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BRCA1 DEFICIENCY EXACERBATES ESTROGEN INDUCED DNA DAMAGE AND GENOMIC INSTABILITY

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Abstract

Germline mutations in BRCA1 predispose carriers to a high incidence of breast and ovarian cancers. BRCA1 functions to maintain genomic stability through critical roles in DNA repair, cell cycle arrest and transcriptional control. A major question has been why BRCA1 loss or mutation leads to tumors mainly in estrogen-regulated tissues, given that BRCA1 has essential functions in all cell types. Here we report that estrogen and estrogen metabolites can cause DNA double strand breaks (DSB) in estrogen receptor- α negative breast cells and that BRCA1 is required to repair these DSBs to prevent metabolite-induced genomic instability. We found that BRCA1 also regulates estrogen metabolism and metabolite-mediated DNA damage by repressing the transcription of estrogen-metabolising enzymes, such as CYP1A1, in breast cells. Lastly, we used a knock-in human cell model with a heterozygous BRCA1 pathogenic mutation to show how BRCA1 haploinsufficiency affects these processes. Our findings provide pivotal new insights into

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why BRCA1 mutation drives the formation of tumours in estrogen-regulated tissues, despite the general role of BRCA1 in DNA repair in all cell types.

Keywords

BRCA1; Estrogen; Estrogen Metabolism; DNA damage; Genomic Instability

INTRODUCTION

BRCA1 is a tumour suppressor protein which functions to preserve genomic stability by regulating key cellular processes including homologous recombination (HR) mediated DNA repair, cell cycle checkpoint control, transcriptional regulation, chromatin remodelling and post-replicative repair (1-3). Despite playing a role in processes essential to all cells, *BRCA1* mutation predisposes to tumours predominantly in estrogen regulated tissues, such as the breasts and ovaries. Indeed, germ-line mutations in a single *BRCA1* allele confer a lifetime risk of up to 90% of developing breast cancer and 30-40% of ovarian cancer (4, 5).

Several observations suggest estrogen has an important role in the development of BRCA1-dependent breast cancer. Pre- or post-menopausal oophorectomy in *BRCA1* mutation carriers significantly reduces the risk of breast cancer onset and recurrence (6-8). Furthermore, pregnancy increases the risk of early-onset breast cancer in *BRCA1* mutation carriers, in contrast to non-carriers for whom pregnancy is protective (9). It has also been reported that BRCA1 represses the expression of CYP19A1 (aromatase), which converts androgens to bioactive estrogens (10). Thus BRCA1 loss may increase CYP19 expression and subsequent estrogen production, further driving tumourigenesis (11).

Estrogen is postulated to promote tumourigenesis directly through stimulation of the estrogen receptor- α (ER α) and the downstream activation of pro-mitogenic transcriptional programs. However, this is confounded by observations that approximately 70-80% of BRCA1 mutated breast tumours are ER α negative (12, 13). Furthermore, BRCA1 drives ER α expression, suggesting the role of estrogen in BRCA1 dependent tumour development may be independent of ER α (14). Consistent with this, estradiol (E₂; the predominant estrogen) induces tumour formation in ER α knockout mice (15). In these mice, reduction of endogenous E₂, by either oophorectomy or treatment with aromatase inhibitors, delayed tumourigenesis, whereas the ER α antagonist fulvestrant had no effect (15).

The endogenous conversion of estrogen to genotoxic metabolites has been reported as an alternative, potentially ER α independent, mechanism for estrogen-dependent breast tumourigenesis. Estrogen is hydroxylated to form the catechol estrogens 2-hydroxyestradiol (2-OHE₁(E₂)) and 4-hydroxyestradiol (4-OHE₁(E₂)), a process which is catalysed by a number of cytochrome (CYP) P450 enzymes, including CYP1A1, CYP1A2, CYP1B1 and CYP3A4. The catechol estrogens are further oxidised (by the same enzymes) into semi-quinone and quinone forms, the latter of which can react with DNA to form adducts. Interestingly, urinary levels of 2-OHE₂ and 4-OHE₂ are elevated in breast cancer patients compared to healthy controls (16) and 4-OHE₂ concentrations have been reported to be up to 3-times higher in breast cancer biopsies compared to normal breast tissue (17). Moreover,

in-vivo studies have demonstrated that exogenous 2-OHE₂ and 4-OHE₂ can induce kidney and uterine cancers in mice (18, 19). The DNA adducts induced by these metabolites produce apurinic sites in the DNA which require repair, error-prone repair of which can lead to A-T to G-C mutations in DNA in the form of G.T heteroduplexes (20-22). Furthermore, high levels of depurinated estrogen adducts have been observed in serum and urine samples from breast cancer patients and women with a strong family history of breast cancer (23, 24). It has been suggested that these depurinating adducts are repaired through the nucleotide excision repair (NER) and base excision repair (BER) pathways, however, a study which examined chromosomal aberrations in DT40 cells after treatment with 4-OHE₂, observed no difference between wild-type cells and cells depleted of XPA, a key protein in NER (25, 26). In contrast, there were enhanced chromosomal breaks following 4-OHE₂ treatment of *rad54* and *ku70* mutant DT40 cells, both of which are required for repair of DSBs by HR and NHEJ, respectively. This suggests that estrogen metabolites may produce DNA DSBs.

The idea that estrogen metabolites may cause DNA DSBs, coupled with the role of BRCA1 in DSB repair, lead us to hypothesise that BRCA1-deficient cells, may be more susceptible to estrogen metabolite induced DNA damage and subsequent genomic instability. We therefore examined whether estrogen and its metabolites 2-OHE₂ and 4-OHE₂ can cause DSBs in human breast cells and examined the role of BRCA1 in both the induction and repair of estrogen metabolite induced DNA damage.

MATERIALS AND METHODS

Cell lines

MCF7 and MCF10A cells were obtained from ATCC and maintained according to the recommended instructions. MCF10A BRCA1 +/- 185delAG and matched control BRCA1 +/+ cells were generated as previously described (27). All cell-lines were verified by STR profiling.

siRNAs

siRNAs were obtained from Qiagen and reverse transfected into cells using RNAiMAX (Invitrogen) to a final concentration of 10nM. See Supplementary methods for sequences.

Immunofluorescence Microscopy

Cells were transfected with siRNAs as above and incubated for 48-hours. Cells were then plated onto coverslips and treated with E₂, 2-OHE₂ or 4-OHE₂ (Sigma), or mock treated with vehicle and incubated for indicated time points. Cells were then fixed and stained with γ -H2AX (Millipore), 53BP1 (Millipore), Cyclin A (SCBT) or pATM^{Ser1981} (Cell Signalling) primary antibodies and imaged using a Nikon Eclipse Ti microscope, using a 60x objective.

Comet assays

Neutral comet assays were carried out using the Cell Biolabs SCGE kit. Comets were scored using CometScore (TriTek Corp).

Western Blotting

Western blotting was carried out as previously described (28).

Metaphase spreads, FISH staining and chromosomal aberrations

Analysis was carried out as described previously (29).

qRT-PCR analysis

qRT-PCR was carried out using Roche LightCycler 480 RealTime ready catalogue assays for each gene (*ACTB*, *CYP11A1*, *CYP11A2*, *CYP3A4*, *CYP11B1*, *COMT* and *NQO1*) as per the manufactures instructions. A matched qRT-PCR reaction was carried out using the RT-ve control for each sample ensuring no genomic DNA contamination.

Chromatin immunoprecipitations (ChIPs)

ChIPs were performed as described previously (28). See supplementary methods for complete protocol and primer sequences.

Immunohistochemical staining of CYP11A1

Immunohistochemical staining was performed using a fully automated BondMax immunostainer with a polymer-based peroxidase detection system. (CYP11A1 (B4) SCBT primary antibody was used at a dilution of 1:50.

