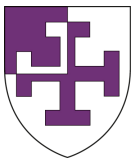
A thesis submitted to the University of Oxford
for the degree of Doctor of Philosophy



ST CROSS COLLEGE
UNIVERSITY OF OXFORD




# BRCA2 loss induces an innate immune response via activation of the cGAS-STING pathway

by

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# BRCA2 loss induces an innate immune response via activation of the cGAS-STING pathway

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Heterozygous germline mutations in *BRCA2* predispose to cancer. Such BRCA2-deficient tumours are sensitive to PARP inhibitors. Yet, tumours acquire resistance and, eventually, relapse. The tumour suppressor BRCA2 plays key roles in genome maintenance. Chromosome instability upon loss of BRCA2 induces senescence and apoptosis. However, it can promote tumourigenesis, if p53 signalling is defective. Still, a p53-deficient tumour cell remains confronted with accumulating chromosome instability even though cell death is evaded.

Here, we investigated how p53-deficient tumour cells react to BRCA2 depletion on a transcriptional level. Expression of BRCA2 was inhibited for 4 or 28 days and differential gene expression was characterised. As an early response to loss of BRCA2 (4 days), genes involved in cell cycle, organelle fission, chromosome segregation, DNA replication and DNA repair were downregulated. Furthermore, cell cycle progression was perturbed which was associated with cell cycle arrest in G1. On the other hand, chronic loss of BRCA2 (28 days) resulted in upregulation of genes associated with an innate immune response. We confirmed that loss of BRCA2 instigated formation of micronuclei activating the cGAS-STING pathway which, in turn, cascaded into a type I IFN response. Moreover, treatment with PARP inhibitors further stimulated the activation of the type I IFN response in cells and tumours lacking BRCA2.

These findings demonstrate that genomic instability upon loss of BRCA2 inflicts a cell-intrinsic type I IFN response which can be further stimulated pharmacologically by treatment with PARP inhibitors.

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## Declaration of authorship

I confirm that this thesis I am submitting is wholly my own work except where otherwise indicated. No portion of this work has been presented previously for another qualification at this or any other university.

Part of this thesis was published in a peer-reviewed journal [1] and some figures are presented here in an adapted format.

## Number of words

This thesis comprises 32,417 words excluding figures.

## Nomenclature of genes, proteins and Latin terms

Human genes are printed in italic and uppercase (e.g. *BRCA2*).

Rodent genes are printed in italic with the first letter in uppercase (e.g. *Brca2*).

Proteins are printed in uppercase (e.g. BRCA2).

Latin terms are printed in italic (e.g. *Drosophila pseudoobscura*, *in vivo*).

## Format of references

References printed before the punctuation refer to the previous sentence.

References printed after the punctuation refer to the previous sentences or paragraph.

References printed within a sentence refer to the previous term.

# List of abbreviations

| | |
|---|---|
| 53BP1 | p53 binding protein 1 |
| aEJ | alternative end joining |
| ATM | ataxia telangiectasia mutated |
| ATR | ATM- and RAD3-related |
| ATRIP | ATR-interacting protein |
| BARD1 | BRCA1-associated RING domain protein 1 |
| BLM | Bloom syndrome protein |
| BRCA | breast cancer gene |
| BSA | bovine serum albumin |
| CDC | cell division cycle |
| CDK1 | cyclin-dependent kinase 1 |
| cDNA | complementary DNA |
| cGAMP | cyclic 2'3'-GMP-AMP |
| cGAS | cGAMP synthase |
| CHD4 | chromodomain-helicase-DNA-binding protein 4 |
| cNHEJ | classical non-homologous end joining |
| CtIP | C-terminal binding protein 1-interacting protein |
| CTLA-4 | cytotoxic T-lymphocyte-associated antigen 4 |
| Ctr | control siRNA |
| D-loop | displacement loop |
| DAPI | 4′,6-diamidino-2-phenylindole |
| DDR | DNA damage response |
| DE | differential gene expression |
| DEPC | diethyl pyrocarbonate |
| DMXAA | 5,6-dimethyllxanthenone-4-acetic acid |
| DNA | deoxyribonucleic acid |
| DNA-PKcs | DNA-dependent protein kinase catalytic subunit |
| DOX | doxycycline |
| DSB | double-strand break |
| DSS1 | deleted in split hand/split foot syndrome 1 |
| EdU | 5-ethynyl-2'-deoxyuridine |
| ERK | extracellular signal-regulated kinase |

# List of abbreviations (continued)

| | |
|---|---|
| EXO1 | exonuclease 1 |
| EZH2 | enhancer of zeste homologue 2 |
| FDR | false discovery rate |
| GO | gene ontology |
| GSEA | Gene Set Enrichment Analysis |
| HISAT | hierarchical indexing for spliced alignment of transcripts |
| HR | homologous recombination |
| IFN | interferon |
| IFNAR | IFNα and β receptor subunit |
| IRF | IFN regulatory factor |
| ISG | IFN-stimulated gene |
| ISGF3 | ISG factor 3 |
| JAK | janus kinase |
| KAP1 | Krüppel-associated box zinc finger protein-associated protein 1 |
| MAPK | mitogen-activated protein kinase |
| MCM | minichromosome maintenance |
| MDA5 | melanoma differentiation-associated protein 5 |
| MDC1 | mediator of DNA damage checkpoint protein 1 |
| MiDAS | mitotic DNA synthesis |
| MMEJ | microhomology-mediated end joining |
| MRE11 | meiotic recombination 11 |
| mRNA | messenger ribonucleic acid |
| $NAD^+$ | nicotinamide adenine dinucleotide |
| NBS1 | Nijmegen breakage syndrome 1 |
| PALB2 | partner and localizer of BRCA2 |
| PAR | poly [ADP-ribose] |
| PARG | PAR glycohydrolase |
| PARP | PAR polymerase |
| PBS | phosphate-buffered saline |
| PBST | 0.05% Tween 20 in PBS |
| PCNA | proliferating cell nuclear antigen |
| PD-1 | programmed cell death 1 |

# List of abbreviations (continued)

| | |
|---|---|
| PD-L1 | programmed death-ligand 1 |
| PDS5 | precocious dissociation of sisters 5 |
| PTIP | Pax transactivation-domain interacting protein |
| qPCR | quantitative polymerase chain reaction |
| RIG-I | retinoic acid-inducible gene I |
| RNA | ribonucleic acid |
| RNA-seq | RNA-sequencing |
| RNF8 | RING-finger-containing nuclear factor 8 |
| RPA | replication protein A |
| RRM2 | ribonucleotide reductase subunit M2 |
| RT | room temperature |
| s.d. | standard deviation |
| SDS-PAGE | sodium dodecyl sulfate-polyacrylamide gel electrophoresis |
| SDSA | synthesis-dependent strand annealing |
| shRNA | short hairpin RNA |
| siRNA | small interfering RNA |
| SMC | structural maintenance of chromosomes |
| SNM1A | sensitive to nitrogen mustard 1A |
| SSA | single-strand annealing |
| SSB | single-strand break |
| ssDNA | single-stranded DNA |
| STAT | signal transducer and activator of transcription |
| STING | stimulator of IFN genes |
| TBK1 | TANK-binding kinase 1 |
| TNF | tumour necrosis factor |
| TNFR | TNF receptor |
| TOPBP1 | DNA topoisomerase II-binding protein 1 |
| TRAF1 | TNFR-associated factor 1 |
| TYK2 | tyrosine kinase 2 |
| WB | western blotting |

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# 1 Introduction

## 1.1 Genome instability – a hallmark of cancer

The human body is made up of an estimated $10^{13}$ cells which collectively operate as an organism [2,3]. Each cell's structure, behaviour and function are governed by information encoded on its genome.

Inevitably, the genome is constantly subject to DNA damage. Sources of DNA damage are of endogenous (e.g. spontaneous base deamination or replication errors) or exogenous (e.g. radiation or genotoxic chemical agents) origin. In fact, the major threat to genome integrity is the physiological process of DNA replication and chromosome segregation. Before cell division, DNA must be accurately and completely copied and subsequently distributed equally into two daughter cells. [4]

Damaging agents and errors during DNA replication alter the DNA chemically. If such an alteration is passed on to the descendent generation before it can be repaired, it is manifested in the genome as a mutation. Mutations may result in a re-wiring of the cellular programme. Dysregulation of a cell's genome resulting in abnormal cell proliferation is the fundamental origin of cancer. Importantly, genome instability is a pervasive hallmark of cancer. It fuels onset and progression of tumourigenesis. Consequently, maintaining genome integrity is a crucial tumour suppressor mechanism. [5,6]

## 1.2 DDR – the DNA damage response

In order to maintain genome integrity, DNA damage must be repaired in a timely fashion and accurately. A plethora of DNA repair pathways have evolved. They

are orchestrated via an intricate surveillance network referred to as the DNA damage response (DDR). [7,8]

In principle, the DDR coordinates several cohering processes. As a first step, the DDR senses the presence and recognises the nature of a DNA damage. In order to avoid transmission of DNA alterations to subsequent generations, the DDR activates cell cycle checkpoints which transiently halt cell cycle progression. That allows time for the DDR to recruit germane repair proteins to the site of damage. In cases where repair fails and DDR signalling persists, the affected cell is removed from the pool of proliferating cells for the sake of the multicellular organism. This is accomplished via apoptosis, which is programmed cell death, or via senescence, which is permanent exit from the cell cycle. Thereby, the DDR functions as a barrier inhibiting tumorigenesis. [9,10]

## 1.3 Response to DNA double-strand breaks

Double-strand breaks (DSBs) represent the most detrimental of all DNA damage. If left unrepaired, they induce apoptosis or senescence. If repaired incorrectly, they can cause gross chromosomal rearrangements leading to genomic instability and, eventually, to tumorigenesis. [11]

In order to react accordingly, an elaborative network of pathways responds to DSBs. Three proteins, meiotic recombination 11 (MRE11), RAD50 and Nijmegen breakage syndrome 1 (NBS1) form the end recognition complex MRN which binds to DSB ends [12,13]. This complex recruits and activates the kinase ataxia telangiectasia mutated (ATM). [14-18] ATM, in turn, has hundreds of substrates [19,20].

An important target of ATM is the tumour suppressor p53 which plays a key role in maintaining genome integrity. Phosphorylation of serine residue 15 activates p53 which, in turn, halts cell cycle progression and promotes entrance into apoptosis or senescence. [21-25] The transcription factor p53 is commonly referred to as 'guardian of the genome' [26]. Its gene, *TP53*, is the most frequently mutated gene in cancer. A pan-cancer analysis across 12 different tumour types revealed that 42% of these cancers bear mutations in *TP53* [27]. In particular, a vast majority of serous ovarian (95%) and serous endometrial carcinomas (89%) as well as a third of breast adenocarcinomas presented mutations in *TP53*. Another target of ATM is the threonine residue 68 of effector checkpoint kinase CHK2 [28-32]. Upon phosphorylation, CHK2 stops cell cycle progression and promotes p53-mediated apoptosis [33-36]. CHK2 phosphorylates serine residue 20 on p53 which inhibits the interaction of p53 with the E3 ubiquitin ligase MDM2. This, in turn, inhibits proteasomal degradation of p53 and thereby promotes its stabilisation. [37-39]

Besides stopping cell cycle progression, ATM signalling initiates DSB repair. To this end, ATM phosphorylates the histone variant H2AX at serine residue 139 (the phosphorylated form is commonly referred to as $\gamma$H2AX) [40-42]. Next, mediator of DNA damage checkpoint protein 1 (MDC1) binds to $\gamma$H2AX and is likewise phosphorylated at threonine residue 4 by ATM [43-47]. Phosphorylated MDC1 forms a dimer [47] which recruits the E3 ubiquitin ligase RING-finger-containing nuclear factor 8 (RNF8). RNF8 ligates K63 poly-ubiquitin chains to histones adjacent to the DSB which, eventually, allows recruitment of DSB repair factors [48-50] such as BRCA1 and p53 binding protein 1 (53BP1) [51-53].

## 1.4 Repair of DNA double-strand breaks

Two main repair pathways have evolved to resolve DSBs: classical non-homologous end joining (cNHEJ) and homologous recombination (HR). The predominant DSB repair pathway is cNHEJ which functions throughout the cell cycle except during mitosis [54-58]. It is based on re-ligation of the broken DNA ends independent of any repair template. cNHEJ is relatively fast (minutes) compared to HR (hours) [59]. Although it has a reputation of being intrinsically error-prone and mutagenic, it has been argued that cNHEJ is mostly accurate [60]. During HR, a homologous sister chromatid is used as a repair template. Therefore, it is the most accurate DSB repair pathway, although it is restricted to S and G2 phases of the cell cycle. In principle, 3'-single-stranded DNA (ssDNA) overhangs are generated by a nucleolytic process called end resection. These 3'-overhangs invade the sister chromatid in order to obtain a template sequence. (Figure 1)

Which DSB repair pathway is chosen depends on several parameters such as the nature of the DSB damage itself and importantly, the current phase of the cell cycle. During G1 phase, when no template for HR is available, 53BP1 inhibits end resection and thereby promotes cNHEJ. During S and G2 phases, end resection, and thereby HR, is promoted. [55,61-75]

Where cNHEJ is promoted, DSB ends are bound rapidly by the non-catalytic heterodimer Ku (consisting of Ku70 and Ku80). The name for Ku derives from the first two letters of the name of a prototype patient whose serum contained anti-Ku autoantibodies [76]. Next, DNA-dependent protein kinase catalytic subunit (DNA-PKcs) is recruited. Together, DNA-PKcs and Ku form the DNA-PK holoenzyme which promotes DNA end-tethering [77]. Eventually, the ends are

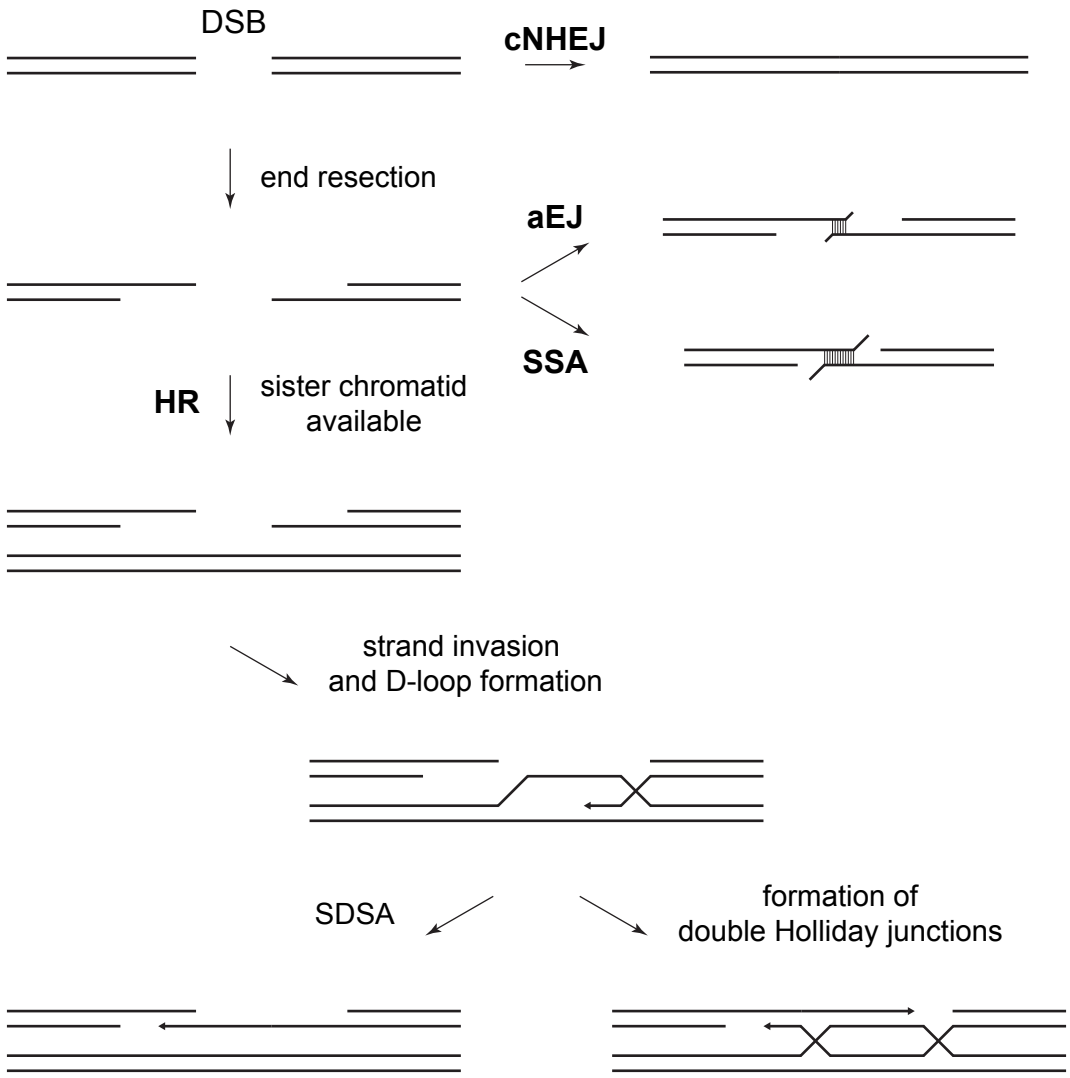



**Figure 1: Illustration of DSB repair pathways**

aligned, processed and ligated. For these processes, the proteins XRCC4 [78], Ligase IV [79,80], XLF [81] (also called Cernunnos [82]), Artemis [83], and PAXX [84] are recruited.

For HR to be promoted, DNA ends must be nucleolytically processed. To this end, the MRN complex interacts with C-terminal binding protein 1-interacting protein (CtIP) and BRCA1 which is constitutively in complex with BRCA1-associated RING domain protein 1 (BARD1) [85-93]. Next, CtIP promotes endo- and exonuclease activities of MRE11. MRE11, in turn, produces

a nick in the DNA and then promotes 3’ to 5’ single-stranded resection towards the DSB end. In a second resection step, the BLM-DNA2 complex or the exonuclease EXO1 promotes extensive 5’ to 3’ resection away from the DSB end [94-98]. End resection results in long 3’-ssDNA overhangs which are rapidly coated by replication protein A (RPA; a heterotrimer consisting of RPA70, RPA32 and RPA14). Coating of ssDNA with RPA protects ssDNA from being degraded and it inhibits formation of secondary DNA structures. [99,100] Next, BRCA1 recruits BRCA2 by interacting with a bridging protein called partner and localizer of BRCA2 (PALB2) [101-104]. The key recombination mediator BRCA2, in association with its constitutive interacting partner deleted in split hand/split foot syndrome 1 (DSS1), loads RAD51 onto the 3’-ssDNA overhangs displacing RPA [105-114].

The generated RAD51-ssDNA nucleofilament searches for a homologous DNA template in the sister chromatid and promotes annealing of the complementary strands facilitated by BRCA1-BARD1 [115-117]. Base pairing of invading and complementary strand displaces the non-complementary strand [118]. The resulted three-stranded structure is called displacement loop (D-loop) [119].

There are multiple mechanisms to resolve that D-loop (Figure 1). The predominant process in mitotic cells is called synthesis-dependent strand annealing (SDSA) [120-123]. This pathway prevents crossover formation preserving genomic integrity. Here, only one nucleofilament of the two DSB ends invade and anneal to the template sequence. The invaded strand is extended by DNA synthesis and then dissociates from the D-loop. Next, it anneals to the 3’-ssDNA overhang of the passive DSB end which has not invaded the template DNA. Eventually, repair is completed by DNA synthesis and ligation.

In an alternative pathway of D-loop resolution, the second DSB end, which has not invaded the template, anneals to the displaced ssDNA strand of the D-loop. The newly formed cruciate structures between invading and template DNA are referred to as double Holliday junctions [123,124]. Here, factors like the BLM-TOPIIIα-RMI1-RMI2 complex [125], the SMX tri-nuclease complex (comprising structure-specific endonuclease dimers SLX1-SLX4, MUS81-EME1, and XPF-ERCC1) [126,127], or GEN1 [128,129] are required to process these double Holliday junctions in order to complete repair. Eventually, these processes lead to either crossover or non-crossover products. The latter compromise genome integrity and are therefore avoided in mitotic cells but desirable in meiotic cells in order to promote genetic diversity [121,123].

Therefore, BRCA1 is involved in multiple steps during HR. Notably, BRCA1 plays a role in the initial end resection step of DSB repair and, additionally, further downstream via its interactions with PALB2 recruiting BRCA2 to load RAD51. Eventually, BRCA1 also interacts with RAD51-ssDNA nucleofilament promoting strand invasion. Both BRCA1 and BRCA2 are essential HR factors being part of the BRCA1-BARD1-PALB2-BRCA2-RAD51 recombinase complex. Loss of any of its components renders a cell HR-deficient.

For such cases when HR is not functional, cells are equipped with two back-up pathways: alternative end joining (aEJ; sometimes referred to as microhomology-mediated end joining or MMEJ) and single-strand annealing (SSA) [106,130-136]. Both pathways are mechanistically similar but require different proteins for their execution (Figure 1). They are based on annealing complementary sequences that might be exposed in the 3’-ssDNA overhangs

upon resection mediated by the MRN complex, CtIP and BRCA1-BARD1 [137-142].

During aEJ, short resection reveals microhomologies (6 to 17 nucleotides) [143] on the 3'-overhangs which are annealed with the help of POLθ [135,136,144]. In contrast, SSA is initiated by extensive resection which exposes long complementary repeat sequences (50 nucleotides or more) [143]. These are annealed with the help of RAD52 [133,134,145,146]. In both cases, the result is a DNA duplex with non-complementary 3′-ssDNA flaps. Eventually, these flaps are removed and the gaps are filled in order to complete repair. The intervening DNA sequence between the complementary nucleotides is lost resulting in deletion. Therefore, these two back-up pathways are intrinsically highly mutagenic. In fact, whole genome sequencing revealed that *BRCA1*- and *BRCA2*-mutated tumours are characterised by numerous large deletions of more than 3 base pairs. At the junctions of these deletions, microhomologies were found underlining the use of back-up pathways in HR-deficient tumour cells. [147] Similar results were described in BRCA-deficient chicken DT40 cell lines *in vitro* [148].

