and subsequently processed for viability assays. (E) Viability assay of H1299+shBRCA2^{DOX} cells treated as in (D). Data are representative of mean of *n* = 3 independent experiments. Error bars represent SEM.

To exclude any cell specific effects of the findings in the H1299 cell line, we examined the effect of DMXAA and Talazoparib on DNA damage and cell viability in RPE1 cells. RPE1 cells were treated for 3 days with 10 μM DMXAA and 10 nM Talazoparib and whole cell extracts were harvested for immunoblotting (Figure 4.6A). We next monitored phosphorylation of KAP1 in order to evaluate DNA damage (Figure 4.6B). Treatment with Talazoparib alone and the combination of Talazoparib and DMXAA led to increased KAP1 phosphorylation in BRCA2-deficient cells compared to vehicle control cells. Compared with DMXAA alone, the combination of DMXAA and Talazoparib led to increased KAP1 phosphorylation in BRCA2-deficient cells. This suggests that the combination treatment increased DNA damage compared to DMXAA treatment alone in BRCA2-deficient cells. However, as observed in the previous cell line, there was no difference between the phosphorylated KAP1 levels in Talazoparib and DMXAA/Talazoparib treated BRCA2-deficient cells.

Next, we monitored cleaved PARP protein levels (Figure 4.6C). Cleaved PARP was observed in BRCA2-depleted cells when they were treated with Talazoparib and DMXAA together or Talazoparib treatment alone. Consequently, we evaluated the viability of RPE1 *BRCA2*$^{+/+}$ and *BRCA2*$^{-/-}$ cells treated with Talazoparib and DMXAA (Figure 4.6D). Consistent with H1299 cells, the sensitivity of RPE1 cells to Talazoparib was unchanged by co-treating cells with DMXAA and Talazoparib (Figure 4.6E).