Ultra-performance-liquid-chromatography-tandem-mass spectrometry (UPLC-MS/MS)

Samples were extracted using liquid-liquid extraction (LLE) with diethyl ether, followed by dansyl chloride derivatisation as described by Xu et al. and analysed using UPLC-MS/MS. See supplementary methods for complete protocol (30).

Isolation and culture of primary breast progenitor cells

See supplementary methods for complete protocol. Ethical approval to obtain primary breast tissue was granted through the Northern Ireland Biobank. Tissue was dissociated and mammospheres cultured as previously described (31) in ultra-low attachment 75 cm² flasks for 7 days. Mammosphere cultured cells were then dissociated and plated into Lab-Tek II CC² treated chamber slides (Nunc) in the media above.

RESULTS

Estrogen metabolites induce DNA DSBs in breast cells

To determine if estrogen metabolites can generate DNA DSBs, we first assessed whether the parent hormone, Estradiol (E₂), induces DSBs in breast cells. Normal like, ER α negative MCF10A breast cells, were treated with either E₂ or mock treated for 3-hours and stained with the DSB markers 53BP1 and γ H2AX. Treatment with E₂ resulted in a significant increase in 53BP1 and γ H2AX foci respectively, compared to controls (p<0.001) (Figure 1a-b). To investigate if estrogen metabolites cause DSBs, we treated MCF10A cells with the metabolic intermediates 2-OHE₂ and 4-OHE₂ for 3-hours and stained with 53BP1 and γ H2AX. Similar to E₂ treatment, we found that both metabolites induced a significant

increase in DSB foci ($p < 0.01$) (Figure 1c-d). These findings were also confirmed in the ER α positive breast cancer cell line, MCF7, indicating that E₂ metabolite induced DSB induction is independent of ER α (Supplementary Figures 1a-b). To further confirm that 2-OHE₂ and 4-OHE₂ induce DSBs, we performed neutral comet assays. Indeed, MCF10A cells treated with 2-OHE₂ and 4-OHE₂ demonstrated a greater percentage of tail DNA, tail moment and tail length, indicative of increased DSBs (Figures 1e-f & Supplementary Figures 1c-d).

DNA DSBs are known to result in activation of the ATM kinase, which is activated through autophosphorylation of ATM at serine-1981. In keeping with this, 2-OHE₂ and 4-OHE₂ treatment resulted in a 2.27-fold and 3.37-fold increase in ATM^{P^{Ser1981}} foci (co-localised with 53BP1 foci) compared to mock controls (Supplementary Figures 1e-f). Taken together, this data suggests that 2-OHE₂ and 4-OHE₂ induce DNA DSBs in breast cells.

Interestingly, when carrying out these experiments, we consistently observed uneven distribution of estrogen metabolite induced DSBs and rather than all cells containing slightly more foci than mock treated cells, we consistently observed that a fraction of cells (approx. 25-30%) incurred a greater number of DNA DSBs following 2-OHE₂ and 4-OHE₂ treatment. For example, in cells treated with 2-OHE₂, 23% had no 53BP1 foci, whereas 26% of cells had 6 or more foci (Figure 1g). This suggested that estrogen metabolite mediated DNA damage may be affected by cell cycle distribution. To test this, we co-stained 2-OHE₂ and 4-OHE₂ treated MCF10A cells with γ H2AX and cyclin-A, which is specifically expressed during S and G2 phases of the cell cycle. We found that the vast majority of estrogen metabolite mediated DNA damage occurred in cyclin-A positive cells, suggesting that 2-OHE₂ and 4-OHE₂ specifically induce DNA damage during S/G2 phase cells (Figures 1h-i). To confirm this, MCF10A cells treated with 2-OHE₂ and 4-OHE₂ were pulse labelled with 5-ethynyl-2'-deoxyuridine (EdU), which is incorporated into DNA during replication. Cells were then co-stained for γ H2AX and EdU. Indeed, significantly more γ H2AX foci were observed in EdU positive cells compared to EdU negative cells following estrogen metabolite treatment (Supplementary Figure 1g-h). This suggests that the DNA DSBs produced by 2-OHE₂ and 4-OHE₂ treatment occur specifically during S-phase.

Estrogen metabolite-mediated DNA damage is exacerbated by BRCA1 loss and not efficiently repaired in BRCA1-deficient cells

BRCA1 is known to play a pivotal role in DNA DSB repair. Additionally, a role for BRCA1 in the post-replicative repair of bulky DNA adducts induced by UV, which are structurally similar to E₂ metabolite adducted bases, has been recently described (3). In light of this, we examined if BRCA1 was required for the repair of estrogen induced DSBs. Endogenous BRCA1 expression was depleted using two independent siRNAs (siBRCA1) compared to a non-targeting scrambled siRNA (siSCR) and the cells treated with 2-OHE₂ and 4-OHE₂ for 3-hours. Cells were then allowed to recover for twenty-four hours following treatment, before being fixed and stained for 53BP1 and γ H2AX (Figure 2a-c & Supplementary Figure 2a-b). No significant difference in 53BP1 and γ H2AX foci number was observed in siSCR cells treated with 2-OHE₂ or 4-OHE₂ relative to untreated control cells, suggesting that DSBs generated during estrogen metabolite treatment had been efficiently repaired. In contrast, significantly more 53BP1 and γ H2AX foci remained in BRCA1-depleted cells

treated with 2-OHE₂ and 4-OHE₂ compared to controls ($p < 0.001$), indicating that BRCA1 is required for efficient repair of E₂-metabolite induced DSBs. This was visualised at multiple time-points following 3-hours of 2-OHE₂ or 4-OHE₂ treatment (Supplementary Figure 2c d). Additionally, given that E₂-metabolites induce DNA damage during S-phase, taken together with the known role for BRCA1 in the post-replicative repair of bulky DNA adducts, we assessed whether depletion of BRCA1, using two independent siRNAs, caused replication fork stalling, marked by residual pS4/8 RPA32 foci (3). Consistent with a role for BRCA1 in repairing E₂-metabolite induced DNA damage in s-phase cells, we observed a dramatic increase in pS4/8 RPA32 positive cells upon BRCA1 depletion following 2OHE₂ and 4OHE₂ treatment (Figure 2d).

We next examined if 2-OHE₂ and 4OHE₂ treatment also induced chromosomal instability in BRCA1-depleted cells. We assessed chromosomal aberrations in control and BRCA1-depleted MCF10A cells 24-hours following treatment with 2-OHE₂ and 4-OHE₂. Structural rearrangements were visualised and quantified in metaphase spreads using chromosome 1 & 2 FISH staining (Figure 2e-f). BRCA1 depletion resulted in a marked increase in chromosomal aberrations following both 2-OHE₂ and 4-OHE₂ treatment, demonstrating that E₂ metabolite treatment induces genomic instability in BRCA1-deficient cells. Intriguingly, when we examined DSB production in these cells immediately following 2-OHE₂ and 4-OHE₂ treatment (3-hours) we found that 2-OHE₂ and 4-OHE₂ treatment resulted in a significant increase in 53BP1 and γ H2AX foci in BRCA1-depleted cells compared to control cells (Figure 2g-h & Supplementary Figure 2e). Additionally, similar to that demonstrated earlier, E₂ metabolite mediated DNA damage occurred specifically in S/G2 phase of the cell cycle in BRCA1-depleted cells (Supplementary Figure 2f). Due to the relatively short treatment time (3-hours), it is unlikely that this increase in DSBs observed in BRCA1-depleted cells is due solely to defective DNA repair. To confirm that this occurred at early time points and was not confounded by the dose and/or time points of E₂-metabolite treatment used, we assessed γ H2AX marked DNA damage in control and BRCA1-depleted cells following treatment with 1nM, 10nM, 100nM and 1 μ M E₂, 2-OHE₂ and 4-OHE₂ at various time-points (Supplementary Figure 3a-c). This revealed increased DNA damage in BRCA1-depleted cells at all doses and treatment time-points assessed.