## 1.5 Replication stress

Before entry into mitosis, the entire genome in the cell must be accurately duplicated. This complex process, referred to as DNA replication, is highly regulated. DNA replication initiates from specific chromosome regions called origins of which thousands are spread throughout the genome. First, origins become 'licensed' during G1 phase and are then 'fired' during S phase, albeit not simultaneously. From each fired origin, two DNA replication forks progress

bi-directionally. Notably, not all licensed origins are fired. Instead, some remain ‘dormant’ and can be utilised as back-up when there are complications during replication. [149,150]

Errors during regular DNA replication are the major source of mutations not least because they are the major source of DSBs and therefore of genome instability [151]. When homeostasis of replication is perturbed, the replication fork slows or stalls. This phenomenon is commonly referred to as replication stress. It can be caused when not enough deoxynucleotide triphosphates [152,153] or histones [154,155] are available for DNA replication. Stalling of the replication fork at obstacles is another cause of replication stress. These obstacles are for example: secondary structures in the DNA; R-loops (three-stranded structures consisting of the RNA-DNA hybrid and the displaced ssDNA strand arising during transcription); DNA-protein adducts. Transient replication stress is usually tolerated and replication restarts once the obstacle has been removed. However, if replication stress is not relieved, the replication fork collapses meaning dissociation of the replication machinery and, eventually, formation of DSBs (Figure 2). [156,157]

Upon replication stress, the DNA polymerase is uncoupled from the hexameric minichromosome maintenance (MCM) helicase complex (comprising MCM2-MCM7) [158,159]. In this scenario, the helicase proceeds unwinding the DNA ahead of the replication fork. That leads to exposure of long stretches of ssDNA between the DNA polymerase and the MCM complex. Extended ssDNA is rapidly bound by RPA. RPA bound ssDNA is recognised by ATR-interacting protein (ATRIP) which is in complex with the kinase ATM- and RAD3-related (ATR) [160-162]. Likewise, the clamp loader RAD17 is recruited to RPA bound

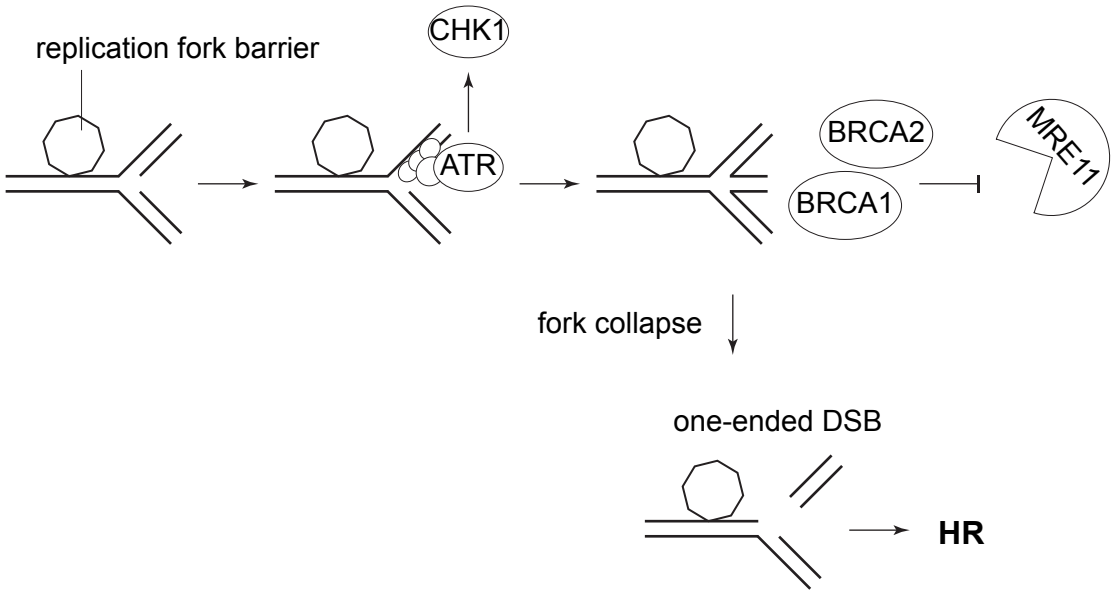



**Figure 2: Illustration of responses to replication stress**

ssDNA which loads the heterotrimeric checkpoint clamp complex 9-1-1 (consisting of RAD9, RAD1, HUS1) [163-165]. Interaction between this clamp complex and DNA topoisomerase II-binding protein 1 (TOPBP1) leads to activation of ATR [166-169].

Upon activation, ATR promotes multiple responses. Progression of the cell cycle is transiently stopped and firing of new origins is suppressed [170-172]. Furthermore, ATR promotes stabilisation and restart of arrested forks [173-177]. If a replication fork obstacle persists, ATR initiates firing of dormant origins [178,179]. The best-characterised targets of ATR are serine residues 317 and 345 of the effector checkpoint kinase CHK1 (Figure 2). Activation of CHK1 promotes cell cycle arrest. [180-182]

Replication stress is also addressed by factors of HR. Visualisation via electron microscopy indicated that stalled forks remodel into a reversed fork [183,184]. In such a scenario, both nascent daughter DNA strands anneal with each other resulting in a cruciate four-way joint structure (Figure 2). Here, RAD51 promotes

fork-reversal independently of BRCA2 [185]. In a next step, BRCA1, BRCA2 and RAD51 were shown to protect the reversed replication forks from nucleolytic degradation by MRE11 [186-191]. To this end, RAD51 protects the regressed arm, which resembles a one-ended DSB, from MRE11 in a BRCA1/2-dependent yet HR-independent manner [192-195]. In this two-step model, the roles of RAD51 in fork reversal and protection are functionally discrete. Eventually, also restart of the stalled replication fork is RAD51-dependent [196].

Additionally, BRCA1 and BRCA2, independently of RAD51, prevent accumulation of R-loops and thereby promote replication fork progression [197,198].

Consistent with these functions, cells deficient for RAD51 or BRCA2 are characterised by slow fork progression which, eventually, leads to problems during mitosis [199,200]. Therefore, BRCA1, BRCA2 and RAD51 are crucial for DSB repair via HR and for smooth DNA replication.

## 1.6 BRCA-associated cancer

Heterozygous germline mutations in either of the two breast cancer genes *BRCA1* [201-203] or *BRCA2* [204-206] are associated with increased risk for breast and ovarian cancer, as well as other cancers such as pancreatic and prostate cancer [207-214]. At some point during the lifetime of a mutation carrier, the wild-type allele is lost in a single cell from which a tumour arises [215,216]. While the tumour is homozygous and therefore BRCA-deficient, healthy tissue is heterozygous and therefore BRCA-proficient. Hence, BRCA1 and BRCA2 are classic tumour suppressors and hereditary mutations in their genes are considered autosomal dominant.

Loss of function mutations in *TP53* and *RB1* as well as *MYC* amplifications are frequently found in *BRCA*-mutated cancers [217]. In BRCA1-deficient cancers, predominantly protein-truncating mutations were described in *TP53* [218,219].

Estimating penetrance is critical for clinical management of mutation carriers. However, risk estimates vary between studies due to methodological differences but also due to complex genetic and environmental factors [220]. In the end, such variations are expected to distribute equally, as long as sample sizes are large enough, making risk estimates more comparable. For example, in one meta-analysis, the results of 22 studies were pooled, independent of family history [221]. In the case of *BRCA1* mutation carriers, the average cumulative risk for developing breast cancer was estimated to be 65% and for ovarian cancer 39%. For *BRCA2* mutation carriers, the estimated risks were 45% and 11%, respectively. A different study selected 1,008 female *BRCA* mutation carriers, also independent of family history [222]. Here, they reported an average cumulative risk of 69% *(BRCA1* mutation) and 74% *(BRCA2* mutation) for developing breast cancer by the age of 70. The respective risks for developing ovarian cancer were 46% for *BRCA1* and 12% for *BRCA2* mutation carriers. A third meta-study analysed data of 10 publications with non-overlapping patient data [223]. Here, the cumulative risks of developing breast cancer by the age of 70 were reported as 55% for *BRCA1* and 47% for *BRCA2* mutation carriers. For ovarian cancer, the risks were 39% and 17%, respectively.

In contrast to high penetrance, prevalence is relatively low in the general population. Around 1-4% of breast and 13-15% of ovarian cancers are associated with germline mutations in either *BRCA1* or *BRCA2* [224-227]. Inherited BRCA2 deficiency was observed in 6% of pancreatic cancer cases [228].

Importantly, in addition to germline *BRCA* mutations, loss of function of BRCA1 or BRCA2 is also present in numerous sporadic cancers [229,230]. Both genetic mutations as well as promoter hypermethylation were found to suppress *BRCA1* gene expression in sporadic cancers [231-242]. In contrast, *BRCA2* was not found to be epigenetically silenced by promoter hypermethylation [237,243,244].

Therefore, a therapy specifically targeting *BRCA*-mutant tumours is not limited to familial cases only but could be also applied against BRCA-deficient sporadic cancers. This significantly expands the number of patients amenable to such targeted therapy.

## 1.7 Targeting BRCA-defective tumours with PARP inhibitors

The term synthetic lethality was coined more than 70 years ago based on genetic observations in *Drosophila pseudoobscura* [245]. Two genes are understood to be synthetically lethal when the functional loss of either gene alone is tolerated by the cell, but the combined (synthetic) loss of both genes together is lethal (Figure 3). It is mostly used to describe the relationship between two genes but could be, in principle, extended to three or even more genes.

The concept of synthetic lethality can be exploited for developing targeted cancer therapeutics [246,247]. For example, genotyping a tumour might reveal a defect in a specific tumour suppressor. Such cancers could then be selectively targeted by inhibiting a gene (or, more practically, its encoded protein) which is synthetically lethal with that missing tumour suppressor. Thereby, healthy tissue will be spared from harm because it expresses a functional copy of that tumour suppressor. The most prominent example of translating synthetic lethality into the clinic is the development of poly [ADP-ribose] (PAR) polymerase (PARP)

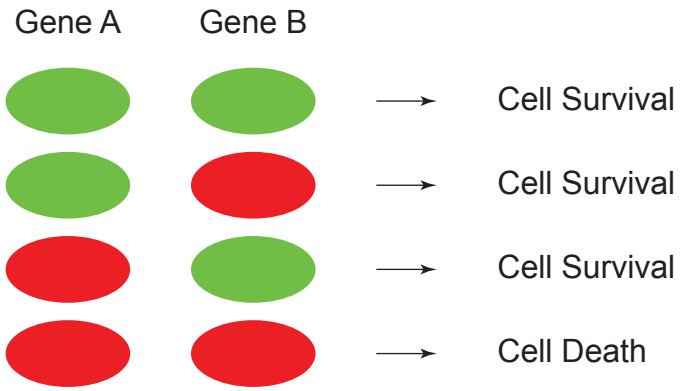



**Figure 3: Illustration of the principle of synthetic lethality**
Green, functional gene; red, non-functional gene.

inhibitors for treatment of *BRCA1/2*-mutated cancers. Initially, two back to back landmark studies reported synthetic lethality of PARP inhibition in BRCA-deficient cells [248,249].

The founding member of the PARP protein family, PARP1, was first purified in the 1970s [250-252] and cloned a decade later [253-255]. Using nicotinamide adenine dinucleotide ($NAD^+$) as a co-factor, PARP catalyses the formation of complex homopolymers of ADP-ribose directly on target proteins. The result are branched chains referred to as PAR which have been identified in cells even before PARP was characterised [256-261]. This post-translational modification on target proteins is referred to as PARylation. The majority of PAR is synthesised by two members of the PARP family, PARP1 (90%-95%) and PARP2 (5%-10%) [262,263].

Activated upon binding to DNA lesions, PARP is an important DDR component. For example, it plays a role in responding to replication stress [184,264-266] and is involved in single-strand break (SSB) repair [267-271]. Branched PAR serves as a scaffold regulating recruitment of DDR proteins [272-274]. Charge repulsion of PARylated histones directly promotes physical relaxation of chromatin

structures. Additionally, PAR promotes recruitment of chromatin remodelers. Resulting chromatin relaxation, in turn, further facilitates access of repair proteins to the lesion site. [275-282] The importance of PARylation is illustrated by the fact that mice deficient for either PARP1 or PARP2 tend to develop spontaneous tumours and are sensitive to radiation and alkylating agents [283-285]. Moreover, simultaneous abrogation of PARP1 and PARP2 is embryonically lethal [285].

The molecular mode of action behind the synthetic lethality between PARP inhibition and BRCA deficiency has not been fully elucidated. Multiple models have been suggested which are not mutually exclusive (Figure 4). Initially, it was proposed that PARP inhibition induced an increase in SSBs which are then converted into one-ended DSBs during DNA replication [248,249,286]. Accumulation of replication-associated one-ended DSBs is tolerated only by HR-proficient cells and therefore lethal in BRCA-deficient cells [287]. It was further proposed that PARP inhibition in HR-deficient cells resulted in deregulation of cNHEJ [288]. That, in the end, induced intolerable levels of genomic instability and, eventually, led to cytotoxicity. However, the most adopted model describes chemical inhibition trapping PARP on the DNA which then acts as a replication fork barrier. Resulting replication stress, in turn, leads to fork collapses and accumulation of DSBs. This model is supported by the observation that the trapping capability of PARP inhibitors correlates with their efficacy. [271,289-293]

Since PARP is involved in numerous cellular processes, the bigger picture of the synthetic lethality is more complex and multifactorial. For example, the helicase RECQ1 promotes restart of reversed replication forks but is inhibited by PARylation. Therefore, inhibition of PARP is associated with premature restart of

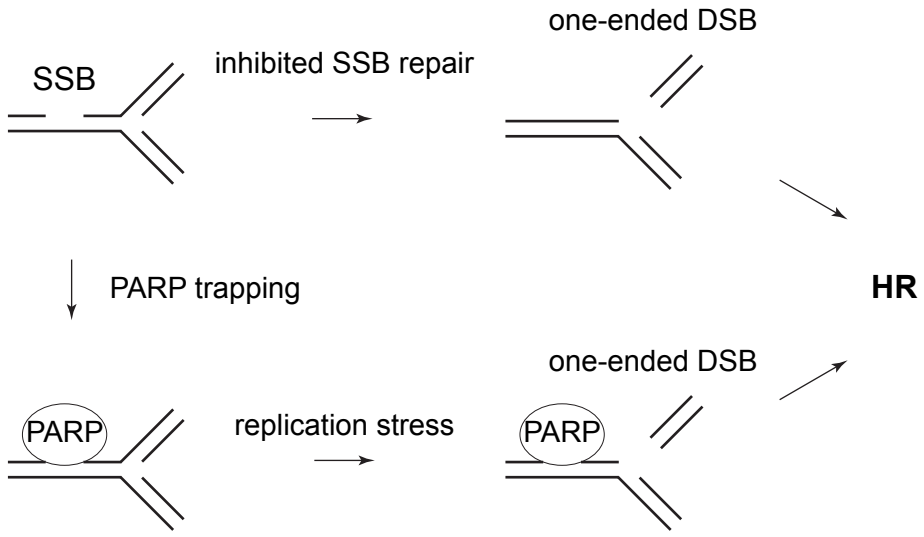



**Figure 4: Illustration of modes of action of PARP inhibitors**

stalled replication forks and therefore accumulation of DNA damage. [294,295] Besides, PARP promotes ligation of Okazaki fragments and, consequently, its inhibition hinders DNA replication [266]. Additionally, PARP recruits POLθ during aEJ. Therefore, treatment with PARP inhibitors impedes a key back-up pathway for DSB repair in HR-deficient cells. [135,136] Furthermore, ribonucleotides are frequently embedded into the genome and removed by ribonucleotide excision repair. If this repair failed, topoisomerase I cleaves those ribonucleotides instead promoting the generation of PARP-trapping DNA lesions. [296]

After clinical studies reported therapeutic efficacy, PARP inhibitor olaparib was approved for patients with germline *BRCA1*- or *BRCA2*-mutant breast [297] and ovarian cancer [298-300]. It was the first approved drug based on the principle of synthetic lethality and the first approved DDR inhibitor. Recently, three other PARP inhibitors were clinically approved for cancers associated with *BRCA* mutations. After clinical investigation, rucaparib [301,302] and niraparib [303] were approved for ovarian cancer. Talazoparib received approval for breast cancer after demonstrating greater efficacy than chemotherapy [304].

Despite these promising reports, PARP inhibitor therapy is, eventually, confronted with tumour relapse. Genomic plasticity allows tumours to evolve mechanisms of resistance to PARP inhibitor treatment. Multiple mechanisms have been identified of which some have been observed clinically and some, so far, only in mouse or cell culture models.

One mechanism is based on restoration of functional BRCA protein. *BRCA2*-mutant tumour cells exposed to PARP inhibitors were shown to gain secondary mutations which restored wild-type *BRCA2* gene expression or promoted expression of a novel yet functional form of BRCA2 [305-309]. A recent study analysed circulating cell-free DNA acquired via liquid biopsies of prostate cancer patients who developed resistance to talazoparib and olaparib [310]. They observed high levels of heterogeneity amongst *BRCA2* reversion mutations within the same patient. One single patient presented 34 different secondary mutations restoring BRCA2.

Using patient-derived xenograft models of *BRCA1*-mutated and *BRCA1* promoter-hypermethylated breast cancers showed that BRCA1 expression could be restored under selection pressure of PARP inhibitor treatment [311]. In *BRCA1*-mutated tumours, secondary mutations led to restoration of BRCA1 expression. In BRCA1 promoter-hypermethylated tumours, demethylation of the promoter was one mechanism of restoration. However, where *BRCA1* promoter was not demethylated, restoration was alternatively achieved via *de novo* gene fusion. Here, *BRCA1* was positioned under transcriptional control of a heterologous promoter.

Another mechanism of resistance is based on restoring functional HR. In BRCA1-deficient cells, HR can be restored via loss of HR antagonist

53BP1 [63,312-315] or one of its downstream factors such as RIF1 [67-69], the Shieldin complex (composed of SHLD1, SHLD2, SHLD3 and REV7) [72,73,316-320] or the CST complex [321,322]. Similarly, overexpression of TRIP13, a negative regulator of REV7, restores HR [323]. Besides, loss of HELB, a 53BP1-independent resection antagonist, restores HR and thereby confers resistance [324]. In contrast, HR deficiency due to defects in PALB2 or BRCA2 is not rescued by loss of 53BP1 [325].

Furthermore, restoration of replication fork protection confers resistance to PARP inhibitors in BRCA-deficient cells. Loss of Pax transactivation-domain interacting protein (PTIP) [326] or nucleosome remodelling factor chromodomain-helicase-DNA-binding protein 4 (CHD4) protects stalled replication forks from degradation by MRE11 in BRCA-deficient cells [327,328]. In BRCA2- but not in BRCA1-deficient cells, loss of methyltransferase enhancer of zeste homologue 2 (EZH2) protects stalled forks from nucleolytic degradation by another nuclease called MUS81 [329].

Upregulation of efflux pumps that eliminate a drug from the cell is a common mechanism for drug resistance [330]. It was also observed in a *Brca1*$^{-/-}$; *Trp53*$^{-/-}$ mouse model upon treatment with olaparib [331].

The enzyme PAR glycohydrolase (PARG) degrades PAR [332,333]. This antagonist of PARP was shown to be mutated in PARP inhibitor resistant cells. Loss of PARG partially rescued PARylation which had been initially inhibited by the drug. Murine mammary tumours and human cancer cells, which did not express PARG nor BRCA2, were resistant to PARP inhibitors. [334]

Despite initial success of treating BRCA-defective tumours with PARP inhibitors, the variety of acquired resistance mechanisms remains challenging for clinicians

as well as for scientists. Consequently, further research is required in order to rationally design more effective mechanism-based therapeutic approaches against BRCA-deficient tumours.

# 2 Aim

The tumour suppressor BRCA2 plays key roles in genome maintenance promoting both DSB repair and DNA replication. Loss of BRCA2 has therefore substantial impact on chromosome integrity. Resulting accumulation of DNA damage activates p53 leading to apoptosis or senescence. In fact, loss of BRCA2 is embryonically lethal [335-338]. Defects in BRCA2 are tolerated by a cell if p53 signalling is not functional. In the course of tumorigenesis, it is believed that loss of BRCA2 chronologically follows loss of p53 signalling. In fact, clinically presented BRCA2-deficient tumours are characterised by defects in p53 signalling. [335,336,338-344]

Even though induction of cell death or senescence is circumvented, a p53-deficient (tumour) cell is confronted with accumulating genome instability upon loss of BRCA2 [340,345-347]. The aim of this study was to investigate how p53-deficient tumour cells react to the loss of BRCA2 on a transcriptional level. To this end, the transcriptome of BRCA2 deficient cells was characterised via whole transcriptome RNA-sequencing (RNA-seq). Expression of BRCA2 was inhibited and samples were collected at two time points: after 4 days and 28 days of BRCA2 depletion. Genes deregulated upon short-term and long-term loss of BRCA2 were identified.

# 3 Results and discussions

## 3.1 Characterising the transcriptome of BRCA2-deficient cells

In order to characterise transcriptional changes of human cells upon loss of BRCA2, we decided not to use terminally adapted *BRCA2*-deleted cells as a primary model. In contrast, we used human non-small cell lung carcinoma H1299 [348] cells and invasive ductal breast cancer MDA-MB-231 [349] cells carrying a doxycycline (DOX)-inducible short hairpin RNA (shRNA) cassette (H1299+BRCA2sh$^{DOX}$ and MDA-MB-231+BRCA2sh$^{DOX}$, respectively). Upon addition of DOX to the growth medium of these cells, a shRNA is transcribed targeting mRNA encoding BRCA2 [350]. We cultured these cells in the presence (+DOX) or absence (-DOX) of DOX for 28 days and collected samples for RNA-seq analysis at two time points, day 4 and day 28 (Figure 5a). By western blotting (WB), we confirmed that expression of BRCA2 was inhibited in cells treated with DOX at both time points (Figure 5b).

In parallel, we conducted a control experiment in order to investigate whether DOX itself might affect the transcriptome in these cell lines. To this end, we used parental H1299 and MDA-MB-231 cells, which do not carry a DOX-inducible shRNA cassette, and treated them with DOX. We performed hierarchical cluster analysis of sample-to-sample distances based on the RNA-seq data and we found that there was neither a clear clustering of +DOX samples nor of -DOX samples which indicated insignificant alterations between these samples (Supplementary Figure 1).