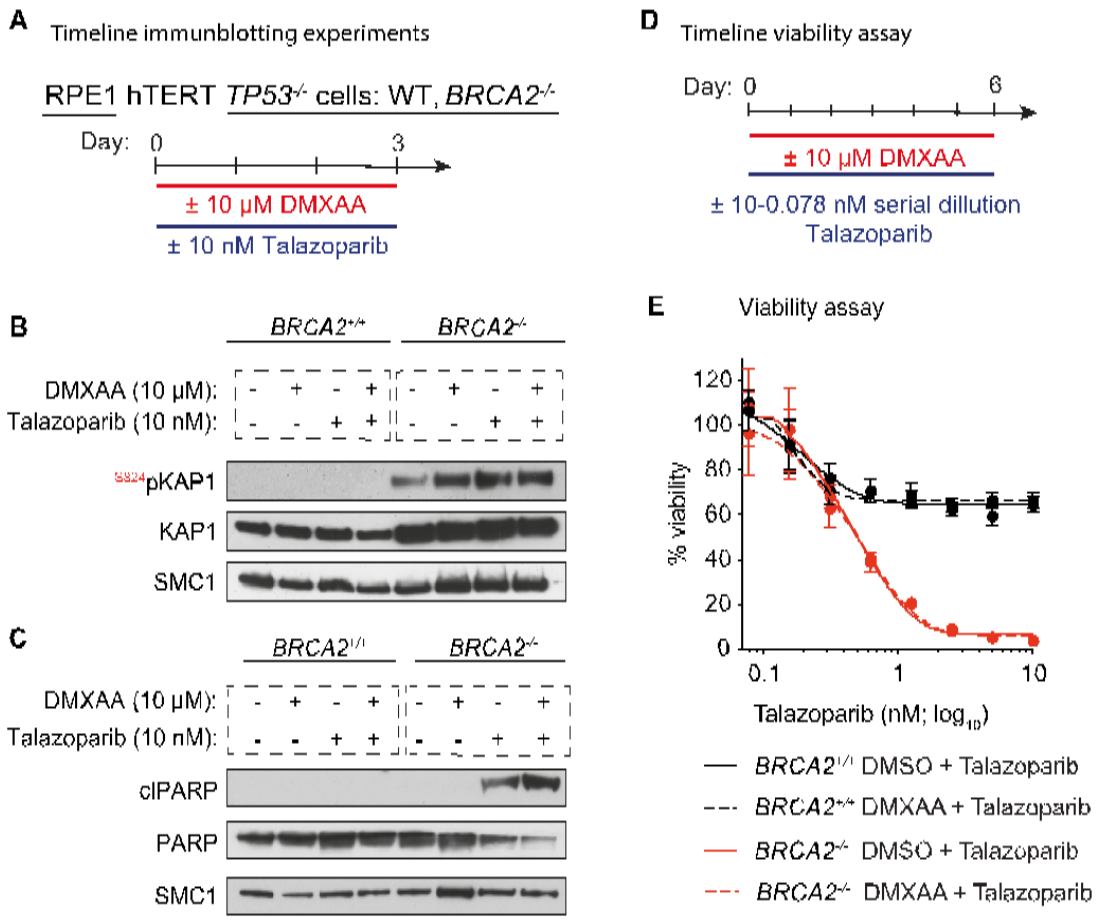


Figure 4.6: Inducing DNA Damage by co-treating BRCA2-deficient cells with DMXAA and Talazoparib.

(A) Experimental timeline for treatment of RPE1 hTERT *TP53*$^{-/-}$ *BRCA2*$^{+/+}$ and *BRCA2*$^{-/-}$ cells treated or not with 10 nM Talazoparib and 10 µM DMXAA for 3 days. After 3 days of treatment the cells were collected and processed for immunoblotting. (B, C) RPE1 hTERT *TP53*$^{-/-}$ *BRCA2*$^{+/+}$ and *BRCA2*$^{-/-}$ cells treated as in (A) were immunoblotted. The site of phosphorylation is indicated in red. SMC1 was used as a loading control. (D) Experimental timeline for treatment of RPE1 hTERT *TP53*$^{-/-}$ *BRCA2*$^{+/+}$ and *BRCA2*$^{-/-}$ treated or not with serial dilution of Talazoparib and 10 µM DMXAA for 6 days and subsequently processed for viability assays. (E) Viability assay of RPE1 hTERT *TP53*$^{-/-}$ *BRCA2*$^{+/+}$ and *BRCA2*$^{-/-}$ cells treated as in (D). Data are representative of mean of *n* = 3 independent experiments. Error bars represent SEM.

5. Discussion

DMXAA, the best characterised STING agonist in the literature, was originally licenced for use in 2001 as a tumour vascular disrupting agent (VDA) as it has been shown to act as a VDA targeting angiogenesis and leading to tumour necrosis (Wallace, LaRosa et al. 2007). Subsequently, DMXAA has been shown to elicit STING-dependent innate immune responses and has been further described to activate the TBK1-IRF-3 signalling axis (Roberts, Goutagny et al. 2007, Conlon, Burdette et al. 2013, Mukai, Konno et al. 2016). In murine models, cGAS-STING pathway activation has been shown to elicit type I IFN responses (Perera, Barber et al. 1994).

We set out to investigate the efficiency of DMXAA alone and in combination with Talazoparib in eliciting antitumour immune responses in BRCA2-deficient cells. We hypothesised that pharmacological activation of the cGAS-STING signalling pathway will further augment antitumour immunity in BRCA2-deficient cells, since DMXAA in other cancer types has been shown to promote antitumour immunity (Zheng, Mo et al. 2020). We observed modest activation of cGAS-STING-dependent innate immune responses and no DNA damage induction upon DMXAA treatment alone in one model of BRCA2 inactivation and no activation of the cGAS-STING pathway in the other model. Suggesting that as a single agent DMXAA has cell specific efficacies in activating the cGAS-STING signalling pathway. Previously, DMXAA has been shown to lead to immune-mediated solid tumour clearance (Corrales, Glickman et al. 2015). In mouse models, DMXAA treatment has been shown to induce cell death and tumour regression (Weiss, Guérin et al. 2017). Given the published literature it was expected to observe that DMXAA alone did not induce DNA damage in BRCA2-deficient cells.

This may be because DMXAA is potent activator of murine but not human STING (Conlon, Burdette et al. 2013).

Combining STING agonists with PARPi was shown to elicit innate immune responses and induce DNA damage (Pantelidou, Jadhav et al. 2022). Therefore, we evaluated the effects of DMXAA in BRCA2-deficient cells in combination with the PARPi Talazoparib. We hypothesised that the combination would activate the cGAS-STING pathway and lead to DNA damage in BRCA2-deficient cells. We found that DMXAA alone and in combination with Talazoparib did indeed induce innate immune activation. As expected, Talazoparib alone and in combination was able to elicit innate immune responses and DNA damage. In particular, the combination treatment resulted in increase in apoptosis in BRCA2-deficient cells compared to Talazoparib treatment alone. However, in contrast to Wang et al where synergism was observed between DMXAA and PARPi, we did not observe a synergistic effect on the viability of different cell lines lacking BRCA2 (Wang, Bergholz et al. 2022). This finding suggests that DMXAA induced STING pathway activation does not increase sensitivity of BRCA2-deficient cells to PARPi. However, to conclude this is the case, these experiments may need to be optimised by altering the drug concentrations or employing a different cell line. Additionally, a different experimental approach may be employed such as clonogenics assay which might provide a higher level of sensitivity.