Taken together, these data indicate that loss of BRCA1 expression results in delayed repair kinetics but also increased levels of DNA damage following treatment with E₂ metabolites. To further confirm this, we examined induction and repair of DNA DSBs following 2-OHE₂ and 4-OHE₂ treatment in BRCA1 mutant MDA-MB-436 cells stably transfected with either empty vector (EV) or a BRCA1 expression plasmid (Supplementary figure 4a-c). Like, BRCA1-depleted cells, this revealed increased DNA damage induction at short time points (3-hours) in the BRCA1-deficient cells (Supplementary figure 4b). Similarly, defective repair of 2-OHE₂ and 4-OHE₂ induced DSBs was observed in BRCA1-deficient cells in comparison to BRCA1 reconstituted cells (Supplementary figure 4c).

Given that BRCA2 is also involved in HR mediated DSB repair and mutations in this gene also predispose to tumours in the breast and ovaries, we examined the effect of BRCA2 depletion on 2-OHE₂ and 4-OHE₂ induced DNA damage in both MCF10A and MCF7 cells. Intriguingly, BRCA2 depletion resulted in slightly increased DSBs following treatment with

2-OHE₂ and 4-OHE₂ for 3-hours, which appeared to remain unrepaired at 24-hours following recovery from 2-OHE₂ and 4-OHE₂ treatment (Supplementary Figure 5). Nevertheless, the increased level of DNA damage observed following treatment with 2-OHE₂ and 4-OHE₂ in BRCA2 depleted cells was minimal in comparison to that observed in BRCA1-depleted cells. Additionally, the defective repair of these DSBs in BRCA2 depleted cells is consistent with BRCA2's role in HR mediated DSB repair.

BRCA1 regulates the expression of estrogen metabolising enzymes

We have previously proposed that BRCA1 may transcriptionally repress the expression of estrogen metabolising genes. Specifically, we have demonstrated that CYP1A1, the enzyme responsible for conversion of E₂ to 2-OHE₂ and semi-quinone/quinone metabolites, is repressed by BRCA1 in a number of breast cancer cell lines (32). Additionally, BRCA1 has been found to transcriptionally activate a number of detoxification/antioxidant genes including NAD(P)H Quinone Oxoreductase 1 (NQO1), which reduces genotoxic quinones to non-reactive hydro-quinones (33). This led us to hypothesise that the exacerbated DNA damage observed in BRCA1-depleted cells at short time points following 2-OHE₂ and 4-OHE₂ treatment may be due, at least in part, to increased estrogen metabolism in these cells, mediated by upregulated expression of estrogen metabolising enzymes, and/or down-regulated expression of detoxification enzymes such as NQO1. To assess this, we examined the expression of a panel of estrogen metabolising and detoxification genes including *CYP1A1*, *CYP1B1*, *CYP1A2*, *CYP3A4*, *NQO1* and *COMT*, using qRT-PCR, in MCF10A and MCF7 cells transfected with control and BRCA1 depleting siRNAs (Figure 3a-b). This revealed that *CYP1A1* and *CYP3A4* are consistently upregulated upon BRCA1 loss, suggesting BRCA1 represses the expression of these genes. Additionally, NQO1 was consistently down-regulated upon BRCA1 depletion in both cell lines. We also examined the expression of these genes in BRCA1-mutant MDA-MB-436 cells, stably transfected with EV or BRCA1 (Figure 3c). Ectopic expression of BRCA1 in these cells repressed expression of CYP1A1, but had a limited effect on CYP3A4 and NQO1 expression.

To assess if BRCA1 regulates the transcription of these genes directly, we performed BRCA1 Chromatin Immunoprecipitation (ChIP)-qPCR assays from MCF10A cells, using primers specific to the promoter regions of *CYP1A1*, *CYP3A4*, *NQO1* and *COMT* as a negative control (Figure 3d). We observed enrichment of BRCA1 at all of these promoters with the exception of *COMT*, which is neither transcriptionally regulated by BRCA1 nor promoter bound by BRCA1.

To confirm that this occurs in BRCA1-deficient tumours, we assessed the levels of CYP1A1, the most highly de-regulated gene upon BRCA1 loss, using immunohistochemistry (IHC) in a panel of 21 BRCA1 mutant and 75 BRCA1 wild-type breast tumours. Intratumoural CYP1A1 expression was scored by a pathologist and an independent scorer as very weak/absent = 1, moderate = 2 or strong = 3 (Figure 3e-f). This revealed that the mean expression of CYP1A1 expression is significantly upregulated in this cohort of BRCA1 mutant tumours compared to BRCA1 wild-type sporadic tumours ($p = 0.0007$).

Given that we observed a slight increase in E₂-metabolite induced DNA damage in BRCA2 depleted cells treated with 2-OHE₂ and 4-OHE₂ we also assessed the role of BRCA2 in regulating the expression of CYP1A1 in both MCF10A and MCF7 cells (Supplementary figure 6a-b). This revealed no significant difference in CYP1A1 expression between control and BRCA2-depleted cells.

BRCA1 suppresses estrogen metabolite mediated DNA damage by suppressing estrogen metabolism

As BRCA1-depletion results in increased levels of CYP1A1 and CYP3A4 enzymes and decreased NQO1 expression, we hypothesised that the levels of 2-OHE₂ and 4-OHE₂ and 2,3/3,4 quinone products would be increased in BRCA1-depleted cells compared to control cells following E₂ treatment. Quinone and semi-quinone metabolites are extremely unstable and have a very limited half-life making them difficult to quantify. In contrast, 2-OHE₂ and 4-OHE₂ (which are generated by CYP1A1 and CYP3A4 in breast cells) are relatively stable. We therefore developed an ultra-performance-liquid-chromatography-tandem-mass spectrometry (UPLC-MS/MS) method for the detection of 2-OHE₂ and 4-OHE₂. The method was optimised for baseline chromatographic separation and the accurate identification and quantification of both isomeric forms of the hydroxyl-estradiol metabolite (Figure 4a). Using this method, we found that the relative concentration of 2-OHE₂ and 4-OHE₂ was significantly higher in BRCA1-depleted cells compared to control cells (p<0.05) (Figure 4b).

As *CYP1A1* was the most robustly up-regulated estrogen metabolising gene upon BRCA1 depletion, we sought to examine the role of this enzyme in estrogen dependent DNA damage. To investigate this, BRCA1 and CYP1A1 were co-depleted in MCF10A cells and DNA DSBs assessed 3-hours following treatment with E₂ (Figure 4c-d). BRCA1 and CYP1A1 depletion was assessed by qRT-PCR (Supplementary Figure 6c-d). Strikingly, CYP1A1 depletion lead to a marked reduction in estrogen mediated DSBs in BRCA1-depleted cells (p<0.001), suggesting that estrogen mediated DNA damage in BRCA1-depleted cells is, at least in part, due to increased CYP1A1 levels in these cells.