Moreover, in H1299, differential gene expression analyses (false discovery rate (FDR) < 0.05) showed only 2 genes differentially expressed at day 4 and none at

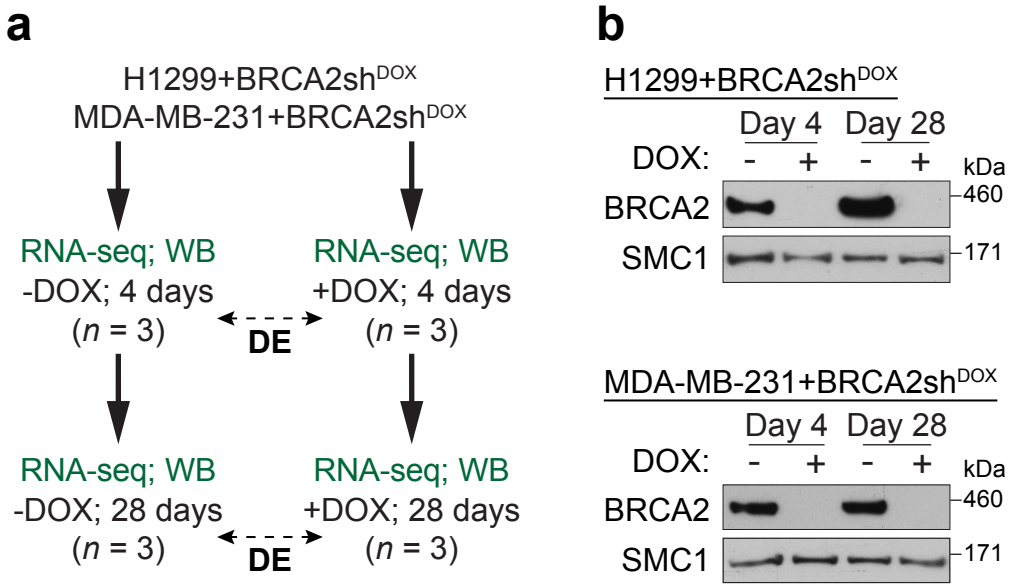



**Figure 5: Transcriptional characterisation of BRCA2-deficient cells**
(a) Illustration of experimental design: H1299+BRCA2sh$^{DOX}$ and MDA-MB-231+BRCA2sh$^{DOX}$ were cultured in presence (+DOX) or absence (-DOX) of 2 µg/ml DOX for 28 days. After 4 and 28 days, samples for RNA-seq and WB were collected. DE, differential gene expression. (b) Whole-cell extracts were immunoblotted as indicated. SMC1 was used as a loading control. *n* = 3 independent experiments.

day 28 (Supplementary Figure 2a). In MDA-MB-231, 3 genes were differentially expressed at day 4 and 7 genes at day 28 (Supplementary Figure 2b). We concluded that DOX itself had a negligible impact on the transcriptome of parental H1299 and MDA-MB-231 cells.

On the contrary, in both H1299+BRCA2sh$^{DOX}$ and MDA-MB-231+BRCA2sh$^{DOX}$, hierarchical clustering of sample-to-sample Euclidean distances revealed a clustering of +DOX samples and of -DOX samples which indicated alterations between BRCA2-deficient and -proficient samples (Supplementary Figure 3).

In H1299+BRCA2sh$^{DOX}$, addition of DOX and thereby abrogation of BRCA2 resulted in significant alteration (FDR < 0.05) in the expression of 1,363 genes at day 4 and of 479 genes at day 28 (Supplementary Figure 4a). In

MDA-MB-231+BRCA2sh$^{DOX}$, 187 genes were significantly (FDR < 0.05) deregulated at day 4 and 480 genes at day 28 (Supplementary Figure 4b).

In summary, these data indicate that addition of DOX to H1299+BRCA2sh$^{DOX}$ and MDA-MB-231+BRCA2sh$^{DOX}$ effectively inhibits expression of BRCA2 over 28 days. Furthermore, DOX treatment by itself does not affect the transcriptome of H1299 and MDA-MB-231 cells which do not carry a DOX-inducible shRNA cassette. However, addition of DOX to H1299+BRCA2sh$^{DOX}$ and MDA-MB-231+BRCA2sh$^{DOX}$ and therefore inhibition of BRCA2 expression substantially alters their transcriptome.

Previous studies pursued similar approaches to assess differential gene expression upon loss of BRCA2 [351-353]. An important difference was that these authors used microarray analysis, while we used RNA-seq. Microarray analysis is limited to a distinct number of genes defined by the probes on the chip. In contrast, RNA-seq allowed us to conduct a transcriptome-wide analysis. In one study [351], BRCA2 was depleted using siRNA in primary human mammary epithelial cells and in the *TP53*-mutated breast cancer cell line BT549. They confirmed reproducible changes in 14 genes upon knockdown of BRCA2. Another study [352] reported on transcriptional alterations in HR-deficient compared to -proficient cells. However, the authors did not use a conditional model to study BRCA2-depletion. In contrast, they identified transcriptional alterations in human breast cancer cell lines with *BRCA2* mutations, HCC1428 and HCC1369. Unlike our model, these are terminally adapted to loss of BRCA2. Similarly, lentiviral infection of human non-malignant, yet immortalised mammary epithelial cells MCF10A with shRNA was used in order to generate cells with a stable knockdown of BRCA2.

Despite differences in experimental design, models and read-out, previous reports together with our whole transcriptome RNA-seq study indicate that loss of BRCA2 leads to changes in the transcriptome.

## 3.2 Acute loss of BRCA2 deregulates cell cycle

Following our observation that loss of BRCA2 inflicts alterations of the transcriptome, we firstly investigated transcriptional alteration in H1299+BRCA2sh$^{DOX}$ and MDA-MB-231+BRCA2sh$^{DOX}$ after 4 days of DOX treatment and therefore upon acute loss of BRCA2. In order to robustly identify genes differentially expressed in BRCA2 deficient cells, we applied the following conditions: FDR < 0.05; $|Log_2(\text{Fold Change})| > 0.5$.

In H1299+BRCA2sh$^{DOX}$, after 4 days of DOX treatment, we identified 574 genes which were significantly downregulated compared to 147 genes which were significantly upregulated (Figure 6a). In MDA-MB-231+BRCA2sh$^{DOX}$, 19 genes were significantly downregulated and 5 genes upregulated upon acute loss of BRCA2 (Figure 7). Also, serving as a control, BRCA2 was downregulated.

Based on curated annotations, we performed gene ontology (GO) enrichment analysis applying the Gene Set Enrichment Analysis (GSEA) method. Upon 4 days of DOX treatment, significantly downregulated genes in H1299+BRCA2sh$^{DOX}$ belonged primarily to cellular processes related to cell cycle, organelle fission, chromosome segregation, DNA replication and DNA repair (Figure 6b). In contrast, GO enrichment analysis of MDA-MB-231+BRCA2sh$^{DOX}$ did not reveal any particular pathways significantly deregulated.

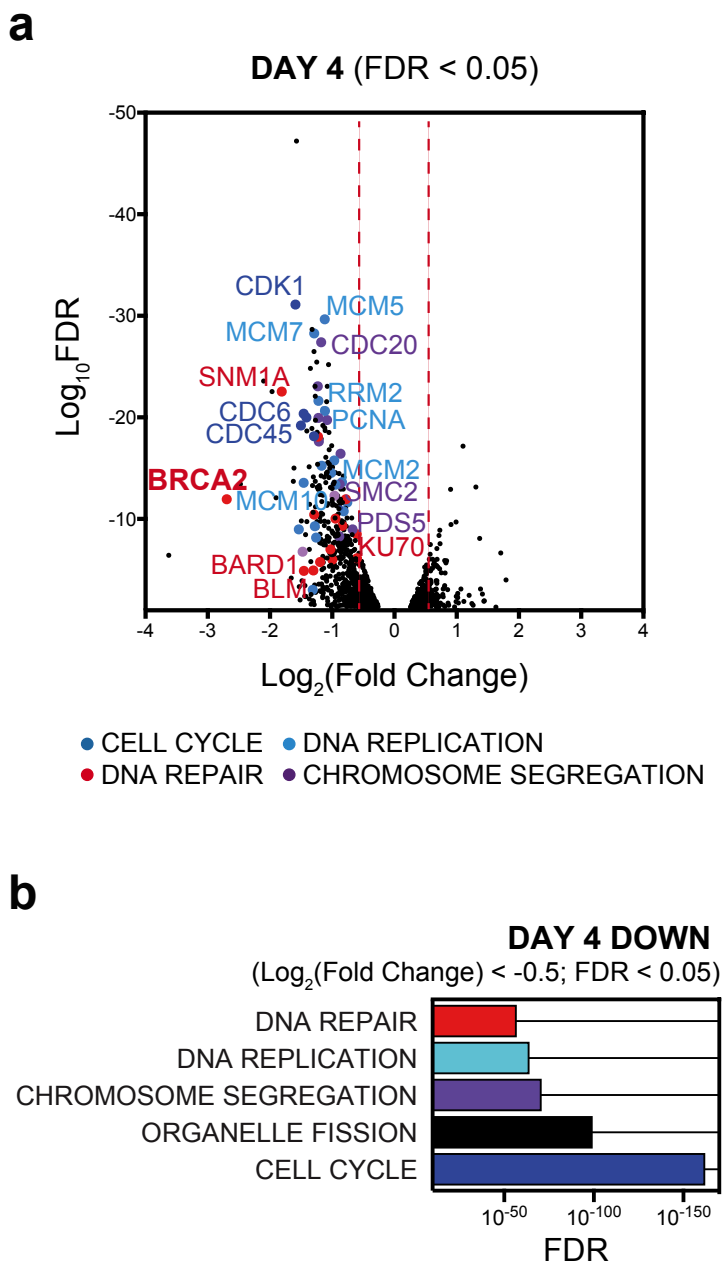



**Figure 6: Genes deregulated in H1299 upon acute loss of BRCA2**
(a) Volcano plot of genes deregulated upon 4 days of DOX treatment of H1299+BRCA2sh$^{DOX}$. Red dashed line represents cut-off at $|Log_2(Fold\ Change)| > 0.5$. (b) GO enrichment analysis of genes downregulated in H1299+BRCA2sh$^{DOX}$ upon 4 days of DOX treatment.

For some genes downregulated in H1299+BRCA2sh$^{DOX}$ upon acute loss of BRCA2, the encoded proteins are highlighted in the volcano plot in Figure 6a. For example, cell division cycle 6 (CDC6) is involved in origin licencing during G1 phase [354]. Cyclin-dependent kinase 1 (CDK1) is a key regulator of the G2-M

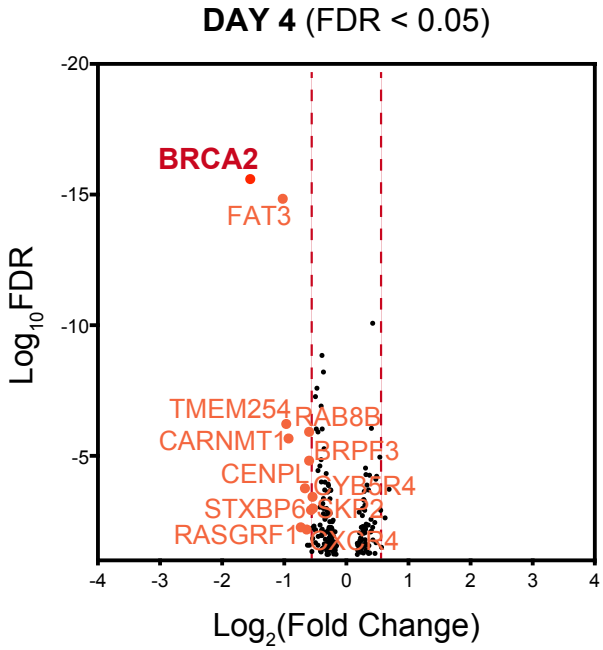



**Figure 7: Genes deregulated in MDA-MB-231 upon acute loss of BRCA2**
Volcano plot of genes deregulated upon 4 days of DOX treatment of MDA-MB-231+BRCA2sh$^{DOX}$. Red dashed line represents cut-off at $|Log_2(Fold\ Change)| > 0.5$.

transition [355]. In addition, genes essential for DNA replication were downregulated such as subunits of the MCM complex (MCM2, MCM5 and MCM7) and its co-factor CDC45 [158]. Also, MCM10 was downregulated which is important for initiation of replication [356,357]. Ribonucleotide reductase subunit M2 (RRM2) is essential for the synthesis of deoxynucleotide triphosphate. Whereas RRM1 expression is constant throughout the cell cycle, RRM2 expression peaks in S phase of unperturbed cells. [358,359] Proliferating cell nuclear antigen (PCNA) is a sliding clamp holding DNA polymerase on the unwound ssDNA during replication [360-362]. Also, genes involved in the DDR were found to be downregulated upon loss of BRCA2. Besides BARD1 and KU70, the exonuclease sensitive to nitrogen mustard 1A (SNM1A) [363] and the helicase Bloom syndrome protein (BLM) [364] were downregulated. Eventually,

also genes important for mitosis were downregulated. Precocious dissociation of sisters 5 (PDS5) interacts with the cohesion complex which is a ring shaped multi-protein structure holding together sister chromatids [365-367]. Structural maintenance of chromosomes 2 (SMC2) is a component of the condensin complex which regulates chromosomes condensation during mitosis [368]. The spindle assembly checkpoint halts anaphase until all chromosomes are attached to the spindle preventing missegregation of chromosomes. This checkpoint is overcome by the anaphase-promoting complex which is activated by CDC20. [369,370]

Some of the downregulated genes have been connected to functions of BRCA2. It was reported that BRCA2 plays a role in controlling chromosome segregation by regulating the anaphase-promoting complex [371]. Another study described PDS5 (also known as APRIN), CDC45 and PCNA interacting with BRCA2 in early S phase [372]. Follow up studies further identified a role of PDS5 in strand invasion during HR [373,374]. Furthermore, a recent study described a role of PDS5 in fork protection against MRE11-mediated fork protection, however independent of BRCA2 [375].

Our findings prompted us to investigate to what extent inhibition of BRCA2 expression affects cell cycle progression. To this end, we cultured H1299+BRCA2sh$^{DOX}$ and MDA-MB-231+BRCA2sh$^{DOX}$ in the presence or absence of DOX for 28 days. At 2-day intervals, we pulse-labelled the cells with 5-ethynyl-2'-deoxyuridine (EdU) and stained DNA with propidium iodide. Eventually, we assessed EdU incorporation and total DNA amount via flow cytometry (Figure 8).

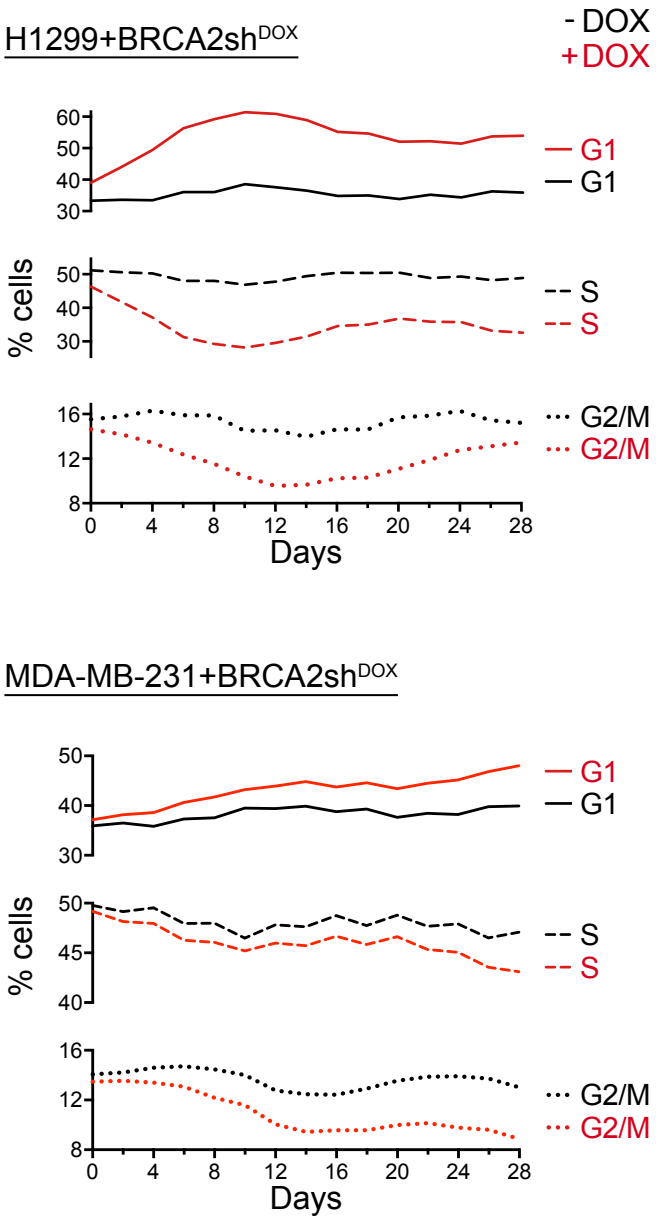



**Figure 8: Cell cycle profile upon loss of BRCA2**
H1299+BRCA2sh$^{DOX}$ and MDA-MB-231+BRCA2sh$^{DOX}$ were cultured in presence (+DOX) or absence (-DOX) of 2 µg/ml DOX for 28 days. At 2-day intervals, cells were pulse-labelled with EdU for 30 min and DNA was stained with propidium iodide. Percentage of cells in G1, S and G2/M phase was determined via flow cytometry analysis. $n = 3$ independent experiments.

Over the duration of the 28-day experiment, the percentage of G1 cells accumulated. In H1299+BRCA2shDOX, it peaked between 10 and 12 days after addition of DOX and then declined but not to the same level as untreated cells.

In MDA-MB-231+BRCA2shDOX, the G1 fraction increased continuously In both cell lines, the percentage of cells in S or G2/M decreased accordingly, remained low and did not recover.

We further monitored the proliferative capacity over eight days upon short- and long-term loss of BRCA2 (Figure 9). Cells treated with DOX proliferated slower compared to untreated cells. Interestingly though, there was no difference in cell proliferation between short- and long-term loss of BRCA2.

These results indicate that loss of BRCA2 inflicts alterations in the cell cycle profile of H1299 and MDA-MB-231. In particular, cells arrest in G1 shortly after loss of BRCA2 in correlation with the GO enrichment analysis of the RNA-seq data. Furthermore, BRCA2-deficient cells proliferate slower than -proficient cells.

These findings are consistent with previous reports [376,377]. Our own group reported a reduction in proliferation and a G1 arrest in H1299+BRCA2sh$^{DOX}$ upon DOX treatment [377]. In an independent study, the authors generated a conditional MCF10A cell line in which one allele of *BRCA2* was knocked out and the other allele was depleted upon induction [376]. These cells are p53-proficient and therefore, loss of BRCA2 induced senescence or apoptosis. Furthermore, upon loss of BRCA2, cell cycle analysis showed an increased number of cells in G1 similar to our results. Interestingly, depletion of p53 diminished the number of senescent and G1-arrested cells. The authors further confirmed that loss of BRCA2 led to replication stress resulting in mitotic abnormalities. They concluded that DNA damage which originated in S and G2 phases persists though mitosis and, eventually, in the subsequent G1 phase, induces p53-dependent G1 arrest. In contrast to that study, we used p53-deficient cells. Therefore, we were not

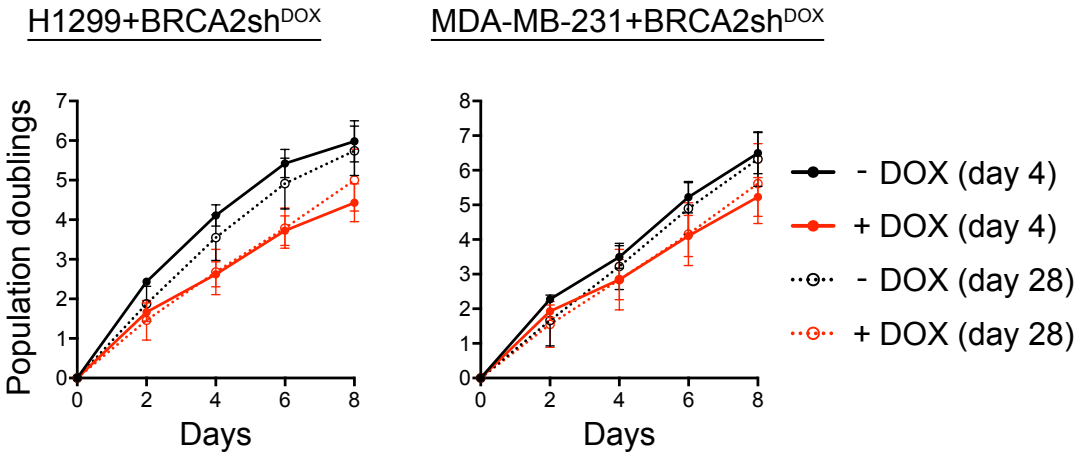



**Figure 9: Proliferative capacity of BRCA2-deficient cells**
H1299+BRCA2sh$^{DOX}$ and MDA-MB-231+BRCA2sh$^{DOX}$ were cultured in presence (+DOX) or absence (-DOX) of 2 µg/ml DOX for 28 days. After 4 and 28 days, cell viability was determined at 2-day intervals for a period of 8 days and reported relative to day 0 in order to calculate population doublings. Day 0 represents either day 4 (solid line) or day 28 (dotted line) after DOX addition. Error bars represent s.d. of $n = 3$ independent experiments each performed in technical triplicates.

restrained by induction of apoptosis or senescence but were able to investigate long-term consequences of BRCA2 loss.

## 3.3 Chronic loss of BRCA2 elicits a type I IFN response

Next, we investigated genes deregulated after 28 days of DOX treatment which means upon chronic loss of BRCA2. In H1299+BRCA2sh$^{DOX}$, 194 genes were significantly downregulated compared to 213 genes which were significantly upregulated (Figure 10a). In MDA-MB-231+BRCA2sh$^{DOX}$, 75 genes were significantly downregulated and 91 genes were significantly upregulated (Figure 11a). Therefore, differential gene expression after chronic treatment differed from acute loss of BRCA2.