STING agonists have been shown to activate cGAS-STING mediated signalling and IFN induction but the direct effect on *STING* expression is not frequently monitored (Johnson, Uchimura et al. 2021). We observed a decrease in *STING* expression upon treatment with DMXAA and Talazoparib in H1299 cells but the downstream target of STING signalling activation, phosphorylated IRF3, was detected. STING is regulated by a negative feedback loop which likely accounts for

these observations. This indicates that monitoring STING pathway activation cannot be done by examining *STING* expression alone and must be combined with monitoring activation of downstream targets such as phosphorylation of IRF3 or the expression of ISGs.

A downstream target of STING is IRF3, which gets phosphorylated upon activation of the pathway and exerts its effects on downstream targets. Importantly, IRF3 has been described as a cofactor essential for induction of ISGs (DeFilippis, Robinson et al. 2006). Interestingly, in H1299 cells, *ISG15* was upregulated by DMXAA treatment alone in BRCA2-deficient cells, but no increase in IRF3 phosphorylation was observed upon DMXAA treatment in BRCA2-deficient H1299 cells. This may be because STING activation can lead to *ISG15* upregulation via the NF-kB signalling axis of the cGAS-STING pathway instead of TBK1-IRF3 axis of the pathway. Highlighting the importance of monitoring ISG expression to confirm STING pathway activation by STING agonists.

Enhanced STING-dependent immune responses, induced by combining PARPi and STING agonist have been examined in several other studies. Compared to monotherapies, combining a STING agonist (ADU-S100) with the PARPi Olaparib in BRCA1-deficient human and mouse cell lines potentiated STING pathway activation and cytokine production (Pantelidou, Jadhav et al. 2021). In mouse models, the combination led to increased CD8+ T cell recruitment, dendritic cell activation, accelerated antigen presentation and therefore improved immune responses. Importantly, compared to monotherapies, the combination therapy led to improved tumour clearance and prolonged survival. Therefore, this study showed that STING agonism led to improved therapeutic efficacy of PARPi, in contrast to our observations

in human BRCA2-deficient cells. This may be because in this study ADU-S100 was employed as a STING agonist while we employed DMXAA.

One limitation of the use of DMXXA is the reduced ability to activate human STING signalling compared to mouse STING. Human STING was shown to fail to bind and respond to DMXAA, and the effects of DMXAA were restricted to mouse STING (Conlon, Burdette et al. 2013). Moreover, this STING agonist did not improve patient outcome in phase III clinical trials (Daei Farshchi Adli, Jahanban-Esfahlan et al. 2018). On the other hand, ADU-S100, has been shown to activate human STING. ADU-S100 has been shown to promote anti-tumour immune responses by eliciting type I IFN response and activating effector CD8+ T cells (Foote, Kok et al. 2017, Zaidi, Kelly et al. 2021). Therefore, the synthetically produced STING agonist ADU-S100 which has greater efficacy at activating human STING should be employed for future experiments and to validate the results of this study.

Overall, we tested the STING agonist DMXAA for its ability to induce innate immune activation alone or in combination with Talazoparib. In response to DMXAA, we observed variability in STING pathway activation and the expression of downstream targets in the two cell lines tested. Moreover, we did not observe a synergistic effect on cell viability when combining DMXAA treatment with Talazoparib in both cell models. To validate these results future experiments may use different drug concentrations or a different cell line or utilise more sensitive assays such as the clonogenic survival assay. It will also be important to examine the effect of recently developed synthetic cyclic STING agonists such as ADU-S100. According to our results, the STING agonist DMXAA did not have notable effect on the DNA damage accumulation and cell viability of human BRCA2-deficient cells. Therefore, preliminary as these results are, they suggest the effects of DMXAA may be cell specific, therefore

they don’t have the same efficacy in BRCA2-deficient cells as observed in other cancers such melanoma.

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