This suggests that BRCA1, apart from mediating repair of E₂-metabolite mediated DNA damage, transcriptionally regulates estrogen metabolising enzymes and subsequently represses estrogen metabolism, thereby protecting cells against estrogen induced DNA damage. This is particularly important in breast and ovarian cells, which are exposed to much higher levels of estrogen than other tissues within the body (34). Nevertheless, to confirm whether this mechanism may drive genomic instability in non-breast cells, we assessed E₂-metabolite induced DNA damage in HEK293, kidney cells (Supplementary Figure 6e-f). This revealed that, although 2-OHE₂ and 4-OHE₂ are capable of inducing DNA DSBs in these cells, they induce much lower levels of DSBs in comparison to breast cells. Additionally, 2-OHE₂ and 4-OHE₂ treatment (3-hours) did not induce increased levels of DSBs in BRCA1-depleted cells (Supplementary Figure 6e). Moreover, we observed similar, low-levels of DNA damage in both BRCA1 and BRCA2 depleted cells. In contrast, consistent with the role of BRCA1 and BRCA2 in DSB repair, both BRCA1 and BRCA2 were required for repair of E₂-metabolite induced DNA damage in these cells

(Supplementary Figure 6f). Given that much lower levels of DNA damage were observed in these cells and that we did not observe any increased DNA damage in BRCA1-depleted cells in comparison to control, or BRCA2 depleted cells, we hypothesised that BRCA1 may not regulate the expression of CYP1A1 in these cells. Intriguingly, we were unable to detect any CYP1A1 transcript in these cells, suggesting that these cells may not metabolise estrogen at the same rate as breast cells (data not shown).

Estrogen metabolite mediated DNA DSBs are exacerbated in BRCA1 heterozygous breast cells

Taken together, our data suggests that in BRCA1-deficient breast cells, de-regulated estrogen metabolism results in increased levels of genotoxic metabolites resulting in increased DNA damage which, coupled with defective DNA repair, leads to genomic instability, a key hallmark of cancer initiation and progression. However, clinical evidence suggests a direct role for estrogen in breast cancer development in BRCA1 mutation carriers, suggesting that heterozygous loss of BRCA1 may result in haploinsufficiency in at least one of BRCA1's functions. Indeed, a number of studies have reported increased sensitivity to ionising radiation in BRCA1 carrier/heterozygous cells suggesting that DNA DSB repair may be impaired in carriers (35). Additionally, a recent study by Konishi et al., using somatic cell gene targeting to introduce the common pathogenic BRCA1 mutation 185delAG into a single BRCA1 allele in MCF10A cells, demonstrated that this heterozygous BRCA1 mutation confers impaired HR mediated DSB repair, hypersensitivity to genotoxic stress and increased genomic instability (27). We therefore set out to determine if heterozygous mutation of BRCA1 impacts estrogen metabolite mediated DNA damage and estrogen metabolism.

Using the same cell line model developed by Konishi et al., heterozygous BRCA1 185delAG (BRCA1^{+/-}) and control (BRCA1^{+/+}) MCF10A cells were treated with 2-OHE₂ and 4-OHE₂ for 3-hours and the media replaced with normal media for 24-hours, before fixing and staining for 53BP1 and γ H2AX. Consistent with a defect in BRCA1 function, a significant number of unresolved DNA damage foci were visible in 2-OHE₂ and 4-OHE₂ treated BRCA1^{+/-} cells in comparison to control BRCA1^{+/+} cells ($p < 0.001$) (Figure 5a). Surprisingly, the level of unrepaired DNA DSBs was similar to that observed in BRCA1-depleted MCF10A cells, suggesting that BRCA1 haploinsufficiency imparts a major defect in repair of E₂-metabolite mediated DNA damage. We next examined if, estrogen metabolites generate more DSBs in BRCA1^{+/-} cells. Indeed, although short term (3-hours) 2-OHE₂ and 4-OHE₂ treatment induced DNA damage in BRCA1^{+/+} cells, significantly more DNA DSB foci were observed in BRCA1^{+/-} cells ($p < 0.01$) (Figure 5b). In support of this, we treated normal primary breast progenitor cells, isolated from breast tissue obtained from a woman undergoing elective breast reduction, as well as BRCA1^{+/-} primary breast progenitor cells from a BRCA1 mutation carrier undergoing a risk-reducing mastectomy, with both 2-OHE₂ and 4-OHE₂ and examined both DNA damage induction at 3-hours post treatment, as well as their ability to repair E₂-metabolite induced DNA damage 24-hours following treatment with 2-OHE₂ and 4-OHE₂. In keeping with our previous findings, 2-OHE₂ and 4-OHE₂ treatment for 3-hours induced more DNA damage in the BRCA1^{+/-} mammary progenitor cells compared to the BRCA1 wild-type cells and this DNA damage

was not repaired as efficiently in the BRCA1^{+/-} cells compared to the BRCA1 wild-type cells (Figure 5c-d). Taken together this data suggests that like BRCA1-depleted cells, BRCA1^{+/-} cells may have increased rates of estrogen metabolism as well as defective repair of E₂-metabolite induced DNA damage.

To examine this, we assessed the expression levels of the same panel of estrogen metabolising and detoxification enzymes in BRCA1^{+/+} and BRCA1^{+/-} cells. Indeed, as in BRCA1-depleted cells, *CYP1A1* and *CYP3A4* were upregulated in BRCA1^{+/-} compared to BRCA1^{+/+} cells (Figure 5e). Intriguingly, *NQO1* expression was maintained at similar levels in both cells lines suggesting that BRCA1 haploinsufficiency does not negatively impact all BRCA1 regulated transcriptional targets. Finally, consistent with the defective DSB repair observed in these cells following 2-OHE₂ and 4-OHE₂ treatment, we found that treatment with either of these metabolites induced genomic instability in BRCA1^{+/-} cells but not BRCA1^{+/+} cells (Figure 5f).

DISCUSSION

One of the most perplexing features of BRCA1 biology, is that despite playing a central role in the DNA damage response and DSB repair pathways in all cells, mutation carriers predominantly develop tumours in the breast and ovaries; both estrogen driven tissues exposed to high levels of estrogen. Here, we show that treatment with both E₂ and the E₂-metabolites 2-OHE₂ and 4-OHE₂, induces DNA DSBs in human breast cancer cells in an ER α independent manner. We also found that E₂-metabolite mediated DSBs occur specifically in S-phase cells, suggesting that induction of these lesions is coupled to DNA replication. We hypothesise that E₂-metabolite adducted DNA bases represent replication barriers, which lead to replication fork stalling during DNA synthesis. Indeed, a number of studies have shown that 4-hydroxyequilenin (4-OHEN) a metabolite of the equine estrogen equilenin (which is almost identical to 4OHE-2 in humans) causes identical DNA-adducts to those caused by 4-OHE₂ and that these adducts cause replication fork stalling (36, 37).

In general, stalled replication forks do not collapse and form DSBs, but are instead stabilised by the ATR kinase through the signalling and recruitment of a plethora of checkpoint signalling and repair proteins, resulting in resolution of the stalled fork through a HR-mediated repair process involving BRCA1. However, recent studies have shown that E₂ inhibits ATR signalling, suggesting that E₂ and its metabolites may lead to replication fork stalling and subsequent fork collapse and DSB formation through the combined effect of replication fork stalling and ATR inhibition (38). Further to this, BRCA1 has been shown to be required for both resolution of stalled replication forks as well as HR mediated repair of DSBs caused following stalled fork collapse (39). BRCA1 is also required for the removal and repair of bulky base adducts, a mechanism through which BRCA1 may suppress adduct induced mutagenesis (3). Consistent with this, we observed a dramatic increase in pS4/8 RPA32 positive cells, upon BRCA1 depletion in 2OHE₂ and 4OHE₂ treated cells.