According to the GO enrichment analysis in H1299+BRCA2sh$^{DOX}$, the top-upregulated genes belonged primarily to pathways involved in immune

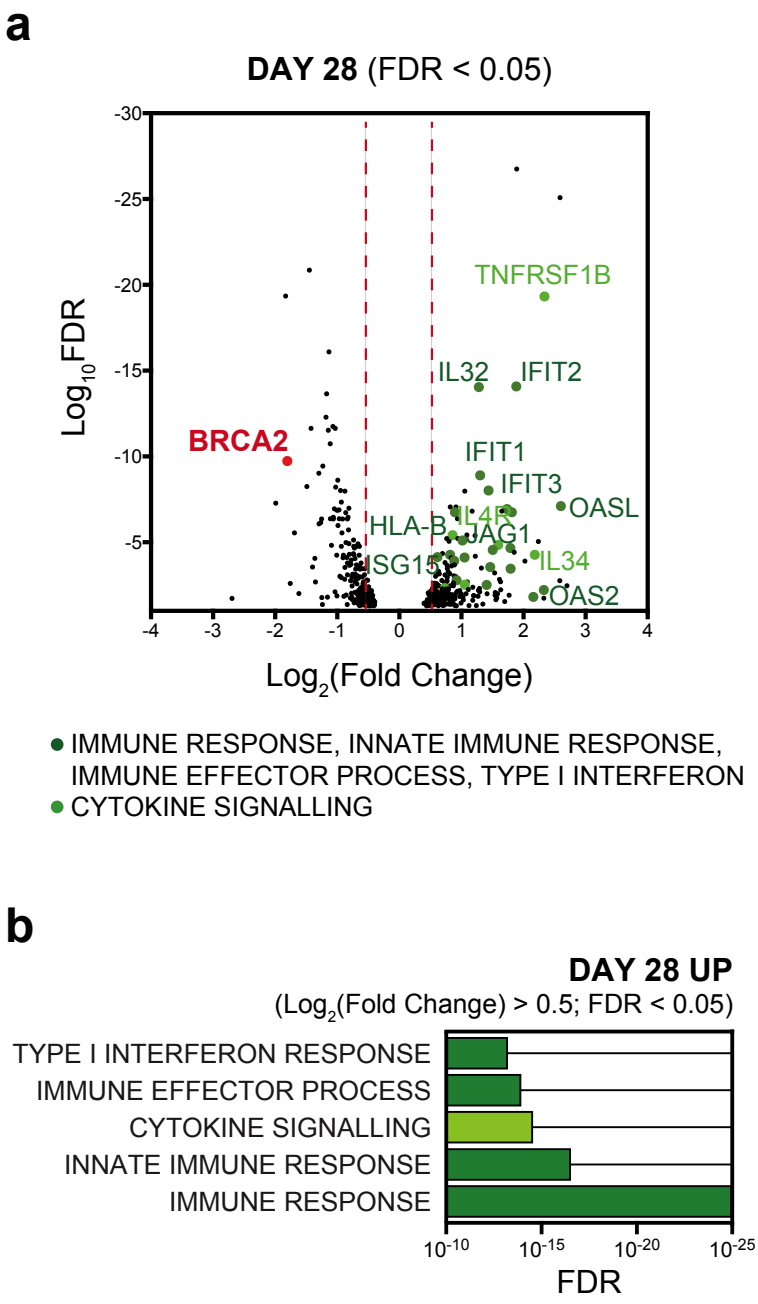



**Figure 10: Genes deregulated in H1299 upon chronic loss of BRCA2**
a) Volcano plot of genes deregulated upon 28 days of DOX treatment of H1299+BRCA2sh$^{DOX}$. Red dashed line represents cut-off at $|Log_2(Fold\ Change)| > 0.5$. (b) GO enrichment analysis of genes upregulated in H1299+BRCA2sh$^{DOX}$ upon 28 days of DOX treatment.

response, innate immune response, cytokine signalling, immune effector process and type I interferon (IFN) response (Figure 10b). In MDA-MB-231+BRCA2sh$^{DOX}$, the top-upregulated genes were primarily attributed

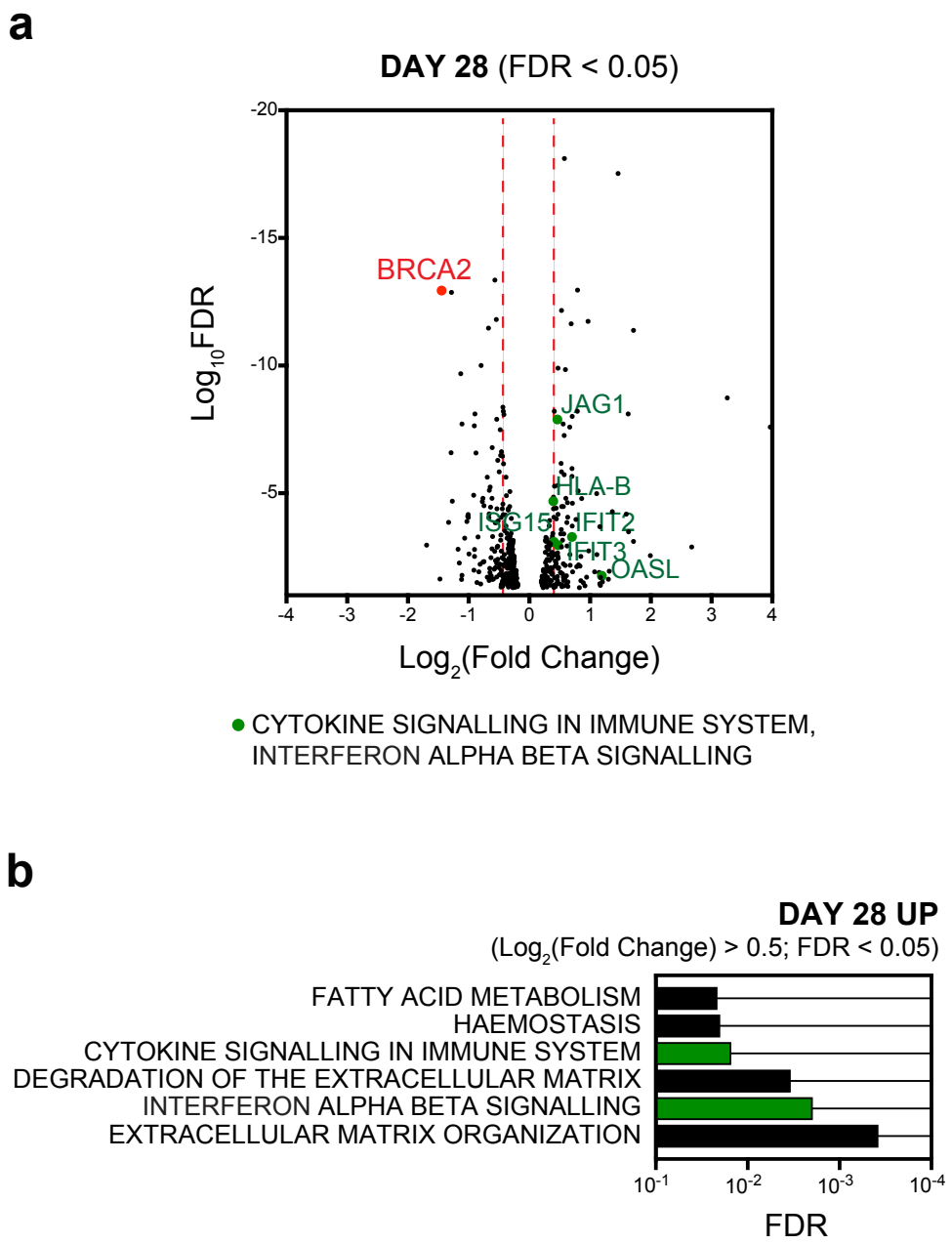



**Figure 11: Genes deregulated in MDA-MB-231 upon chronic loss of BRCA2**
(a) Volcano plot of genes deregulated upon 28 days of DOX treatment of MDA-MB-231+BRCA2sh$^{DOX}$. Red dashed line represents cut-off at $|Log_2(Fold\ Change)| > 0.5$. (b) GO enrichment analysis of genes upregulated in MDA-MB-231+BRCA2sh$^{DOX}$ upon 28 days of DOX treatment.

to extracellular matrix organisation, IFN signalling, degradation of the extracellular matrix, cytokine signalling in immune system, haemostasis and fatty acid metabolism (Figure 11b).

We next determined genes commonly upregulated in both cell lines, H1299+BRCA2sh$^{DOX}$ and MDA-MB-231+BRCA2sh$^{DOX}$, after chronic loss of BRCA2. To assess similarities between two different cell lines, we adapted the cut-off of fold change to $Log_2$(Fold Change) > 0.3. Under these conditions, we identified 28 genes significantly (FDR < 0.05) upregulated in both cells lines (Figure 12a). GO enrichment analysis revealed that these 28 genes belonged primarily to pathways involved in cytokine signalling, type I IFN response, response to cytokine, immune response and immune effector process (Figure 12b).

Our findings are consistent with a previous report [353]. Here, the authors used microarray analysis in order to assess the transcriptome of human colorectal carcinoma cell line HCT116 after knockout of *BRCA2*. First, they extended their results of deregulated genes by adding functionally similar genes identified by network analysis. Thereby, they found genes upregulated which are predominantly associated with the type I IFN response such as *IFIT2*, *ISG15* and *OAS1*. They further confirmed their findings with the *BRCA2*-mutant ovarian cancer cell line PEO1 and with BRCA2-depleted mouse mammary tumour cells. Furthermore, they described accumulation of endogenous DNA damage in BRCA2-deficient HCT116. Induction of DNA damage by treatment with ionising radiation, phleomycin, camptothecin or aphidicolin induced a comparable type I IFN response in BRCA2-proficient HCT116.

Consistent with this published data, our data indicate that chronic loss of BRCA2 leads to an upregulation of genes involved in the innate immune response in both cell lines, H1299 and MDA-MB-231. We further committed to investigating how the type I IFN response had been activated by chronic loss of BRCA2.

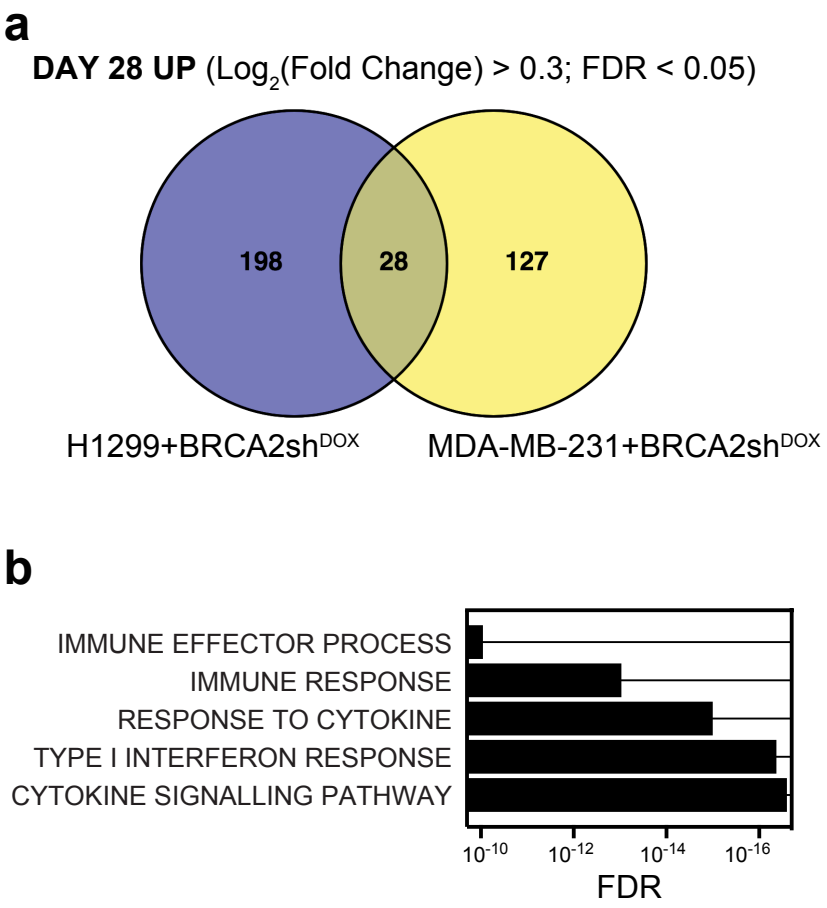



**Figure 12: Genes commonly upregulated in H1299 and MDA-MB-231 upon chronic loss of BRCA2**
a) Venn diagram of genes upregulated in H1299+BRCA2sh$^{DOX}$ and MDA-MB-231+BRCA2sh$^{DOX}$ upon 28 days of DOX treatment. Cut-off was defined as $Log_2$(Fold Change) > 0.3. (b) GO enrichment analysis of 28 genes commonly upregulated in H1299+BRCA2sh$^{DOX}$ and MDA-MB-231+BRCA2sh$^{DOX}$ upon 28 days of DOX treatment.

## 3.4 The type I IFN response due to loss of BRCA2 depends on the cGAS-STING and JAK-STAT pathway

First, we set out to validate top-upregulated genes and to monitor their stimulation in time. To this end, we performed quantitative polymerase chain reaction (qPCR) analysis at 2-day intervals over 28 days treating H1299+BRCA2sh$^{DOX}$ or MDA-MB-231+BRCA2sh$^{DOX}$ with DOX.

Upon addition of DOX to H1299+BRCA2sh$^{DOX}$, mRNA levels of *IFIT1*, *IFIT2*, *IFIT3*, *IFI6*, *OAS1*, *OAS2*, *ISG15* and *TNFRSF1B* increased gradually over 28 days (Figure 13). A similar trend was observed in MDA-MB-231+BRCA2sh$^{DOX}$ for mRNA levels of *IFIT1*, *IFIT2*, *OAS2* and *ISG15* although to a lesser extent than in H1299+BRCA2sh$^{DOX}$ (Figure 14). In MDA-MB-231+BRCA2sh$^{DOX}$, mRNA levels of *IFIT3* and *TNFRSF1B* were not monitored but when measuring mRNA levels of *IFI6* and *OAS1*, no increase was observed.

Those upregulated genes belong to a group called IFN-stimulated genes (ISGs) and are characteristic for a type I IFN response [378-381]. This is a key signalling pathway of the innate immune response (Figure 15). It is activated cell-intrinsically upon invasion of pathogens and characterised by secretion of IFNα and IFNβ which are major cytokines in the anti-viral response. Virtually all cell types are capable of producing and secreting type I IFNs. They, in turn, act autocrine or paracrine binding to a heterodimeric receptor consisting of IFNα and β receptor subunit 1 and 2 (IFNAR1 and IFNAR2) [382]. Ligand-receptor interaction activates the IFNAR1-bound tyrosine kinase 2 (TYK2) [383] which phosphorylates IFNAR2-bound janus kinase 1 (JAK1) [384] which, in turn, phosphorylates members of the signal transducer and activator of transcription (STAT) [385] family [386-390]. Phosphorylation of tyrosine residue 701 of STAT1 [391] and of tyrosine residue 690 of STAT2 [392] induces their heterodimerisation. The newly formed STAT1/2 heterodimer and IFN regulatory factor 9 (IRF9) form a complex referred to as ISG factor 3 (ISGF3) which translocates into the nucleus where it acts as a transcription factor inducing expression of ISGs [386,387,392,393].

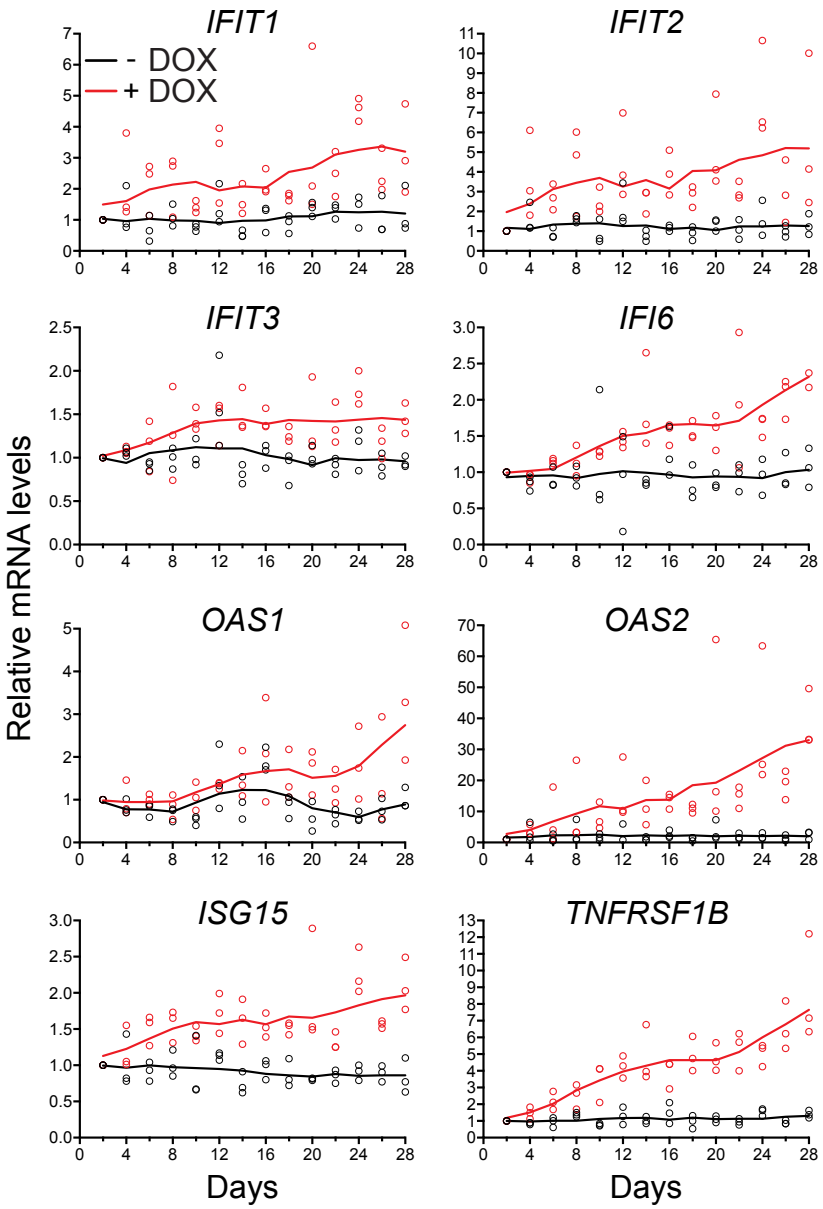



**Figure 13: Validation of upregulated genes in H1299 upon chronic loss of BRCA2**
H1299+BRCA2sh$^{DOX}$ were cultured in presence (+DOX) or absence (-DOX) of 2 µg/ml DOX for 28 days. At 2-day intervals, total RNA was extracted and subjected to qPCR analysis using primers specific for indicated genes. mRNA levels were expressed relative to mRNA levels encoding β-actin and to day 2 samples. Values were expressed as $2^{-\Delta\Delta C_t}$. $n = 3$ independent experiments, each performed in technical triplicates. Each dot represents mean of technical triplicates.

Recently, two back to back studies established micronuclei formation as a link between DNA damage and innate immunity [394,395]. Both groups independently observed that progression through mitosis upon induction of DNA

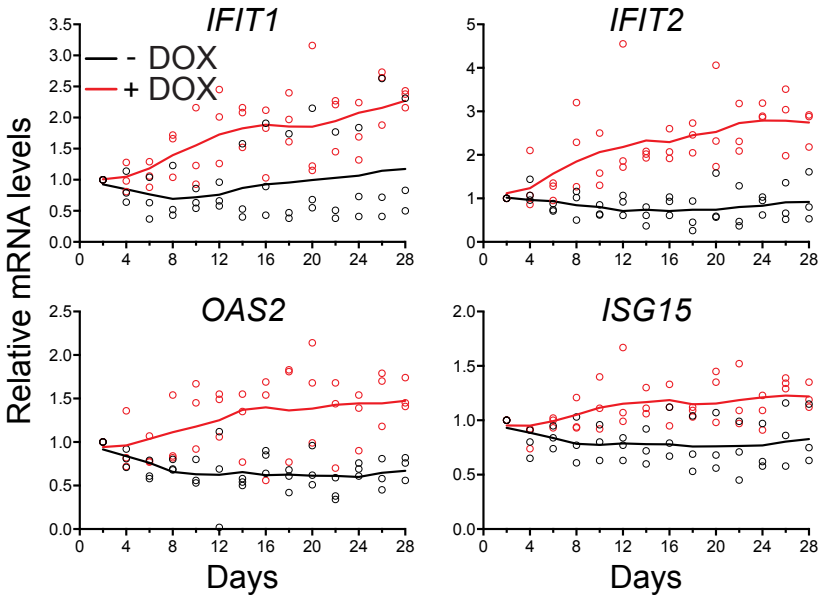



**Figure 14: Validation of upregulated genes in MDA-MB-231 upon chronic loss of BRCA2**
MDA-MB-231+BRCA2sh$^{DOX}$ were cultured in presence (+DOX) or absence (-DOX) of 2 µg/ml DOX for 28 days. At 2-day intervals, total RNA was extracted and subjected to qPCR analysis using primers specific for indicated genes. mRNA levels were expressed relative to mRNA levels encoding β-actin and to day 2 samples. Values were expressed as $2^{-\Delta\Delta C_t}$. *n* = 3 independent experiments, each performed in technical triplicates. Each dot represents mean of technical triplicates.

damage resulted in formation of micronuclei which co-localised with a cytosolic DNA sensor called cyclic 2'3'-GMP-AMP (cGAMP) synthase (cGAS). Upon viral Infection for instance, cGAS binds viral DNA in the cytoplasm which, in turn, induces a type I IFN response [396-399]. Similarly, genomic DNA in micronuclei is detected by cGAS [394,395]. Interestingly, cGAS translocates predominantly into micronuclei with a compromised membrane [395]. Activated cGAS synthesises the second messenger cGAMP which, in turn, activates stimulator of IFN genes (STING) [400-404] (also known as MPYS [405], ERIS [406] or MITA [407]). Next, STING recruits the kinase TANK-binding kinase 1 (TBK1) which, in turn, phosphorylates numerous residues of transcription factor IRF3 such as serine residue 386 which induces its homodimerisation and translocation

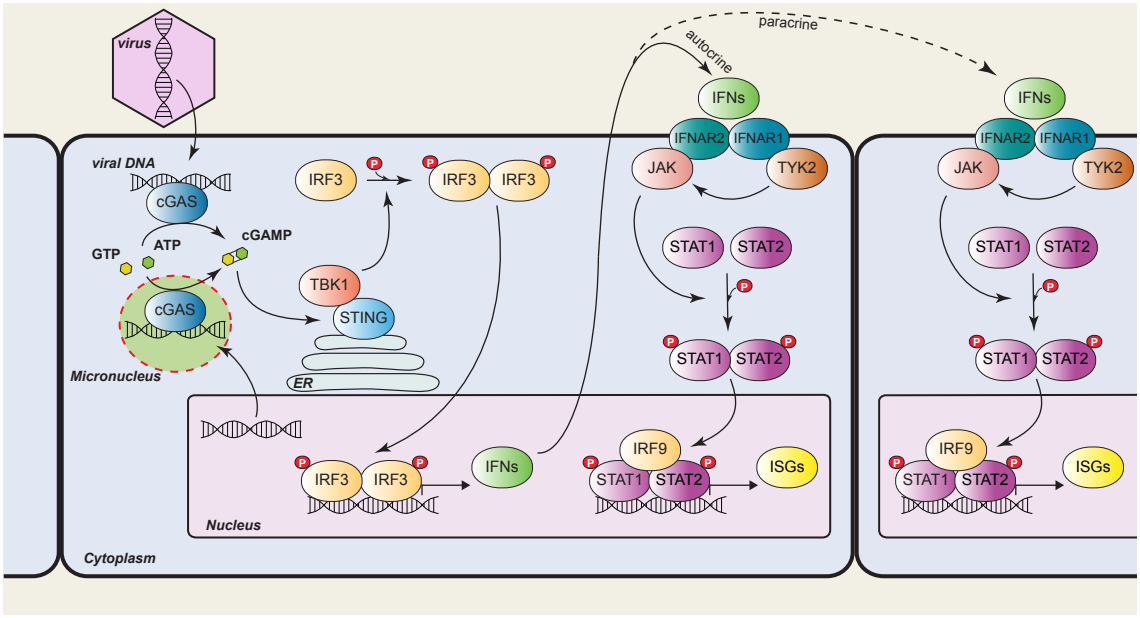



**Figure 15: Illustration of type I IFN response activation**

into the nucleus driving expression of IFNα and IFNβ [407-409]. The result is a sterile type I IFN response induced by genome instability as opposed to a viral infection.