Interestingly, we found that BRCA1 depletion also resulted in increased levels of E₂-metabolite induced DNA damage, even at very early time points. This suggested that BRCA1 may also play a more direct role in regulating the physical levels of DNA damage

induced by estrogen metabolites. Previous data from our laboratory had indicated that BRCA1 loss leads to upregulation of *CYP1A1*, a major regulator of E₂ metabolism in breast tissues (32). We therefore tested whether BRCA1 may also regulate the expression of other estrogen metabolising enzymes, thereby regulating the levels of estrogen derived metabolites. This analysis revealed that BRCA1 directly represses the transcription of *CYP1A1* and *CYP3A4* and promotes the expression of the NAD(P)H:quinone oxidoreductase, *NQO1*. We confirmed, using IHC in a cohort of BRCA1 mutant and matched BRCA1 wild-type tumours, that *CYP1A1*, the major enzyme involved in conversion of E₂ to 2-OHE₂ in breast tissues, is significantly upregulated in BRCA1 mutant tumours. This is consistent with the increased levels of both 2-OHE₂ and 4-OHE₂ observed in BRCA1-depleted cells. We also demonstrated that depletion of *CYP1A1* significantly reduces the amount of DNA damage induced in BRCA1-depleted cells exposed to short term E₂ treatment, confirming that E₂-mediated DNA damage in BRCA1-depleted cells is, at least in part, due to increased estrogen metabolism. Intriguingly, when examining the impact of BRCA2 on estrogen metabolite-induced DNA damage, we found that although BRCA2 is required for the repair of these breaks, loss of BRCA2 does not lead to deregulated estrogen metabolism and the associated increased DNA damage. Perhaps this explains why BRCA2 mutations are less penetrant than BRCA1 mutations in predisposing carriers to breast and ovarian cancers.

We have also demonstrated that 2-OHE₂ and 4-OHE₂ treatment leads to increased DSB production in MCF10A cells and primary breast cells harbouring a pathogenic heterozygous BRCA1 mutation, and that like BRCA1-depleted cells, these cells are unable to repair E₂-metabolite mediated DNA damage leading to increased genomic instability. Importantly, we found that BRCA1 heterozygous mutant cells also have upregulated levels of *CYP1A1* and *CYP3A4*, suggesting that increased estrogen metabolism may contribute to E₂-mediated DNA damage in these cells. Consistent with this, higher levels of urinary excreted 2-OHE₂ and 4-OHE₂ have been observed in BRCA1 carriers compared to healthy control women with no BRCA1 mutation (40).

Taken together, these findings suggest that exposure to estrogen and its subsequent metabolism in BRCA1-deficient breast cells, is capable of driving genomic instability, a well-defined early event in breast cancer development. Given that estrogen levels in normal/benign breast tissue are known to be 6-7 times that of circulating estrogen levels, our findings suggest a mechanism through which BRCA1 carriers, through enhanced production of DNA damaging estrogen metabolites, may acquire the genetic alterations that initiate neoplastic transformation in breast tissue (34). Similarly, levels of estrogen in ovarian tissues greatly exceed that of circulating estrogen, suggesting that this model may also explain the substantially increased risk of ovarian cancer in BRCA1 carriers (41).

A phase III trial termed, Prevention of Breast Cancer by Letrozole in Postmenopausal Women carrying a BRCA1/2 Mutation (LIBER), (ClinicalTrials.gov number, NCT00673335) is currently enrolling postmenopausal women for treatment with letrozole, an Aromatase Inhibitor (AI), to evaluate its ability to prevent the development of breast cancer in patients with a BRCA1/2 mutation. Our results coupled with the finding that

aromatase levels are substantially higher in prophylactic mastectomy and oophorectomy tissue from BRCA1 carriers (10), provides further mechanistic data to support this approach.

However, AIs may have little preventative effect in premenopausal women, in whom the majority of BRCA1-linked tumours develop, and in whom estrogen production occurs predominantly in the ovaries through an aromatase independent biosynthesis pathway. In these women, oophorectomy has been shown to reduce the risk of breast cancer by up to 60% (42). Taking our findings into account, it may also be worth considering the use of AIs as an additional chemopreventative strategy in premenopausal women, whom have undergone risk-reducing oophorectomy without mastectomy.

Finally, in premenopausal women who have opted not to undergo risk-reducing oophorectomy or mastectomy, Luteinising hormone releasing hormone agonists (LHRHa's), may prove useful as chemopreventative agents. These drugs cause reversible ovarian suppression/ablation and are currently used in combination with tamoxifen or AIs for the treatment of premenopausal women with ER α positive breast cancer (43, 44).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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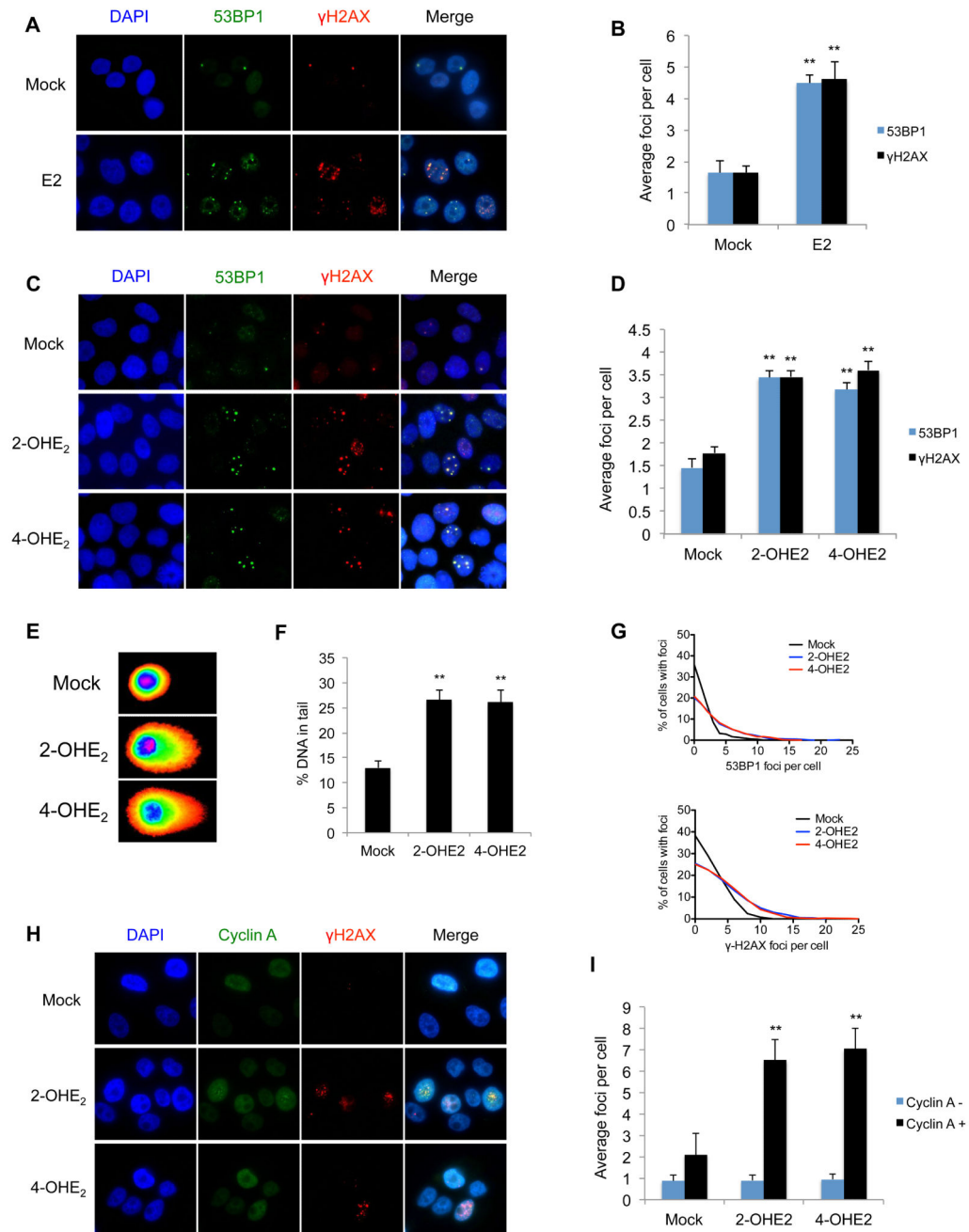


Figure 1. Estrogen and its metabolites cause DNA double strand breaks in S/G2 phase cells
 A) Representative images of 53BP1 and γ -H2AX marked DNA DSBs in MCF10A cells 3-hours after mock treatment or treatment with 10nM Estradiol (E2). B) Quantification of 53BP1 and γ -H2AX foci in cells described above. Bars represent mean number of foci per cell \pm SEM from three independent experiments (>200 cells were counted per experiment). Significance of changes in foci numbers were assessed using students two-tailed t-test C) Representative images of 53BP1 and γ -H2AX marked DNA DSBs in MCF10A cells 3-hours after mock treatment or treatment with 1 μ M 2-OHE₂ or 4-OHE₂. D)

Quantification of 53BP1 and γ -H2AX foci in cells described above. E) Representative comet images of MCF10A cells treated as above and subjected to neutral single cell electrophoresis. Fluorescence intensity mapping of comet images has been applied using CometScore. F) Quantification of tail DNA in comets from above. Tail DNA was quantified using CometScore. Bars represent mean percentage of DNA in comet tails, \pm SEM, from three independent experiments (>100 comets were scored per experiment). G) Frequency distribution of 53BP1 and γ -H2AX foci in MCF10A cells treated with 2-OHE₂ or 4-OHE₂. H) Representative images of γ -H2AX marked DNA DSBs in MCF10A cells 3-hours after mock treatment or treatment with 1 μ M 2-OHE₂ or 4-OHE₂. Cells were also stained with Cyclin-A antibodies to identify cells in S/G2-phase. I) Quantification of γ -H2AX foci in Cyclin-A positive and negative cells treated as above.

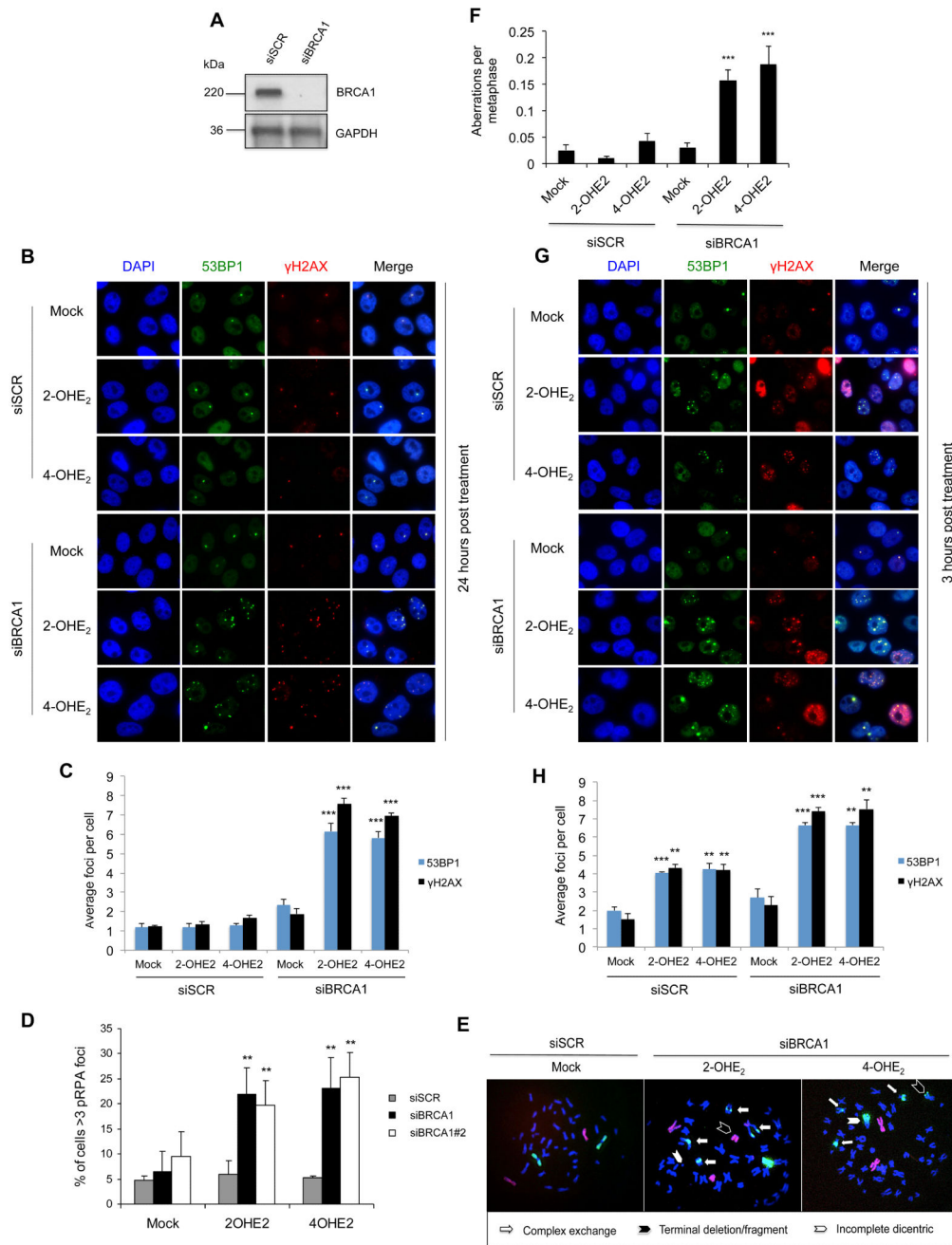


Figure 2. BRCA1 represses estrogen metabolite mediated DNA DSBs and is required for their repair

A) Representative western blot demonstrating BRCA1 knockdown in MCF10A cells. B) Representative images of 53BP1 and γ -H2AX marked DNA DSBs in control (siSCR) and BRCA1-depleted (siBRCA1) MCF10A cells, 24-hours after treatment with 2-OHE₂ or 4-OHE₂ (1 μ M 3-hours). C) Quantification of 53BP1 and γ -H2AX foci in cells described above. Bars represent mean number of foci per cell \pm SEM from three independent experiments (>200 cells were counted per experiment). D) Quantification of cells containing >3 pS4/8-RPA foci (indicative of stalled replication forks) in control (siSCR) and BRCA1-

depleted cells (siBRCA1 & siBRCA#2) treated with 2-OHE₂ or 4-OHE₂ (1μM 3-hours). E) Representative metaphase spreads of control (siSCR) and BRCA1-depleted (siBRCA1) MCF10A cells 24-hours after treatment with 2-OHE₂ or 4-OHE₂ (1μM 3-hours). Spreads were stained with FISH probes against chromosome 1 (Green) and Chromosome 2 (Red) in order to visualise gross chromosomal aberrations. F) Quantification of chromosomal aberrations in metaphase spreads from cells described above (>100 metaphases were scored per experiment). G) Representative images of 53BP1 and γ-H2AX marked DNA DSBs in MCF10A cells 3-hours after mock treatment or treatment with 1μM 2-OHE₂ or 4-OHE₂. H) Quantification of 53BP1 and γ-H2AX foci in cells described above.