These reports prompted us to investigate whether induction of ISG expression upon loss of BRCA2 was due to activation of the cGAS-STING pathway.

Compared to H1299+BRCA2sh$^{DOX}$ (Figure 13), addition of DOX to MDA-MB-231+BRCA2sh$^{DOX}$ (Figure 14) induced a weaker expression of ISGs. In a previous study, ISG expression was compared in different breast cancer cell lines upon genotoxic treatment with mafosfamide [410]. Compared to other cell lines, MDA-MB-231 did not show an increased expression of ISGs. Consistently, in a recent study, genomic instability was induced in MDA-MB-231 by inhibiting the attachment of microtubules to chromosomes leading to chromosome missegregation during anaphase [411]. The resulting chromosomal instability

was shown to inflict cGAS-infiltrated micronuclei. However, also here the authors could not detect a strong induction of ISG expression in MDA-MB-231.

In line with our findings, these reports indicate that MDA-MB-231 do not respond to the same extent to activation of the cGAS-STING pathway compared to other cell lines. Therefore, we focused on H1299 cells for the remainder of our *in vitro* studies.

First, we set out to assess a link between loss of BRCA2 and putative activation of cGAS. Therefore, we monitored formation of micronuclei formation. To this end, we treated H1299+BRCA2sh$^{DOX}$ with DOX for 28 days and assessed the number of cGAS-positive micronuclei after 4 days and 28 days (Figure 16a). At both time points, we found a significantly larger amount of cGAS-positive micronuclei in BRCA2-deficient cells compared to BRCA2-proficient cells. Importantly, in BRCA2-deficient cells, we found a significantly larger amount of cGAS-positive micronuclei at day 28 compared to day 4. In cells not treated with DOX, the number of cGAS-positive micronuclei was not significantly different at day 28 compared to day 4.

These results are consistent with previous studies [200,376,412,413]. Taken together, these studies demonstrated that loss of BRCA2 leads to replication stress resulting in mitotic abnormalities such as chromosome missegregation and lagging chromosomes. Chromatid fragments or even whole chromosomes, which are not integrated into the nuclear envelope, remain in the cytoplasm. Here they are enclosed by a membrane noticeable as micronuclei in daughter cells. Eventually, cGAS enters micronuclei with a compromised membrane. We further concluded that the temporal increase in cGAS-positive micronuclei (Figure 16a)

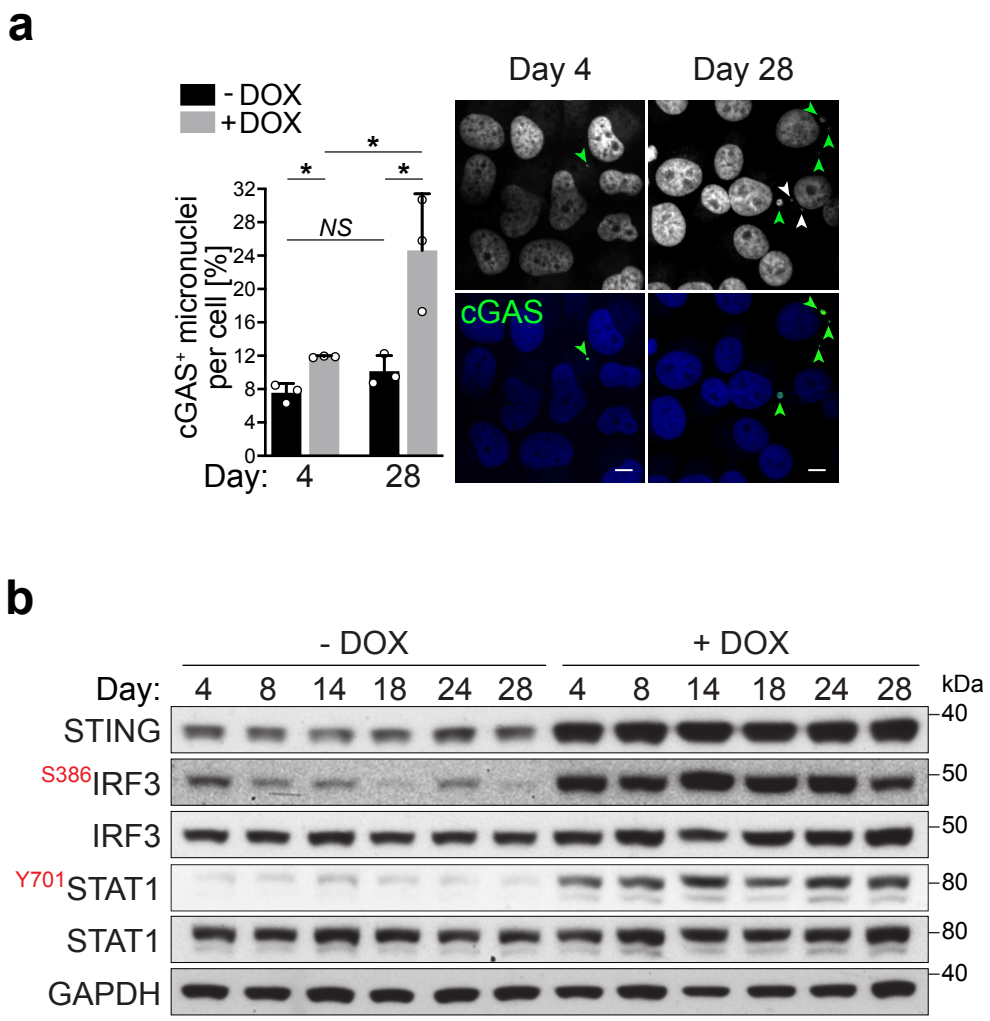



**Figure 16: Loss of BRCA2 results in micronuclei formation and activation of cGAS-STING and JAK-STAT signalling**
H1299+BRCA2sh$^{DOX}$ were cultured in presence (+DOX) or absence (-DOX) of 2 µg/ml DOX for 28 days. (a) After 4 and 28 days, cells were fixed and stained with antibody against cGAS (green) and counterstained with DAPI (blue). Shown are representative images of cells treated with DOX. Green arrows indicate cGAS-positive micronuclei and white arrows indicate cGAS-negative micronuclei. Scale bar represents 10 µm. Error bars represent s.d. of $n = 3$ independent experiments. *NS*, $p > 0.05$; *, $p < 0.05$ (unpaired two-tailed t-test). (b) At indicated time points, whole-cell extracts were immunoblotted as indicated. GAPDH was used as a loading control.

correlated with the temporal increase in ISG expression observed by qPCR (Figure 13).

As a next step, we monitored markers of activation of the cGAS-STING and JAK-STAT pathway. To this end, we assessed phosphorylation of serine

residue 386 of IRF3 [409] and of tyrosine residue 701 of STAT1 [391] via WB (Figure 16b). We observed induction of IRF3 phosphorylation and STAT1 phosphorylation upon loss of BRCA2 already at early time points. Furthermore, we assessed expression levels of STING since it is an ISG itself and therefore under control of the JAK-STAT pathway [414]. We observed that protein levels of STING were upregulated in cells treated with DOX. These results indicate that the cGAS-STING and the JAK-STAT pathway were activated upon loss of BRCA2.

Next, we tested whether cGAS and STING were required for induction of IRF3 and STAT1 phosphorylation upon loss of BRCA2. To this end, we cultured H1299+BRCA2sh$^{DOX}$ in the presence of DOX for 28 days and transfected cells in parallel with small interfering RNA (siRNA) against cGAS (Figure 17a) or STING (Figure 17b) every 4 days. As indicated by WB, transfection with siRNA effectively inhibited expression of cGAS and STING, respectively. Moreover, inhibition of either cGAS or STING expression abrogated phosphorylation of IRF3 and STAT1 in cells treated with DOX. These results indicate that both, cGAS and STING, are required for induction of IRF3 and STAT1 phosphorylation upon loss of BRCA2.

Next, we assessed whether ISG induction upon loss of BRCA2 depends on STAT1. To this end, we inhibited expression of STAT1 using siRNA in H1299+BRCA2sh$^{DOX}$ after chronic treatment with DOX and assessed expression of ISGs via qPCR (Figure 17c). As indicated by WB, transfection with siRNA effectively inhibited expression of STAT1. Moreover, we observed that inhibition of STAT1 diminished the mRNA levels of *IFIT1*, *IFIT2*, *IFIT3*, *OAS1* and *OAS2* in

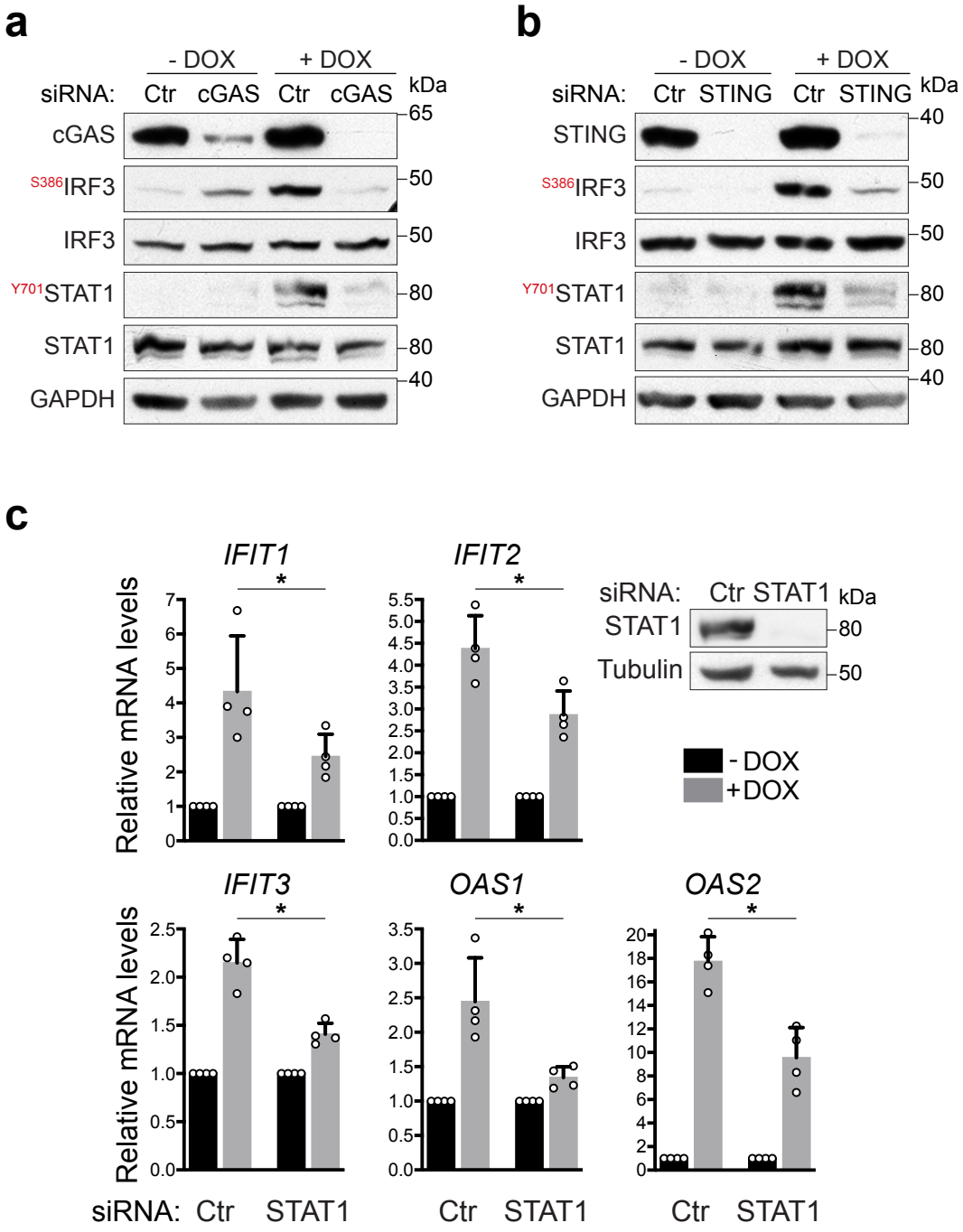



**Figure 17: Activation of type I IFN response upon loss of BRCA2 depends on cGAS, STING and STAT1**

H1299+BRCA2sh$^{DOX}$ were cultured in presence (+DOX) or absence (-DOX) of 2 µg/ml DOX for 28 days. (a, b) At 4-day intervals, cells were transfected with siRNA targeting (a) cGAS or (b) STING. After 28 days, whole-cell extracts were immunoblotted as indicated. GAPDH was used as a loading control. (c) After 28 days, cells were subjected to single transfection with siRNA targeting STAT1. Total RNA was extracted and subjected to qPCR analysis using primers specific for indicated genes. mRNA levels were expressed relative to mRNA levels encoding β-actin and to -DOX samples. Values were expressed as $2^{-\Delta\Delta C_t}$. Error bars represent s.d. of $n = 4$ independent experiments, each performed in technical triplicates. *NS*, $p > 0.05$; *, $p < 0.05$ (unpaired two-tailed t-test). Each dot represents mean of technical triplicates. Whole-cell extracts were immunoblotted as indicated. Tubulin was used as a loading control.

BRCA2-deficient cells. These results indicate that STAT1 is involved in expression of ISGs upon loss of BRCA2.

Taken together, these data indicate, that the cGAS-STING and the JAK-STAT pathway are required for induction of a type I IFN response upon loss of BRCA2.

However, a type I IFN response can also be elicited by invasion of RNA viruses [415] and under certain circumstances also by overexpressed shRNA [416-418]. Here, RNA sensors such as retinoic acid-inducible gene I (RIG-I) [419] and melanoma differentiation-associated protein 5 (MDA5) [420] (also known as Helicard [421]) are the counterparts of DNA sensor cGAS.

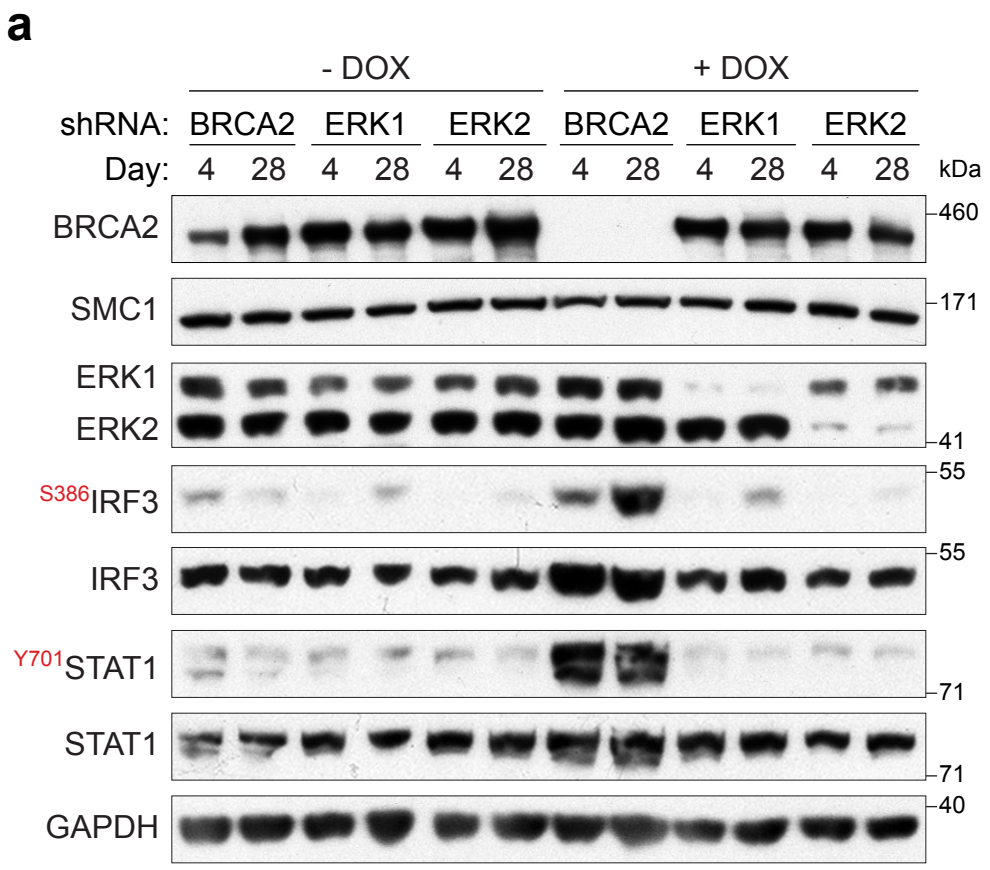
These reports prompted us to assess whether the mere expression of shRNA in our cell model could induce a type I IFN response independent of BRCA2 loss. To this end, we used H1299 cells carrying a DOX-inducible shRNA against extracellular signal-regulated kinase 1 or 2 (ERK1 or ERK2) referred to as H1299+ERK1sh$^{DOX}$ and H1299+ERK2sh$^{DOX}$, respectively. Importantly, ERK1 [422] and ERK2 [423] do not share roles with BRCA2 in HR nor in replication fork protection. In contrast, ERK1 and ERK2 are effector kinases of the mitogen-activated protein kinase (MAPK) pathway which integrate external stimuli, for example by growth factors, promoting cell proliferation and survival. These two isoforms share a 90% identical protein sequence [423] and are mostly functionally redundant [424,425]. Therefore, we expected inhibition of one single isoform not to have major cellular effects.

We cultured these cells for 28 days in parallel to the H1299+BRCA2sh$^{DOX}$. At day 4 and day 28, we collected samples for WB and qPCR analysis. As shown by WB, protein levels of BRCA2, ERK1 and ERK2 were reduced in the respective cell line upon treatment with DOX at both time points (Figure 18a). This is

indicative for the respective shRNA to be effectively transcribed upon addition of DOX. Importantly, we monitored phosphorylation of IRF3 and STAT1 only upon expression of shRNA against BRCA2 but neither against ERK1 nor ERK2. On mRNA level, expression levels of ISGs *IFIT1*, *IFIT2*, *OAS1* and *OAS2* were only upregulated upon expression of shRNA against BRCA2 but neither against ERK1 nor ERK2 (Figure 18b). These data support the idea that the type I IFN response in our cell model is not due to accumulation of shRNA but induced specifically by abrogation of BRCA2.

Our findings are consistent with a very recent report [426]. Similar to our model, the authors used cells harbouring a DOX-inducible shRNA against BRCA2. However, the parental cells were triple negative breast cancer cell lines BT-549 and HCC38. They further characterised the proteasome and transcriptome of these cells after 48 h or 72 h of DOX treatment, respectively. They found, similar to our results, that abrogation of BRCA2 induced formation of micronuclei which induced cGAS-STING signalling cascading into a type I IFN response.

In summary, our data together with the report mentioned above support the following model (Figure 15): genomic instability due to loss of BRCA2 inflicts formation of micronuclei. Genomic DNA in micronuclei is then detected by cGAS which, in turn, activates STING to promote phosphorylation and nuclear translocation of IRF3 transcription factor. In the nucleus, IRF3 initiates transcription of type I IFNs which are secreted and, in turn, activate via their receptors the JAK-STAT pathway. Finally, STAT1 and STAT2 are phosphorylated by JAK and dimerise. The heterodimer is bound by IRF9 to form the ISGF3 complex which translocates into the nucleus to act as a transcription factor initiating transcription of ISGs.


a
- DOX
+ DOX
shRNA: BRCA2 ERK1 ERK2 BRCA2 ERK1 ERK2
Day: 4 28 4 28 4 28 4 28 4 28 4 28
kDa
BRCA2
460
SMC1
171
ERK1
ERK2
41
S386IRF3
55
IRF3
55
Y701STAT1
71
STAT1
71
GAPDH
40

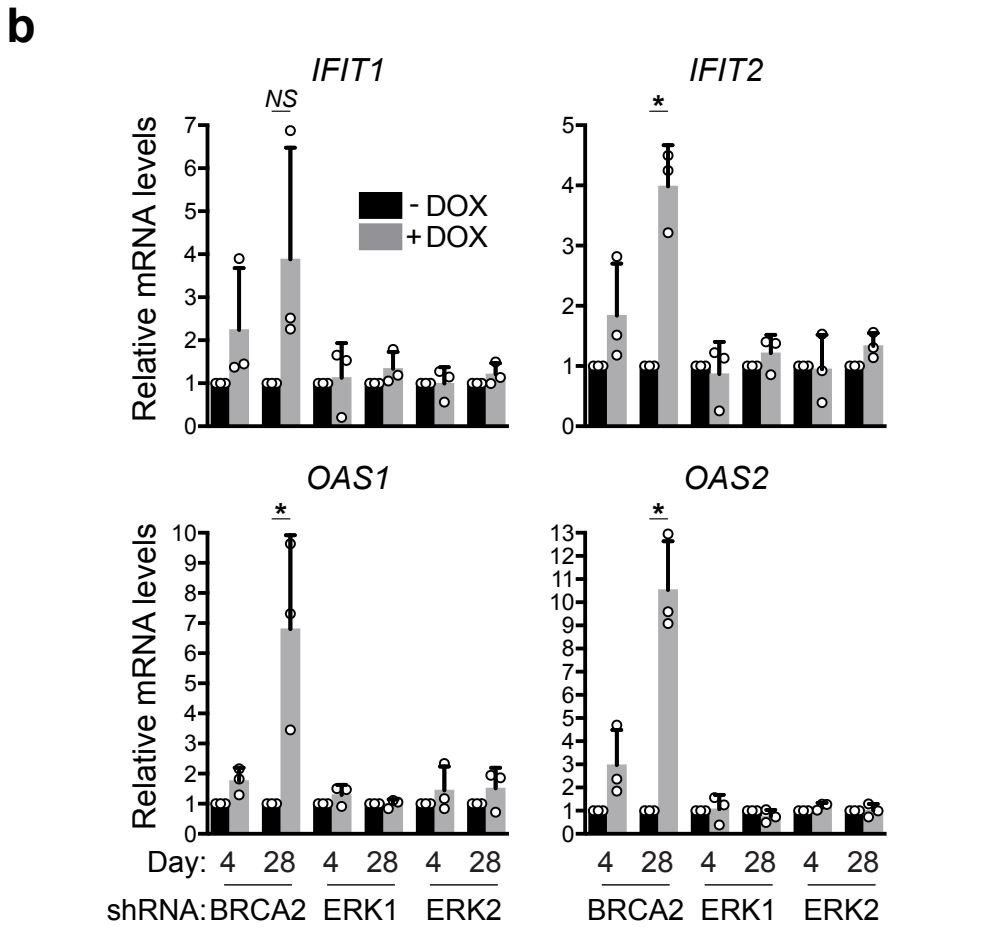

b
IFIT1
NS
IFIT2
*
Relative mRNA levels
- DOX
+ DOX
OAS1
*
OAS2
*
Day: 4 28 4 28 4 28 4 28 4 28 4 28
shRNA: BRCA2 ERK1 ERK2 BRCA2 ERK1 ERK2

**Figure 18: Expression of shRNA against ERK1 or ERK2 does not activate a type I IFN response in H1299**
H1299+BRCA2sh$^{DOX}$, H1299+ERK1sh$^{DOX}$ and H1299+ERK2sh$^{DOX}$ were cultured in presence (+DOX) or absence (-DOX) of 2 µg/ml DOX for 28 days. (a) After 4 and 28 days, whole-cell extracts were immunoblotted as indicated. GAPDH and SMC1 were used as loading controls. (b) After 4 and 28 days, total RNA was extracted and subjected to qPCR analysis using primers specific for indicated genes. mRNA levels were expressed relative to mRNA levels encoding β-actin and to -DOX samples. Values were expressed as $2^{-\Delta\Delta C_t}$. Error bars represent s.d. of $n = 3$ independent experiments, each performed in technical triplicates. *NS*, $p > 0.05$; *, $p < 0.05$ (unpaired two-tailed t-test). Each dot represents mean of technical triplicates.