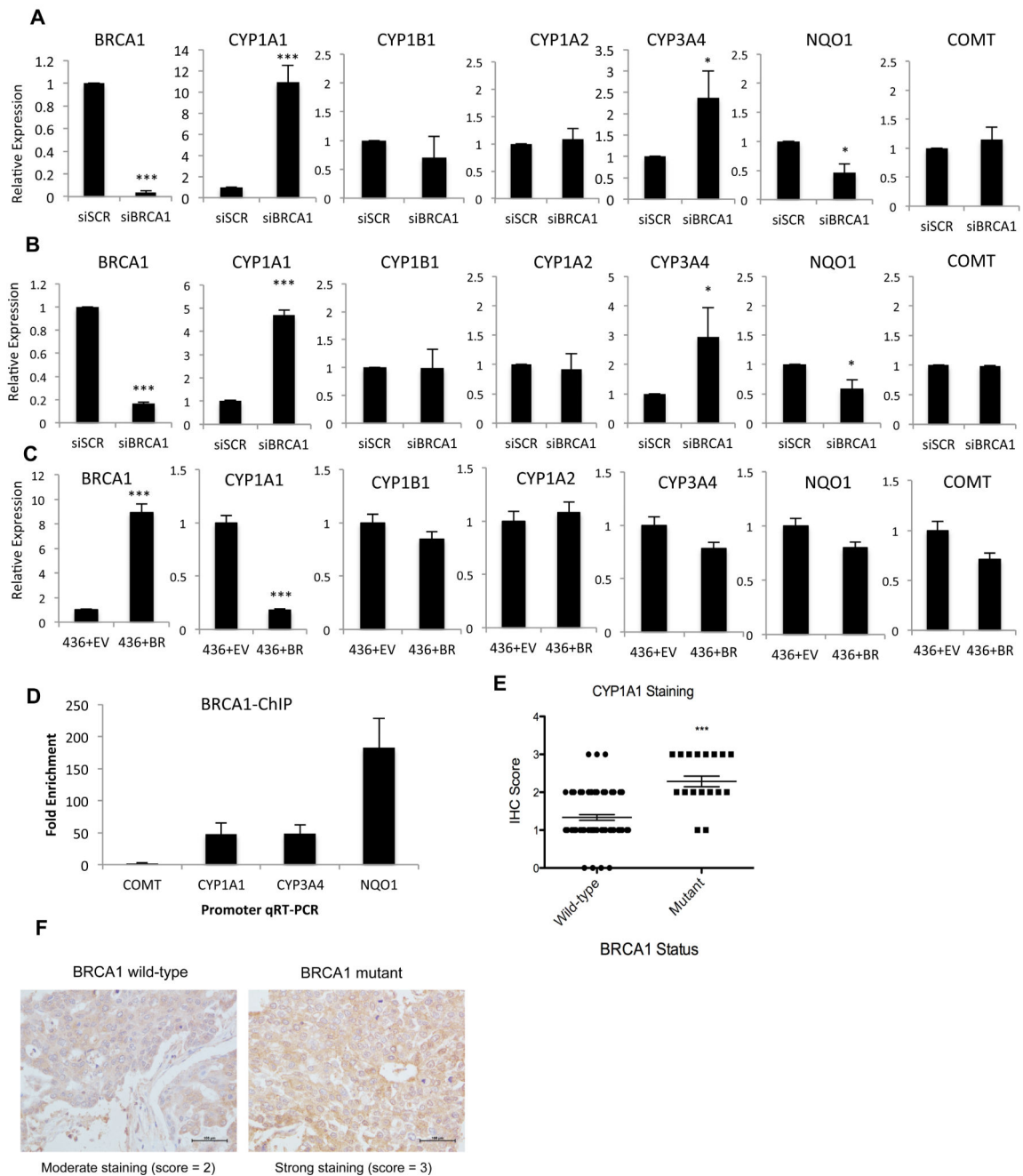


Figure 3. BRCA1 transcriptionally regulates estrogen metabolizing genes

A-B) qRT-PCR determined expression of genes involved in estrogen metabolism in control (siSCR) and BRCA1-depleted (siBRCA1) MCF10A (a) and MCF7 (b) cells. Gene expression was normalized to ACTB expression and is shown relative to expression in control (siSCR) cells. Bars represent mean relative expression \pm SEM from three independent experiments. C) qRT-PCR determined expression of genes as above in the BRCA1-deficient MDA-MB-436 cells stably transfected with either empty vector (+EV) or a BRCA1 expression plasmid (+BRCA1). D) BRCA1 Chromatin Immunoprecipitation

(ChIP) qPCR using primers targeting the promoters of COMT (not regulated by BRCA1), CYP1A1, CYP3A4 and NQO1. Quantified amounts of immunoprecipitated DNA were normalised to inputs and reported relative to the amount quantified at a non-specific control region. Bars represent mean fold enrichment \pm SEM from three independent experiments. E) Immunohistochemistry (IHC) determined expression of CYP1A1 in BRCA1 mutant, and matched BRCA1 wild-type breast tumours. CYP1A1 expression in each tumour was scored as 0= absent, 1 = low, 2 = moderate, or 3 = high. Significance of changes in gene expression were assessed using students two-tailed t-test - ***p = 0.0007. F). Representative images of moderate and high CYP1A1 staining in BRCA1 wild-type and BRCA1 mutant breast tumours.

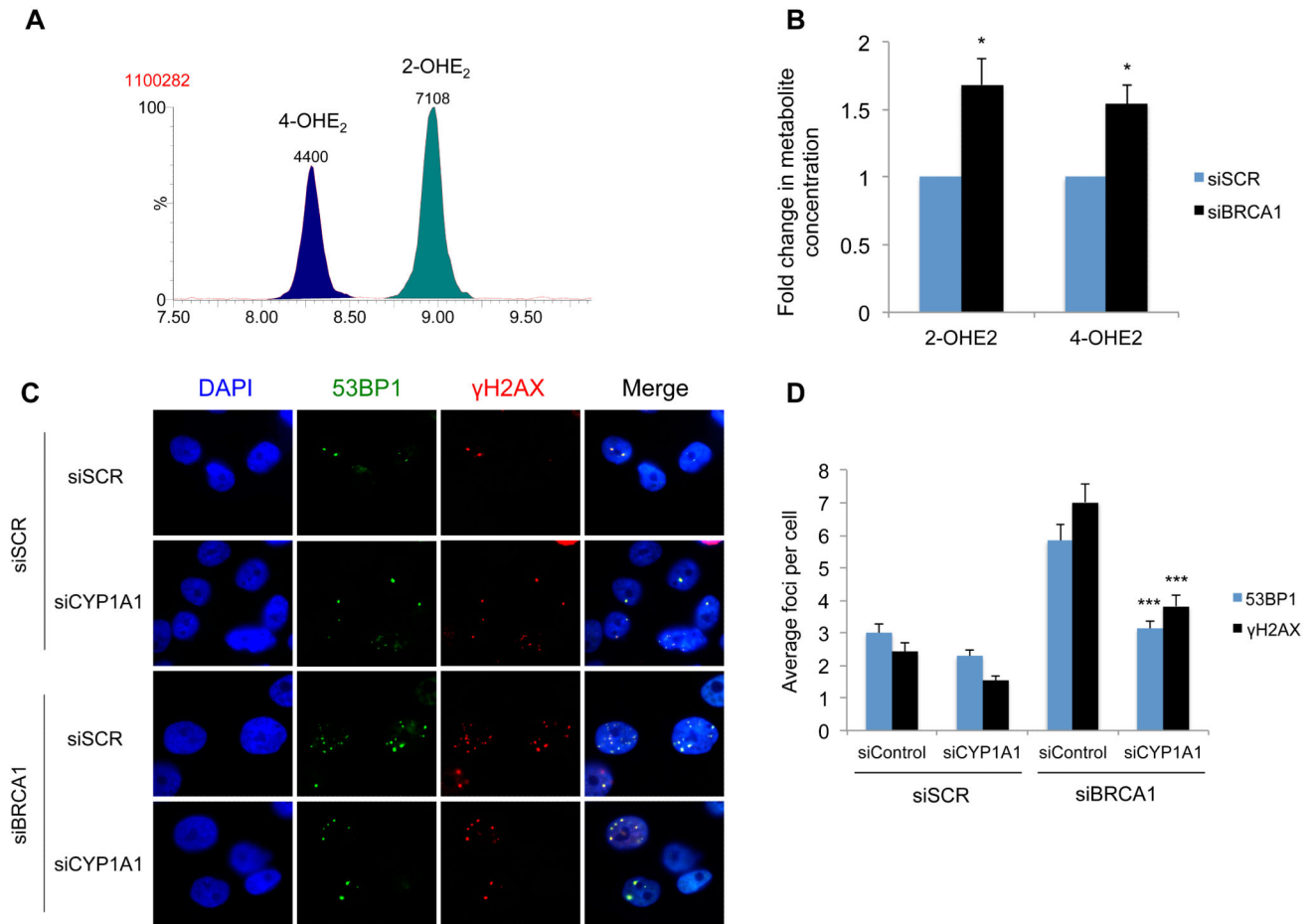


Figure 4. BRCA1 Suppresses Estrogen Metabolite Mediated DNA Damage by Suppressing Estrogen Metabolism