## 3.5 BRCA2-depleted cells rely on TNFR2 overexpression

One of the top-upregulated genes in H1299 upon chronic loss of BRCA2, *TNFRSF1B*, encodes for tumour necrosis factor (TNF) receptor 2 (TNFR2) (Figure 10a and 13). This cell surface receptor is activated upon binding of the pleiotropic cytokine TNF which is also the ligand of the structurally different receptor TNFR1 [427,428]. Upon binding of TNF, both receptors activate distinct as well as overlapping downstream pathways. These can result in activation of different partly opposing outcomes such as promoting apoptosis, cell survival, cell proliferation, cell differentiation and inflammation. Whereas TNFR1 is ubiquitously expressed in nearly all tissues, expression of TNFR2 is restricted to a subset of cell types, in particular immune cells such as macrophages. Furthermore, TNFR2 is upregulated in several cancers. [429,430]

Next, we investigated whether overexpression of this receptor is essential for BRCA2-deficient cells. To this end, we used H1299+BRCA2sh$^{DOX}$ and treated them with DOX for 28 days. Then, we transfected them with siRNA against TNFR2 and monitored population doubling. Both BRCA2-proficient and -deficient

cells proliferated slower upon loss of TNFR2. Especially BRCA2-deficient cells suffered from loss of TNFR2 (Figure 19a). Since we did not have an antibody against TNFR2, we did not assess knockdown efficiency via WB but via qPCR (Figure 19b). Transfection of siRNA effectively reduced levels of TNFR2 mRNA in BRCA2-proficient and -deficient cells. However, even upon transfection, levels of TNFR2 mRNA were still higher in BRCA2-deficient compared to -proficient cells.

Our results indicate that TNFR2 is upregulated in BRCA2-deficient H1299 in order to promote viability. These findings are consistent with a previous report which showed that TNFR-associated factor 1 (TRAF1), a TNFR2 downstream signalling factor, was essential for H1299 cells [431]. Here, inhibiting TRAF1 expression using siRNA diminished proliferation of H1299 cells and induced apoptosis.

Interestingly, a recent study reported a relation between TNF-signalling and loss of BRCA2 [426]. Here, they inhibited BRCA2 via a DOX-inducible system similar to ours but in the near-haploid human myeloid leukaemia cell line KBM-7. Albeit p53-deficient, these cells died upon induction of shBRCA2. This phenotype was rescued by loss of TNFR1. Furthermore, treatment with recombinant TNF was selectively toxic to numerous BRCA2-deficient cell lines.

Our preliminary data together with those published reports indicate that loss of BRCA2 might affect expression of and dependability on TNFR1- and TNFR2-signalling components dependent on the cell type.

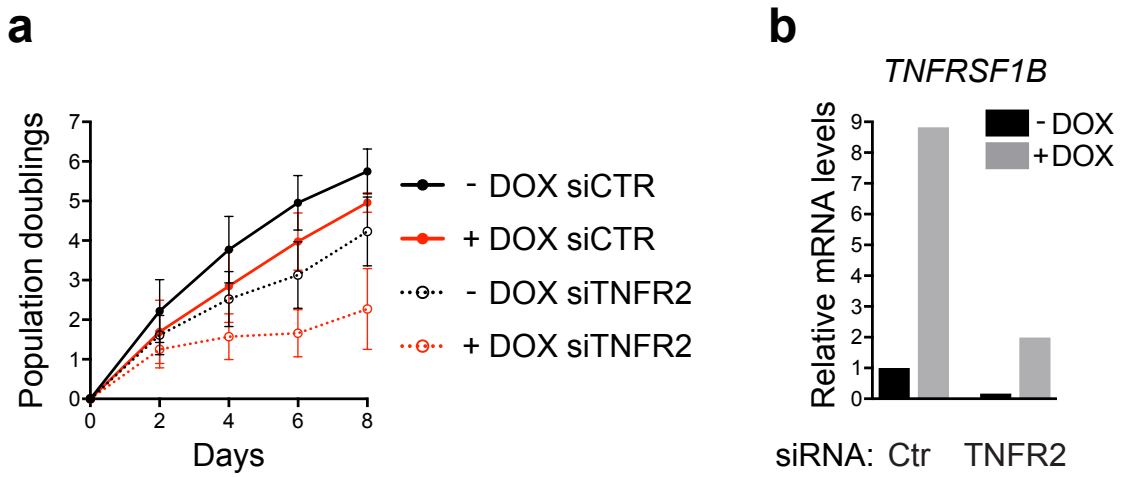



**Figure 19: Proliferative capacity of BRCA2- and TNFR2-deficient cells** H1299+BRCA2sh$^{DOX}$ were cultured in presence (+DOX) or absence (-DOX) of 2 µg/ml DOX for 28 days. After 28 days, cells were transfected with siRNA targeting TNFR2. (a) Viability was determined at 2-day intervals for a period of 8 days and reported relative to day 0 in order to calculate population doublings. Error bars represent s.d. of $n = 3$ independent experiments, each performed in technical triplicates. (b) Total RNA was extracted and subjected to qPCR analysis using primers specific for *TNFRSF1B*. mRNA levels were expressed relative to mRNA levels encoding β-actin and to -DOX samples transfected with control siRNA. Values were expressed as $2^{-\Delta\Delta C_t}$. $n = 1$ experiment performed in technical triplicates.

## 3.6 Treatment with PARP inhibitors potentiates the type I IFN response in BRCA-deficient cells and tumours

Next, we investigated the effect of PARP inhibitor treatment on the activation of type I IFN response in BRCA2-deficient cells. To this end, we cultured H1299+BRCA2sh$^{DOX}$ for 4 days in the presence of DOX followed by a 72 h treatment with 1 or 10 µM olaparib and, eventually, collected samples for qPCR, WB and EdU labelling.

After 4 days of DOX treatment, BRCA2 expression was inhibited (Figure 5b) but we did not observe a major upregulation of ISGs in H1299+BRCA2sh$^{DOX}$ (Figure 13). In contrast, upon treatment with olaparib, we observed a

dose-dependent increase in expression of *IFIT1*, *IFIT2* and *IFI6* in BRCA2-deficient cells treated with olaparib (Figure 20a).

Treatment with olaparib also induced phosphorylation of IRF3 and STAT1 in a dose-dependent manner in both BRCA2-proficient and -deficient cells but to a greater extent in BRCA2-deficient cells (Figure 20b). We also monitored phosphorylation of serine residue 824 of Krüppel-associated box zinc finger protein-associated protein 1 (KAP1) as a surrogate marker for DNA damage [432]. Olaparib treatment inflicted DNA damage in BRCA2-deficient cells already at the lower concentration whereas BRCA2-proficient cells required a high concentration of olaparib to induce enough damage to be detected by the phospho-KAP1 read out.

A recent report [433] investigated the effect of olaparib treatment on BRCA1-deficient murine and human mammary carcinoma cell lines. Similar to our observations, treatment inflicted DNA damage followed by cGAS-STING activation more prominently in BRCA1-deficient than -proficient cells.

Similarly, a previous study was published in which treatment of the BRCA2-proficient ovarian cancer cell line HOC1 with talazoparib was described to induce cGAS-STING signalling [434]. However, the authors did not directly compare the effect of talazoparib on BRCA2-proficient with -deficient cells.

Next, we investigated the effect of inhibiting PARP on cell cycle progression (Figure 20c). In the absence of treatment, upon loss of BRCA2, cells arrested in G1 and, accordingly, fewer cells were in S or G2/M as described above (Figure 8). Independent of the BRCA2 status, treatment with olaparib increased the numbers of cells in G2/M in a dose-dependent manner consistent with previous reports [249,435-437].

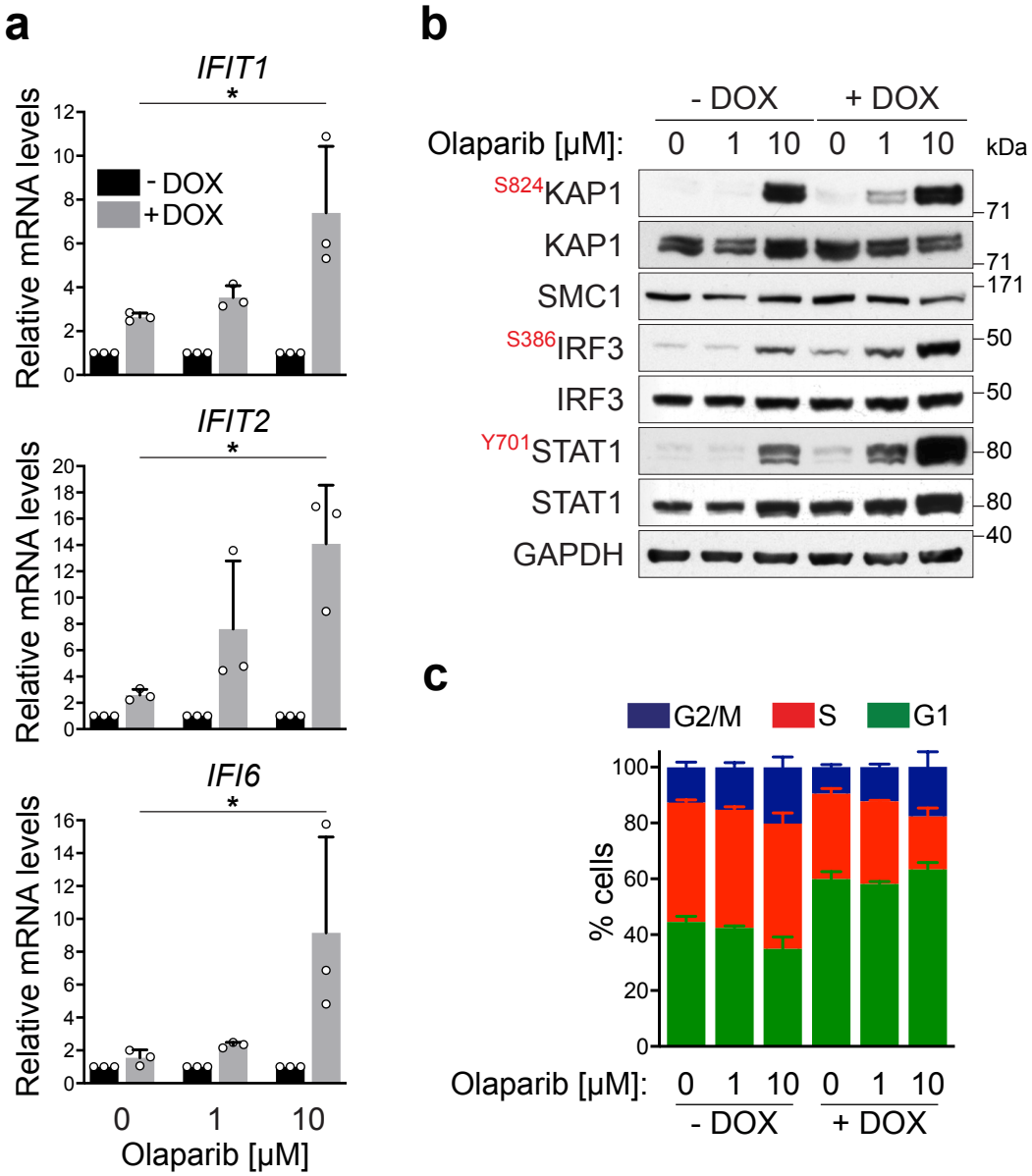



**Figure 20: Effect of PARP inhibitor treatment on activation of type I IFN response in BRCA2-deficient cells**

H1299+BRCA2sh$^{DOX}$ were cultured in presence (+DOX) or absence (-DOX) of 2 μg/ml DOX for 4 days and then treated with olaparib at indicated concentrations for 3 days. (a) Total RNA was extracted and subjected to qPCR analysis using primers specific for indicated genes. mRNA levels were expressed relative to mRNA levels encoding β-actin and to -DOX samples. Values were expressed as $2^{-\Delta\Delta C_t}$. Error bars represent s.d. of $n = 3$ independent experiments, each performed in technical triplicates. *, $p < 0.05$ (unpaired two-tailed t-test). Each dot represents mean of technical triplicates. (b) Whole-cell extracts were immunoblotted as indicated. GAPDH and SMC1 were used as loading controls. (c) Cells were pulse-labelled with EdU for 30 min and DNA was stained with propidium iodide. Percentage of cells in G1, S and G2/M phase was determined via flow cytometry analysis. Error bars represent s.d. of $n = 3$ independent experiments.

In summary, these data together with the published reports suggest that olaparib inflicts more DNA damage in BRCA2-deficient than in -proficient cells. The resulting damage induces a G2/M arrest. Conceivably, if such damage is carried over into mitosis, chromosomal missegregation and micronuclei formation will follow. Thereby, olaparib promotes induction of the type I IFN response. Importantly, activation of cGAS-STING signalling requires lower concentrations of olaparib in BRCA2-deficient compared to -proficient cells.

We showed that treatment with PARP inhibitor promotes a type I IFN response in BRCA2-deficient cells *in vitro*. Next, we investigated whether this effect can be recapitulated *in vivo*. To this end, we generated two different xenograft models.

In the first model, we used isogenic *BRCA2*$^{+/+}$ and *BRCA2*$^{-/-}$ human colorectal carcinoma HCT116 cells [353] and injected them intramuscularly into the hind leg muscles of male CB17/SCID mice (Figure 21). In the second model, we injected BRCA1-compromised MDA-MB-436 mammary breast cancer cells into the mammary fat pad of female CB17/SCID mice. A 5396+1G>A mutation in the *BRCA1* gene of these cells abrogates protein expression (Figure 22) [438].

In each model, talazoparib was administered daily for five days. Then, the mice were given a 2-day break followed by another five days of daily drug administration. Eventually, tumours were removed and processed for qPCR analysis. Upon treatment with PARP inhibitor, expression of ISGs *IFIT1*, *IFIT2*, *IFI6*, *OAS1*, *OAS2* and *ISG15* was upregulated specifically in BRCA2-deficient tumours but not in BRCA2-proficient tumours (Figure 21). In concordance, treatment of BRCA1-deficient tumours induced an upregulation of ISGs *IFIT1*, *IFI6*, *OAS1*, *OAS2* and *ISG15* (Figure 22).

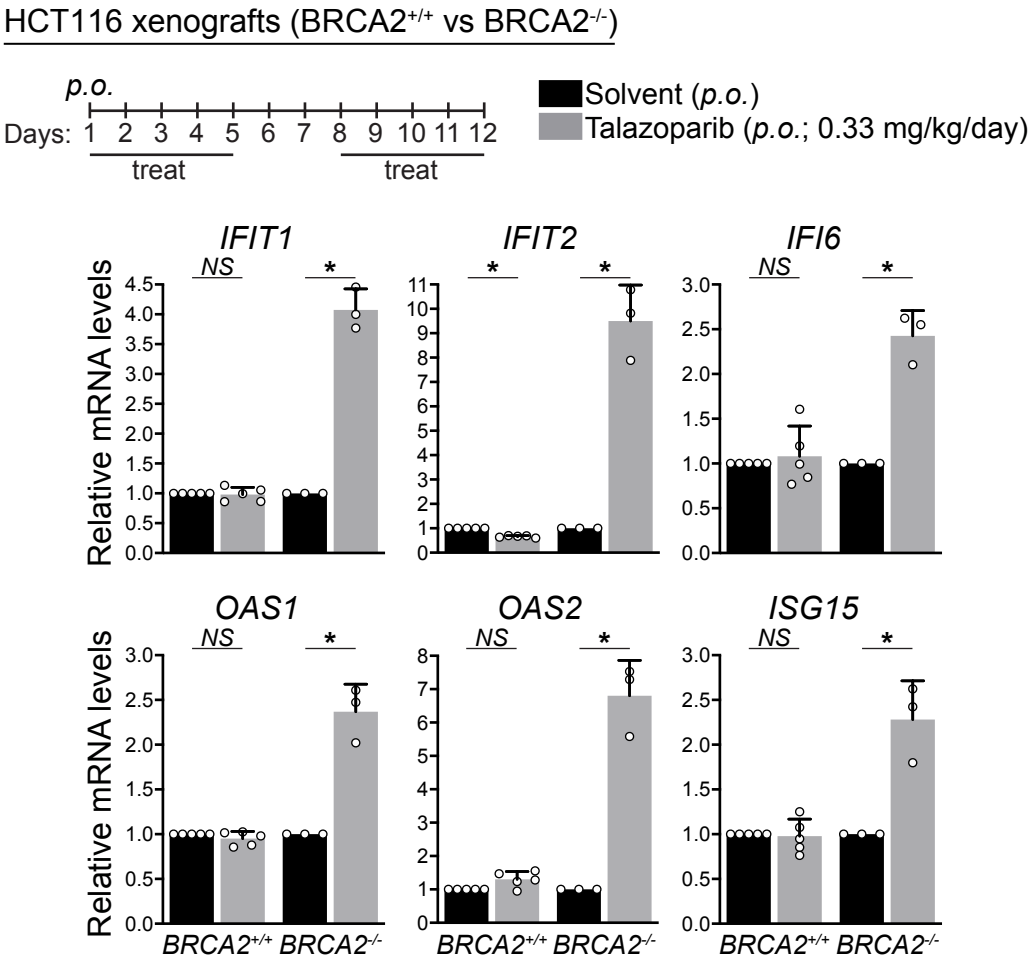



**Figure 21: Effect of PARP inhibitor treatment on ISG expression in *BRCA2*-mutated xenograft tumours**
HCT116 (*BRCA2*$^{+/+}$ and *BRCA2*$^{-/-}$) were injected intramuscularly into the hind leg muscles of male mice. Talazoparib was administered orally (*p.o.*) at a dose of 0.33 mg/kg per day for five consecutive days. After a 2-day break, administration was repeated for another five days. Tumours were excised, total RNA was extracted and subjected to qPCR analysis using primers specific for indicated genes. mRNA levels were expressed relative to mRNA levels encoding β-actin and to solvent-treated samples. Values were expressed as $2^{-\Delta\Delta C_t}$. Error bars represent s.d. of $n = 5$ (*BRCA2*$^{+/+}$) or $n = 3$ (*BRCA2*$^{-/-}$) independent experiments, each performed in technical triplicates. *, $p < 0.05$ (unpaired two-tailed t-test). Each dot represents mean of technical triplicates.

These data suggest that treatment with PARP inhibitor talazoparib promotes activation of the type I IFN response in BRCA1- and BRCA2-deficient tumours. Therefore, these *in vivo* experiments recapitulate our *in vitro* observations.

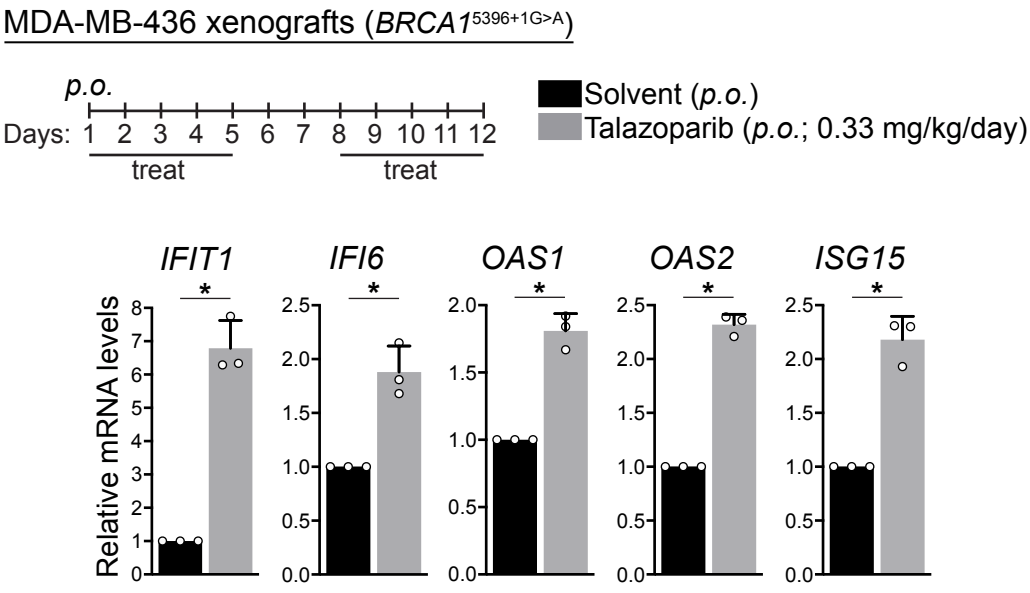



**Figure 22: Effect of PARP inhibitor treatment on ISG expression in *BRCA1*-mutated xenograft tumours**

MDA-MB-436 (*BRCA1*$^{5396+1G>A}$) were injected into the mammary fat pad of female mice. Talazoparib was administered orally (*p.o.*) at a dose of 0.33 mg/kg per day for five consecutive days. After a 2-day break, administration was repeated for another five days. Tumours were excised, total RNA was extracted and subjected to qPCR analysis using primers specific for indicated genes. mRNA levels were expressed relative to mRNA levels encoding GAPDH and to solvent-treated samples. Values were expressed as $2^{-\Delta\Delta C_t}$. Error bars represent s.d. of $n = 3$ independent experiments, each performed in technical triplicates. *, $p < 0.05$ (unpaired two-tailed t-test). Each dot represents mean of technical triplicates.