A) UPLC-MS/MS MRM transition (m/z 755.1>521.1) chromatogram from control (siSCR) MCF10A cell culture medium. The transition (representing loss of one dansyl group at the O-S bond) detects both isobaric species 2- & 4-OHE₂-bisdansyl with baseline chromatographic separation. Isomer identification was confirmed by ratio of a secondary ion transition (m/z 755.1>170.2) in comparison with analytical standards. Quantification accuracy was enhanced by use of isotopically labelled internal standards. B) Levels of 2- & 4-OHE₂ in culture medium from control (siSCR) and BRCA1-depleted (siBRCA1) MCF10A cells quantified by an isotope dilution UPLC-MS/MS method. Cells were cultured in medium containing 10 nM E2 for 24h prior to media sampling. All values were normalised to cell counts. Bars represent mean concentration \pm SEM from three independent experiments (8 replicate cultures). Significance of changes in gene expression were assessed using Student's two-tailed t-test with significant changes in concentration data indicated by; * = $p < 0.05$. C) Representative images of 53BP1 and γ -H2AX marked DNA DSBs in BRCA1 and CYP1A1 co-depleted MCF10A cells, 3-hours after mock treatment or treatment with 10nM Estradiol. Depletion of BRCA1 and CYP1A1 was confirmed by qRT-PCR (Supplementary figure 6c-d) (E2). D) Quantification of 53BP1 and γ -H2AX foci in

cells described above (>200 cells were counted per experiment). Significance of changes indicated by; *** = $p < 0.001$.

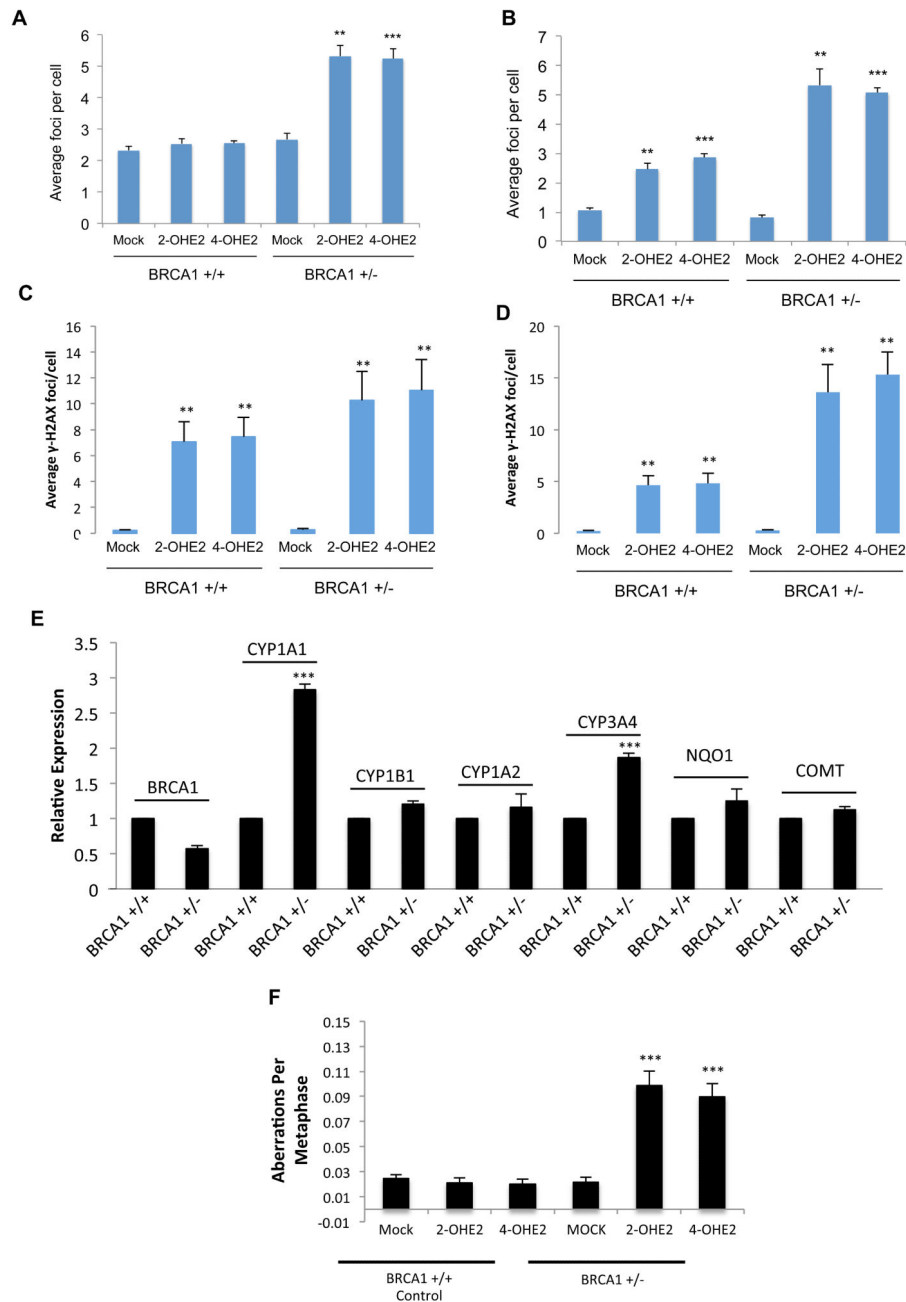


Figure 5. BRCA1 heterozygosity leads to increased estrogen metabolite mediated DNA damage, defective DNA repair, and genomic instability and loss of repression of estrogen metabolizing enzymes

A-B) Quantification of γ -H2AX marked DNA DSBs in BRCA1 wild-type (BRCA1 +/+) and BRCA1 heterozygous 185delAG (BRCA1 +/-) MCF10A cells, 24-hours after treatment with 2-OHE₂ or 4-OHE₂ (a) or immediately following 3-hours of treatment with 2-OHE₂ or 4-OHE₂ (b). Bars represent mean number of foci per cell \pm SEM from three independent experiments (>200 cells were counted per experiment). C-D) Quantification of γ -H2AX marked DNA DSBs as above in BRCA1 wild-type (BRCA1 +/+) and BRCA1 heterozygous

primary breast progenitor cells 24 hours after treatment with 2-OHE₂ or 4-OHE₂ (c) or immediately following 3-hours of treatment with 2-OHE₂ or 4-OHE₂ (d). E) qRT-PCR determined expression of genes involved in estrogen metabolism in BRCA1 wild-type (BRCA1 +/+) and BRCA1 heterozygous 185delAG (BRCA1 -/+) MCF10A cells. Bars represent mean relative expression +/- SEM from three independent experiments. F) Quantification of chromosomal aberrations in metaphase spreads from BRCA1 wild-type (BRCA1 +/+) and BRCA1 heterozygous 185delAG (BRCA1 -/+) MCF10A cells, 24-hours after treatment with 2-OHE₂ or 4-OHE₂. Bars represent mean aberrations per metaphase +/- SEM, from three independent experiments (>100 metaphases were scored per experiment).