# 4 Conclusion and outlook

The tumour suppressor BRCA2 is an essential protein. As a key factor of the HR pathway, it is involved in DSB repair. Additionally, it promotes DNA replication particularly by protecting stalled replication forks from nucleolytic degradation. Therefore, loss of BRCA2 is associated with accumulation of DNA damage and induction of senescence or apoptosis.

Monoallelic germline mutations in *BRCA2* predispose to breast, ovarian and other cancers. Loss of heterozygosity is believed to promote tumourigenesis due to inflicted genome instability. Here, loss of BRCA2 is paradoxically associated with accelerated proliferation. Induction of senescence or apoptosis is evaded by preceding mutations disrupting p53 signalling and thereby permitting tumourigenesis.

Although loss of p53 allows proliferation of tumour cells upon loss of BRCA2, accumulation of chromosomal instability is not circumvented. Therefore, tumours deficient for BRCA2 are characterised by gross genome instability. We set out to investigate how p53-deficient cells react to the loss of BRCA2 on a transcriptional level. To this end, we employed a cell model which allowed us to induce inhibition of BRCA2 expression. Upon short-term (4 days) and long-term (28 days) BRCA2 depletion, we assessed transcriptional changes via RNA-seq.

We found that acute loss of BRCA2 (4 days) disturbs cell cycle progression. According to our RNA-seq data, genes associated with cell cycle, organelle fission, chromosome segregation, DNA replication and DNA repair were significantly downregulated. Furthermore, we confirmed that BRCA2 abrogation inflicted G1 arrest and that BRCA2-deficient cells proliferated slower than

their -proficient counterparts. Many open questions remain and it will be interesting to further investigate the downregulated genes in the context of BRCA2 deficiency. What molecular pathways triggered the observed downregulation? Why are these genes in particular downregulated upon loss of BRCA2? Does a downregulation of these genes sustain viability of BRCA2-deficient cells? As an immediate next step, validation of the downregulated genes via qPCR is necessary though.

It has been described before that replication stress due to loss of BRCA2 is associated with DNA under-replication which results in abnormalities during mitosis [376,377]. In the future, it will be interesting to further understand in detail how a BRCA2-deficient cell reacts to under-replicated DNA during mitosis. A rescue pathway called mitotic DNA synthesis (MiDAS) was described to enable DNA replication of such under-replicated DNA regions before chromosome segregation [439]. Furthermore, our group has reported before that BRCA2-deficient cells rely on endonuclease activity of MUS81 to perform MiDAS [377]. It will be further interesting to fully elucidate this process. In particular, it is unknown which sites of the genome are prone to under-replication in BRCA2-deficient cells.

In contrast to the acute response to loss of BRCA2, our RNA-seq analysis revealed that the transcriptome of chronically BRCA2-depleted cells (28 days) was characterised by an upregulation of ISGs. These genes are associated with a type I IFN response. We committed to investigating the mechanism behind this observation. Earlier publications have established micronuclei formation as a link between genome instability and the type I IFN response [394,395]. The authors described a cytosolic DNA sensor called cGAS which translocates into

micronuclei where it is activated by binding to genomic DNA. This, in turn, cascades into a type I IFN response.

Since BRCA2 is involved in HR and DNA replication, it is important during S and G2 phases of the cell cycle. Loss of BRCA2 inflicts DNA damage, in particular in these cell cycle phases. When DNA damage is carried over into mitosis, lagging chromosomal fragments might not be integrated into the newly formed daughter nucleus during telophase but remain in the cytoplasm. Here, they are enclosed by a fragile membrane. The resulting cytosolic compartment in the daughter cell is referred to as a micronucleus. We further confirmed that loss of BRCA2 leads to formation of micronuclei which co-localise with cGAS on a sub-cellular level. Furthermore, the number of cGAS-infiltrated micronuclei increased over time which correlated with a temporal increase in ISG transcripts. In a previous report, cell cycle arrest in G0 via serum starvation abolished both formation of micronuclei and induction of a type I IFN response [395]. Together with our temporal description, this highlights the requirement of proliferation for induction of a type I IFN response upon loss of BRCA2.

Now, activation of the cGAS-STING pathway results in expression of type I IFNs which are secreted and activate via their receptors the JAK-STAT pathway driving expression of ISGs. The function of ISG products as anti-viral proteins is well characterised [381]. In the future, it will be interesting to investigate whether some of these ISG products act directly in response to genome instability. In order to elucidate potential novel functions of ISGs, an initial systematic approach could involve a CRISPR- or siRNA-screen using a library against ISGs.

In this study, we began investigating the role of one of the top-upregulated genes, *TNFRSF1B*, encoding for TNFR2. Our preliminary data indicate that TNFR2

expression promotes cell viability of BRCA2-depleted H1299. Importantly, further experiments are necessary to validate this observation. Also, the relation of TNFR1 and TNFR2 must be further taken into consideration since they share the same ligand but the outcome of their activation can be contradictive. Interestingly, data from a recent report indicated that TNFR2 signalling promotes repair of DNA damage induced by topoisomerase II inhibitor doxorubicin in MDA-MB-231 and MCF-7 cell lines [440]. It will be further interesting to investigate the role of TNFR2 signalling upon loss of BRCA2.

Malignant transformation is driven by genomic instability. Therefore, early detection and elimination of cells with accumulating genomic instability is a crucial barrier against tumourigenesis. As described above, the DDR serves as a key cell-intrinsic surveillance mechanism counteracting genome instability. An alternative but not mutually exclusive surveillance mechanism is operated by the immune system. Upon genome instability, a cell-intrinsic type I IFN response is activated which promotes clearance of the faulty cell by the immune system. [441,442] Conceivably, upon loss of heterozygosity in a single cell of a *BRCA2* germline mutation carrier, accumulating genomic instability induces clearance of that cell. Under certain circumstances, such a cell evades its fate and undergoes malignant transformation. For example, surveillance by the DDR could be evaded via a preceding mutation in *TP53*. Similarly, evasion of immune surveillance could be achieved via mutations in factors of the type I IFN response such as cGAS or STING. In the future, it will be interesting to investigate whether breast and ovarian tissue is more prone to immune evasion. In the end, this could shed light on tissue predisposition of cancers in *BRCA2* mutation carriers.

Due to their selective cytotoxicity, PARP inhibitors are approved for treatment of *BRCA2*-mutated tumours. However, acquired resistance remains a challenge in the clinic. Therefore, it is important to fully elucidate the cellular consequences of PARP inhibitor treatment. We set out to investigate its effect on the type I IFN response in BRCA2-deficient cells. To this end, we treated cells with the PARP inhibitor olaparib upon acute loss of BRCA2 when the type I IFN response has not been majorly upregulated. Treatment with PARP inhibitors inflicted DNA damage in BRCA2-proficient and -deficient cells in a dose-dependent manner. However, in BRCA2-deficient cells, lower doses were sufficient to induce damage. Furthermore, this damage correlated with an induction of a type I IFN response. At a lower dose of PARP inhibitor, the cGAS-STING and JAK-STAT pathway were upregulated in BRCA2-deficient cells only but not in -proficient cells. At a higher concentration of inhibitor, the type I IFN response was also inflicted in BRCA2-proficient cells. Moreover, we confirmed these observations in xenograft models of *BRCA1*- and *BRCA2*-mutated tumours. Therefore, treatment with PARP inhibitors might allow a selective induction of a type I IFN response in BRCA-deficient tumours in the clinic.

Consequently, there is a direct translational aspect of our findings. The main function of the type I IFN response is to promote and activate the adaptive immune system [442]. Promoting adaptive immunity, especially an anti-tumour T cell response, has proven beneficial outcomes in the clinic. This approach is mainly based on inhibition of immune checkpoint factors, such as cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), programmed cell death 1 (PD-1), or programmed death-ligand 1 (PD-L1). [443-445] Furthermore, combination of

inhibitors of immune checkpoint factors together with targeted induction of an innate immune response might be a promising treatment regime.

Promoting a type I IFN response has great potential to promote an anti-cancer immune response. In fact, many pharmaceutical companies are developing STING agonists. For example, the compound 5,6-dimethylIxanthenone-4-acetic acid (DMXAA) is an agonist of murine STING [446]. In a melanoma mouse model, DMXAA promoted T cell infiltration and tumour rejection [447]. However, it does not activate human STING which was the reason for its failure in a phase III clinical trial [446]. Further effort led to the discovery of human-specific STING agonists such as ADU-S100 [448]. By activating a type I IFN response, ADU-S100 was shown to promote an anti-tumour response by T cells. Furthermore, PD-1 inhibition synergised with STING activation.

However, in order to avoid anticipated systemic inflammatory side effects of STING agonists, a tumour targeted induction of the cGAS-STING pathway is arguably more desirable. Enhancing cell-intrinsic DNA damage in tumours allows such a targeted approach. As shown by our study, induction of DNA damage in BRCA2-deficient tumour cells, for example by PARP inhibitor treatment, potentiates the innate immune response. Similarly, another research group investigated the efficacy of olaparib in a BRCA1-deficient tumour mouse model [433]. They used immuno-competent and -compromised mice and observed that PARP inhibitor treatment was more effective against tumours in mice with an intact immune system. They further showed that treatment increased the numbers of infiltrating lymphocytes and that their recruitment relied on cGAS-STING activation. Another study reported that PARP inhibitor niraparib and anti-PD-1 antibody pembrolizumab synergised in both BRCA1-proficient

and -deficient murine tumour models [449]. Furthermore, combination treatment conferred a prolonged response. Even tumours implanted after the end of treatment, when mice were tumour-free, did not grow. The same combination was efficient in a clinical study on ovarian cancer including BRCA-proficient and -deficient tumours [450].

Targeted induction of DNA damage in tumour cells could provide a novel neoadjuvant strategy for cancer immunotherapy. Therefore, fully understanding the link of genome instability and induction of an innate immune response might elucidate a powerful tool for future cancer treatments.

# 5 Material and methods

## 5.1 Cell lines

Cell lines were cultivated as monolayers at 37˚C in 5% $CO_2$. Human non-small cell lung carcinoma cells H1299 and invasive ductal breast cancer cells MDA-MB-231 were grown in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich #D5796) supplemented with tetracycline-free 10% foetal bovine serum (Clontech #631106, sterilely filtered). For induction of shRNA expression against BRCA2 (GGG AAA CAC UCA GAU UAA A), ERK1 (GCC ATG AGA GAT GTC TAC A) or ERK2 (GAG GAT TGA AGT AGA ACA G), DOX (Sigma-Aldrich #D9891) was added to growth medium at 2 µg/ml. [350]

## 5.2 RNA extraction and sequencing

The RNeasy® Mini Kit (Qiagen #74104 and #74106) was used according to the manufacturer's instructions. First, RNA samples were quantified using RiboGreen (Invitrogen) and the FLUOstar® Omega plate reader (BMG Labtech). Size profile and integrity were analysed using the Agilent 2200 and 4200 TapeStation system (Agilent). For all samples, RNA integrity number estimates were between 9.0 and 10.0. Prior to library preparation, input material was normalised to 1 µg.

The TruSeq® Stranded mRNA kit (Illumina) was used according to the manufacturer's instructions for poly(A) transcript enrichment and strand-specific library preparation. Libraries were amplified over 15 cycles on a Tetrad® (Bio-Rad) using in-house unique dual indexing primers [451]. Individual libraries were normalized using Qubit assays. The size profile was analysed using the Agilent 2200 and 4200 TapeStation system (Agilent). For storage, individual

libraries were pooled together and diluted to approximately 10 nM. Prior to loading on the sequencer, each library aliquot was denatured and further diluted. Using a HiSeq4000 75 base pair platform (Illumina, HiSeq 3000/4000 PE Cluster Kit and 150 cycle SBS Kit), paired-end sequencing was performed resulting in a raw read count of > 22 x $10^6$ reads per sample.

## 5.3 RNA-seq data processing

A version [452] of the algorithm hierarchical indexing for spliced alignment of transcripts (HISAT), HISATx2, was used in order to align the sequencing reads to a human reference genome (hg19/GRCh37). Duplicated reads were removed using the Picard tool MarkDuplicates (Broad Institute). In order to uniquely assign reads to annotated genes, the programme *featureCounts* was used [453]. For further analysis, Bioconductor was used [454]. Genes with less than approximately 10 reads in more than three samples were neglected in order to control for bias in the subsequent differential expression analysis. This resulted in sets of approximately 14,000 to 15,000 genes which were assessed for differential gene expression.

## 5.4 Differential gene expression analysis

For RNA-seq data analysis, *DESeq2* was used (version 1.18.1) [455]. In order to apply a FDR < 0.05, Benjamini-Hochberg procedure was used to adjust *p*-values. For each comparison, hierarchical clustering analysis was applied for the top 500 differentially expressed genes using Euclidean distances. Differentially expressed genes common to various data sets were identified by performing gene set intersections and assessed using hypergeometric tests. The Gene Set

Enrichment Analysis (GSEA) method was used in order to perform gene ontology (GO) enrichment analysis [456].

## 5.5 Population doubling

A resazurin-based assay was used to determine population doubling. Cells were incubated with 10 µg/ml resazurin (Sigma-Aldrich #R7017) in growth medium at 37˚C for 2 h. Afterwards, fluorescence was measured (544 nm excitation and 590 nm emission) using POLARstar® Omega plate reader (BMG Labtech). For population doubling, measurements were repeated at 2-day intervals and related to day 0.

## 5.6 Transfection with siRNA

Before transfection, siRNAs were re-suspended in siRNA Buffer (Thermo Fisher Scientific #B-002000-UB-100 and #B-003000-WB-100) to 40 µM (Table 1). For transfection, DharmaFECT-1 (Dharmacon™ #T-2001-03) was mixed 1:100 with Opti-MEM™ (Thermo Fisher Scientific #31985-062) and incubated at room temperature (RT) for 5 min. Re-suspended siRNA (in siRNA buffer at 40 µM) was mixed 1:100 with Opti-MEM™. Both solutions were mixed 1:1 and incubated at RT for 20 min. The final transfection solution was added to cells in growth medium (1:5, leading to a final siRNA concentration of 40 nM). Medium was replaced the next day with growth medium. AllStars Negative Control siRNA (Ctr) was used (Qiagen #1027281).

**Table 1: siRNA – SMARTpools provided by Dharmacon™**

| siRNA - SMARTpool | Sense Sequences |
|---|---|
| Human MB21D1 (115004) #M-015607-01-0005 | |
| D-015607-01 | GAA GAA ACA UGG CGG CUA U |
| D-015607-02 | GAA GAG AAA UGU UGC AGG A |
| D-015607-03 | GUA AGG AAU UUC UGA CAA A |
| D-015607-04 | CAA CAC UCG UGC AUA UUA C |
| Human STAT1 (6772) #L-003543-00-0005 | |
| J-003543-06 | GCA CGA UGG GCU CAG CUU U |
| J-003543-07 | CUA CGA ACA UGA CCC UAU C |
| J-003543-08 | GAA CCU GAC UUC CAU GCG G |
| J-003543-09 | AGA AAG AGC UUG ACA GUA A |
| Human TMEM173 (340061) #L-024333-00-0005 | |
| J-024333-05 | GGU CAU AUU ACA UCG GAU A |
| J-024333-06 | AAC AUU CGC UUC CUG GAU A |
| J-024333-07 | GCA UCA AGG AUC GGG UUU A |
| J-024333-08 | GCA CCU GUG UCC UGG AGU A |
| Human TNFRSF1B (7133) #M-0039034-00-0005 | |
| D-003934-01 | GCA CAU GCC GGC UCA GAG A |
| D-003934-02 | CAU CAG ACG UGG UGU GCA A |
| D-003934-03 | UCA CUU GCC UGC CGA UAA G |
| D-003934-04 | UAA UAG GAG UGG UGA ACU G |

## 5.7 Western blotting (WB)

Cells were lysed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (0.16 M Tris pH 8 (Thermo Fisher Scientific #BPE1762-1), 4% SDS (Thermo Fisher Scientific #BP1311-200), 20% glycerol (MP Biomedical #02193996), 0.01% bromophenol blue (Thermo Fisher Scientific #B392-5), 100 mM dithiothreitol (VWR #441494N)). Loading buffer was

supplemented with 1 tablet of phosphatase inhibitor cocktail (Roche #04 906845 001) together with 1 tablet of protease inhibitor cocktail (Roche #04 693 159 001) per 8-10 ml loading buffer. Samples were sonicated for 2 to 5 s using the Ultrasonic Homogeniser 300 V/T (Biologics Inc.) and then incubated at 70˚C for 10 min. Samples were centrifuged at > 20,000 x g for 7 min. Protein concentration was determined using Spectrophotometer ND-1000 (NanoDrop Technologies Inc.). Equal amounts of protein were loaded onto Bis-Tris or Tris-Acetate gels (Thermo Fisher Scientific #NP0322, #EA03752 or #EA03785) submerged in MOPS or Tris-Acetate SDS running buffer, respectively (Thermo Fisher Scientific #N0001 or #LA0041). A voltage of 180 V was applied using the XCell SureLock™ Mini-Cell (Thermo Fisher Scientific #EI0001). Proteins were transferred onto a nitrocellulose membrane (GE Healthcare Life Sciences #10600001) applying 30 V in transfer buffer (Thermo Fisher Scientific #NP0006-1) supplemented with 10% methanol. Membrane was blocked with 5% dried skimmed milk in PBST (0.05% Tween 20 (Sigma-Aldrich #P7949) in phosphate-buffered saline (PBS, Thermo Fisher Scientific #BR0014G)) at RT for 1 h and subsequently incubated in primary antibody diluted in 2% bovine serum albumin (BSA, Sigma-Aldrich #A7906) and 0.05% azide in PBST at 4˚C overnight (Table 2). The membrane was washed with PBST three times at RT for 10 min and subsequently incubated in secondary antibody coupled to horseradish peroxidase diluted 1:5,000 in blocking buffer at RT for 1 h (Table 3). The membrane was washed in PBST three times at RT for 10 min. Enhanced chemiluminescence WB detection reagent (Thermo Fisher Scientific #32106; Millipore #WBKLS0100/-500; GE Healthcare #RPN2235) was applied on the membrane followed by detection on X-ray films (Fujifilm #4741019289; Thermo

Fisher Scientific #34089) using the CP1000 developer (AGFA) or the X-ray film processor ECOMAX™ (PROTEC #1186-3-4000).

**Table 2: Primary antibodies used for WB**

| Specificity | Host and Isotype | Clone | Provider | Catalogue Number | Dilution |
|---|---|---|---|---|---|
| BRCA2 | Mouse IgG2b | 2B | Merck | OP95 | 1:1,000 |
| cGAS | Rabbit IgG | D1D3G | Cell Signaling | 15102 | 1:1,000 |
| ERK1/2 | Rabbit IgG | 137F5 | Cell Signaling | 4695 | 1:5,000 |
| GAPDH | Mouse IgG1 | 6C5 | Novus Biologicals | NB600-502 | 1:30,000 |
| IRF3 | Rabbit IgG | EP2419Y | Abcam | AB76409 | 1:1,000 |
| Phospho-IRF3 | Rabbit IgG | EPR2346 | Abcam | AB76493 | 1:1,000 |
| KAP1 | Rabbit IgG | polyclonal | Bethyl Laboratories | A300-274A | 1:5,000 |
| Phospho-KAP1 | Rabbit IgG | polyclonal | Bethyl Laboratories | A300-767A | 1:1,000 |
| SMC1 | Rabbit IgG | polyclonal | Bethyl Laboratories | A300-055A | 1:5,000 |
| STAT1 | Rabbit IgG | 42H3 | Cell Signaling | 9175 | 1:1,000 |
| Phospho-STAT1 | Rabbit IgG | 58D6 | Cell Signaling | 9167 | 1:1,000 |
| STING | Rabbit IgG | D2P2F | Cell Signaling | 13647 | 1:1,000 |
| α-Tubulin | Mouse IgG | TAT-1 | CRUK Monoclonal Antibody Service | - | 1:30,000 |

**Table 3: Secondary polyclonal antibodies used for WB**

| Specificity | Host and Isotype | Provider | Catalogue Number | Dilution |
|---|---|---|---|---|
| Mouse | Goat IgG | Dako | P0447 | 1:5,000 |
| Rabbit | Goat IgG | Dako | P0448 | 1:5,000 |

## 5.8 Quantitative polymerase chain reaction (qPCR)

For RNA reverse transcription, the SYBR™ Green Cells-to-$C_T$™ Kit (Thermo Fisher Scientific #4402954) was used according to manufacturer's instructions. Resulting complementary DNA (cDNA) was diluted 1:10 with diethyl pyrocarbonate (DEPC)-treated water (Thermo Fisher Scientific #AM9906). Per

reaction of qPCR, 2 µl of cDNA was mixed with 5 µl SensiMix™ SYBR® No-ROX Kit (Bioline #QT650-05), 0.8 µl of primers (mix of forward primer and reverse primer at 10 µM (Table 4)) and 2.2 µl of DEPC-treated water (Thermo Fisher Scientific #AM9906). Reactions were performed using QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific #A28140) programmed as in Table 5.

**Table 4: Primer pairs used for qPCR**

| Target | Sense | Sequence |
|---|---|---|
| β-actin | Forward | ATT GGC AAT GAG CGG TTC |
| | Reverse | GGA TGC CAC AGG ACT CCA T |
| GAPDH | Forward | GAC AGT CAG CCG CAT CTT CT |
| | Reverse | ACC AAA TCC GTT GAC TCC GA |
| IFI6 | Forward | TCG CTG ATG AGC TGG TCT GC |
| | Reverse | ATT ACC TAT GAC GAC GCT GC |
| IFIT1 | Forward | TAC CTG GAC AAG GTG GAG AA |
| | Reverse | GTG AGG ACA TGT TGG CTA GA |
| IFIT2 | Forward | TGT GCA ACC TAC TGG CCT AT |
| | Reverse | TTG CCA GTC CAG AGG TGA AT |
| IFIT3 | Forward | CTT CAG TAT TTA CTT GAG GCA GAC |
| | Reverse | CTT GGT GAC CTC ACT CAT GAC |
| ISG15 | Forward | GCG AAC TCA TCT TTG CCA GTA |
| | Reverse | CCA GCA TCT TCA CCG TCA G |
| OAS1 | Forward | CGC CTA GTC AAG CAC TGG TA |
| | Reverse | CAG GAG CTC AGG GCA TA |
| OAS2 | Forward | TCC AGG GAG TGG CCA TAG |
| | Reverse | TCT GAT CCT GGA ATT GTT TTA AGT C |
| TNFRSF1B | Forward | CGG GAG CTC AGA TTC TTC CC |
| | Reverse | GTC TCC AGC TGT GAC CGA AA |

Gene expression was normalised to housekeeping genes β-actin or GAPDH applying the Livak method [457]. To this end, the $C_t$ (threshold cycle) values were determined and normalised as followed:

$$\Delta C_t = C_t \text{ (target gene)} - C_t \text{ (housekeeping gene)}$$

In order to determine differential gene expression between two samples, $\Delta C_t$ values were relativised as followed:

$$\Delta\Delta C_t = \Delta C_t \text{ (sample 1)} - \Delta C_t \text{ (sample 2)}$$

Finally, values were exponentially transformed and expressed as $2^{-\Delta\Delta C_t}$.

**Table 5: Programming of qPCR reaction**

| Stage | Ramp Rate | Temperature [˚C] | Hold Time |
|---|---|---|---|
| Enzyme activation | 1.6 ˚C/s | 95 ˚C | 10 min |
| PCR cycles (40x) | 1.6 ˚C/s | 95 ˚C | 15 sec |
| | 1.6 ˚C/s | 60 ˚C | 1 min |
| Dissociation curve | 1.6 ˚C/s | 95 ˚C | 15 sec |
| | 1.6 ˚C/s | 60 ˚C | 1 min |
| | 0.075 ˚C/s | 95 ˚C | 15 sec |

## 5.9 EdU incorporation

Asynchronous cells were incubated with 25 µM EdU for 30 min. Cells were then fixed with 90% methanol and incorporated EdU was detected using the Click-iT™ Plus EdU Alexa Fluor™ 647 Flow Cytometry Assay Kit (Thermo Fisher Scientific #C10634) according to manufacturer's instructions. Cells were re-suspended in PBS supplemented with 20 µg/ml propidium iodide (Sigma-Aldrich #P4864) and 400 µg/ml RNase A (Thermo Fisher Scientific #12091-021) and then assessed

using the BD FACSCalibur flow cytometer (BD Biosciences #342975). Per condition, 10,000 events were analysed using FlowJo® software.

## 5.10 Immunofluorescence

Cells were seeded on coverslips and cultured as described above. After washing with PBS, a hypotonic solution (85.5 mM NaCl and 5 mM $MgCl_2$, pH 7.0) was used to swell the cells at RT for 5 min. Cells were then fixed using 4% paraformaldehyde (Electron Microscopy Sciences #15710 or Alfa Aesar #43368) at RT for 10 min, before permeabilising them with 4% paraformaldehyde supplemented with 0.03% SDS (Thermo Fisher Scientific #BP1311-200). Cells were rinsed two times with PBS supplemented with 0.4% Photo-Flo 200 (Sigma-Aldrich #P7417-1PT), before blocking with blocking buffer (1% goat serum (Sigma-Aldrich #G6767), 0.3% BSA (Sigma-Aldrich #A7906), 0.05% Triton™ X-100 (Sigma-Aldrich #X100) in PBS) at RT for 15 min. Rabbit monoclonal antibody against cGAS (1:200, clone D1D3G, Cell Signaling #15102) in blocking buffer was added to the cells at RT overnight. The next day, cells were washed first with blocking buffer supplemented with 0.4% Photo-Flo 200, then with blocking buffer enriched with Triton™ X-100 (0.0625% instead of 0.05%) and finally again with blocking buffer supplemented with 0.4% Photo-Flo 200 at RT for 5 min each. Then, cells were incubated with an Alexa Fluor® 488-conjugated secondary antibody (1:400, Thermo Fisher Scientific #A11008) in blocking buffer at RT for 1 h. Cells were washed two times with PBS supplemented with 0.4% Photo-Flo 200 for 5 min each and then rinsed with distilled $H_2O$ supplemented with 0.4% Photo-Flo 200. Coverslips were dried at RT and finally mounted on microscope slides (Thermo Fisher Scientific #12372098) using ProLong® Gold

antifade reagent with 4′,6-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific #P36935). Images were acquired using a Nikon Plan Apochromat 60x 1.4 NA oil immersion lens on the Dragonfly imaging system (Andor technologies) equipped with 405 nm and 488 nm lasers for visualising DAPI and cGAS. The system is built on a Nikon Ti2-E microscope body with the Nikon Perfect Focus System (Nikon Instruments). Data was collected using the Spinning Disk 40 µm pinhole mode of the Dragonfly on a Zyla 4.2 sCMOS camera using Fusion v2.0.0.15 software (Andor technologies). Resulting image files were first blinded and then each image was quantified manually using Fiji software (≥ 412 nuclei were counted per *n* per condition).

## 5.11 Xenograft models

CB17/SCID mice were 6 weeks old and weighed 26-28 g. They were purchased from Charles River Laboratories (Calco, Italy). All animal procedures complied with the national and international directives (D.L. March 4, 2014, no. 26; directive 2010/63/EU of the European Parliament and of the council; Guide for the Care and Use of Laboratory Animals, United States National Research Council, 2011).

Human colorectal carcinoma cells HCT116, *BRCA2*$^{+/+}$ and *BRCA2*$^{-/-}$, (3 x $10^6$ cells) were injected intramuscularly into the hind leg muscles of male mice. When a tumour volume of approximately 250 $mm^3$ was evident, treatment was initiated. Mammary breast cancer cells MDA-MB-436 with a *BRCA1*$^{5396+1G>A}$ mutation ($10^6$ cells) were injected into the mammary fat pad of female mice. When tumours were palpable (seven days after cell injection), treatment was initiated. Each experimental group included five mice.

Talazoparib (solvent: 6% Solutol HS 15, 10% dimethylacetamide in PBS) was administered orally at a dose of 0.33 mg/kg per day for five consecutive days. After a 2-day break, administration was repeated for another five days. Tumours were excised and total RNA was extracted using TRIzol™ (Thermo Fisher Scientific). Of the 5 animals carrying HCT116 *BRCA2*$^{-/-}$ tumours and the 5 animals carrying MDA-MB-436 tumours, treatment reduced tumour volume to an extent that only 3 out of 5 samples from each model could have been obtained for qPCR analysis.

## 5.12 Statistical analyses

Statistical analyses for all experiments except of RNA-seq analysis were performed using GraphPad Prism 8. Values are reported as mean ± s.d. (standard deviation). For comparison of means, unpaired two-tailed Student's t-test was applied. For statistical analysis of qPCR results, mean $\Delta C_t$ or $\Delta\Delta C_t$ values were compared. A *p*-value of $p < 0.05$ was considered as statistically significant.

# 6 References


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# 7 Appendix

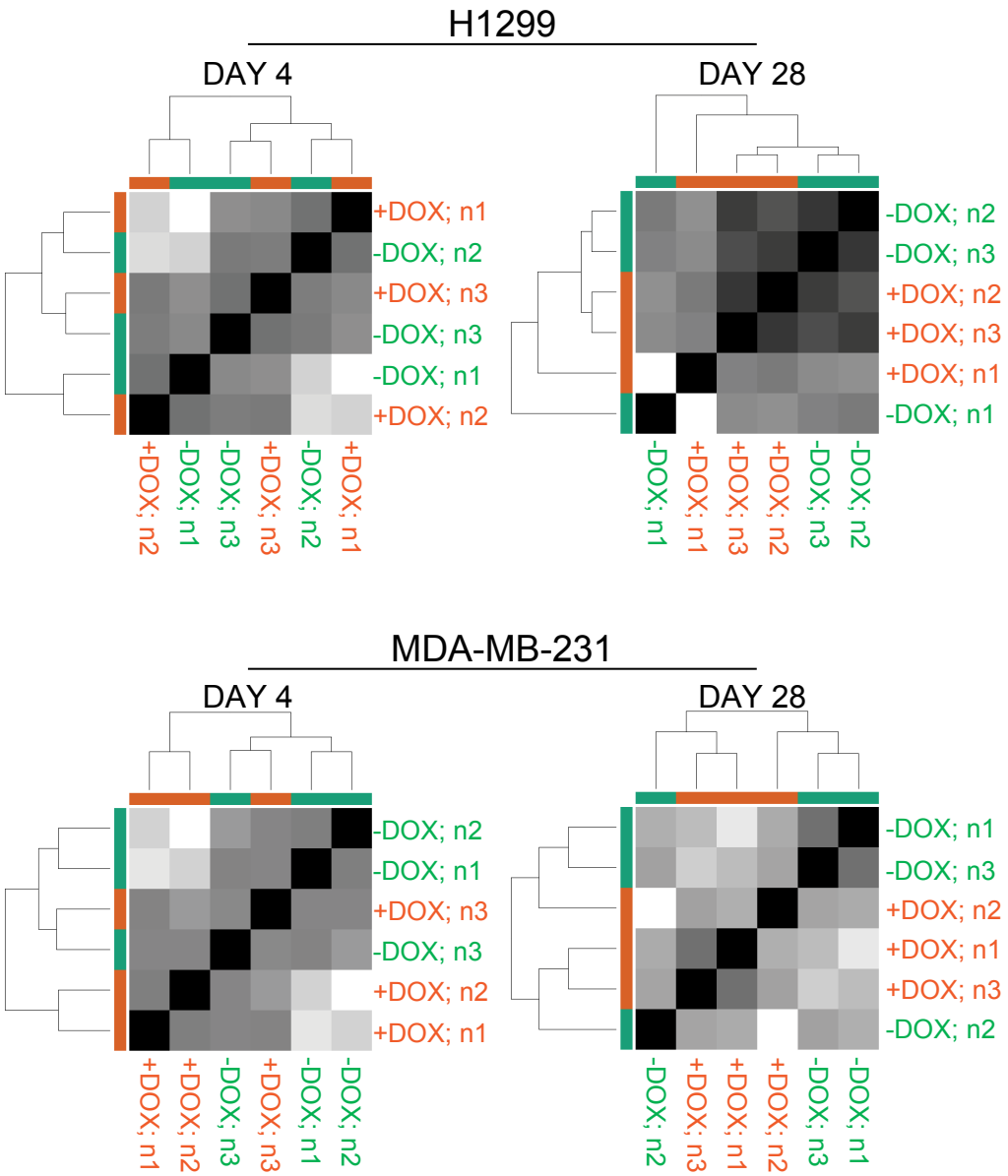
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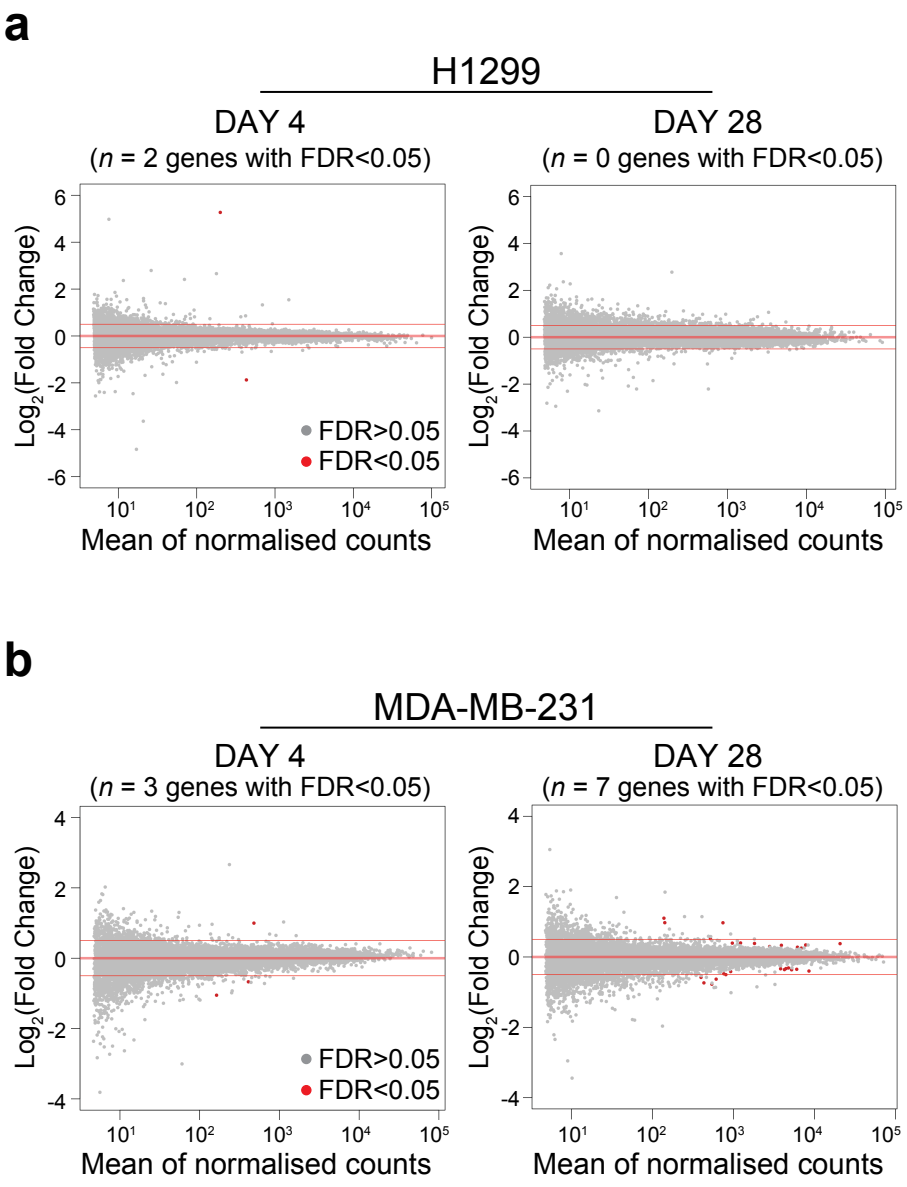
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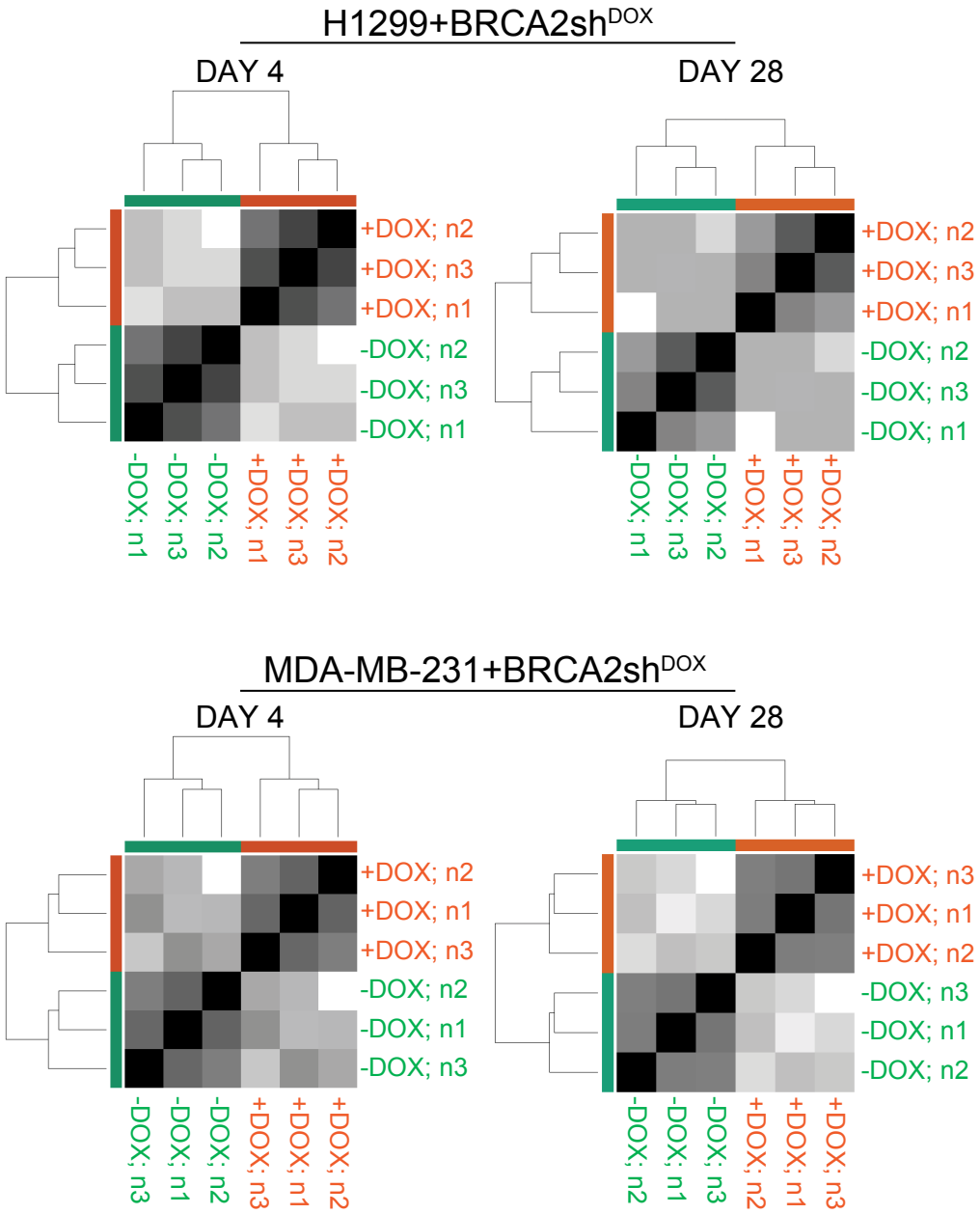
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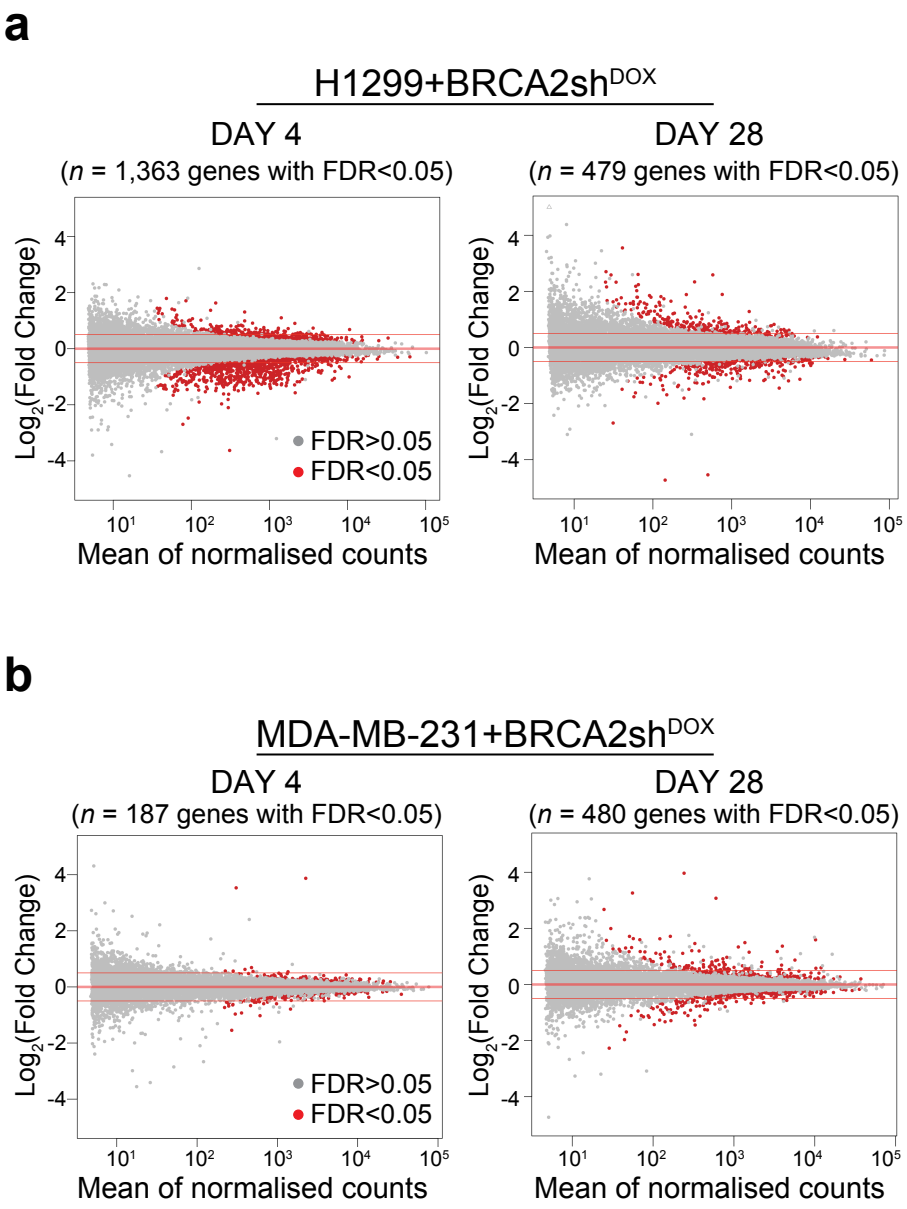

**Supplementary Figure 1: Hierarchical cluster analysis of differentially expressed genes in H1299 and MDA-MB-231 upon DOX treatment**
H1299 and MDA-MB-231 were cultured in presence (+DOX) or absence (-DOX) of 2 µg/ml DOX for 28 days. After 4 and 28 days, samples for RNA-seq were collected. Hierarchical cluster analysis of sample-to-sample distances was performed based on the RNA-seq data. *n* = 3 independent experiments.




**Supplementary Figure 2: Differentially expressed genes in H1299 and MDA-MB-231 upon DOX treatment**
(a) H1299 and (b) MDA-MB-231 were cultured in presence (+DOX) or absence (-DOX) of 2 µg/ml DOX for 28 days. After 4 and 28 days, samples for RNA-seq were collected. Each dot of the MA plot represents a differentially expressed gene. Red, significantly altered expression value (FDR < 0.05). Grey, not significantly altered expression value (FDR > 0.05). *n* = 3 independent experiments.




**Supplementary Figure 3: Hierarchical cluster analysis of differentially expressed genes in H1299+BRCA2sh$^{DOX}$ and MDA-MB-231+BRCA2sh$^{DOX}$ upon DOX treatment**
H1299+BRCA2sh$^{DOX}$ and MDA-MB-231+BRCA2sh$^{DOX}$ were cultured in presence (+DOX) or absence (-DOX) of 2 µg/ml DOX for 28 days. After 4 and 28 days, samples for RNA-seq were collected. Hierarchical cluster analysis of sample-to-sample distances was performed based on the RNA-seq data. *n* = 3 independent experiments.




**Supplementary Figure 4: Differentially expressed genes in H1299+BRCA2sh$^{DOX}$ and MDA-MB-231+BRCA2sh$^{DOX}$ upon DOX treatment**

(a) H1299+BRCA2sh$^{DOX}$ and (b) MDA-MB-231+BRCA2sh$^{DOX}$ were cultured in presence (+DOX) or absence (-DOX) of 2 µg/ml DOX for 28 days. After 4 and 28 days, samples for RNA-seq were collected. Each dot of the MA plot represents a differentially expressed gene. Red, significantly altered expression value (FDR < 0.05). Grey, not significantly altered expression value (FDR > 0.05). *n* = 3 independent experiments.