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Deletion of BRCA2 exon 27 causes defects in response to both stalled and collapsed replication forks

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Abstract

BRCA2 is a tumor suppressor that maintains genomic integrity through double strand break (DSB) repair and replication fork protection. The BRC motifs and an exon 27-encoded domain (Ex27) of BRCA2 interact with the recombinase RAD51 to respectively facilitate the formation and stability of a RAD51 filament on single strand DNA. The BRC-RAD51 associations enable DSB repair while the Ex27-RAD51 association protects the nascent replication strand from MRE11-mediated degradation. MRE11 is a nuclease that facilitates the generation of 3' overhangs needed for homologous recombination (HR)-mediated DSB repair. Here we report the dynamics of replication fork maintenance in mouse embryonic stem (ES) cells deleted for Ex27 (*brca2^{lex1/lex2}*) after exposure to hydroxyurea (HU) that depletes nucleotides. HU conditions were varied from mild to severe. Mild conditions induce an ATR-response to replication fork stalling while severe conditions induce a DNA-PK_{CS}-response to replication fork collapse and a DSB. These responses were differentiated by replication protein A (RPA) phosphorylation. We found that Ex27 deletion reduced MRE11 localization to stalled, but not collapsed, replication forks and that Ex27-deletion caused a proportionately more severe phenotype with HU dose. Therefore, the BRCA2 exon 27 domain maintains chromosomal integrity at both stalled and collapsed replication forks consistent with involvement in both replication fork maintenance and double strand break repair.

Keywords

BRCA2; RAD51; replication fork stalling; double strand break; chromosomal instability

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Conflict of interest statement

The authors declare there is no conflict of interest with regard to the publication of this paper.

1. Introduction

BRCA2 and RAD51 maintain the genome to suppress cancer. In humans, *BRCA2* heterozygous mutations lead to familial breast cancer after loss of heterozygosity [1]. In addition, biallelic mutations that generate truncated proteins cause Fanconi anemia characterized by congenital abnormalities, progressive bone marrow failure and increased incidence of acute myeloid leukemia and squamous cell carcinoma [2]. Furthermore, suppression of *BRCA2* transcription causes sporadic breast and ovarian cancer [3, 4]. RAD51 is often overexpressed in tumors causing resistance to chemotherapeutics and increasing genomic instability that further contributes to cancer development and progression [5]. Thus, BRCA2 and RAD51 influence cancer etiology.

BRCA2 and RAD51 belong to the homologous recombination (HR) pathway that repairs DNA DSBs [6]. HR repairs DSBs independent of replication (DNA damage induced) or single-ended DSBs within replication. MRE11 enables the generation of a 3' overhang at a DSB [7, 8]. Initially, RPA binds to this strand. Then BRCA2 assists RAD51 in replacing RPA to form a filament on the single DNA strand. The RAD51 filament serves as a catalytic center for strand annealing to a homologous template usually provided by the sister chromatid during replication. Thus, HR-mediated DSB repair is usually high fidelity.

Besides HR-mediated DSB repair, RAD51 and BRCA2 also stabilize replication forks without a DSB [9-12]. For this activity RAD51 is likely recruited to single strand DNA at stalled replication forks. This early recruitment is independent of MRE11 nuclease activity [13]. To support the possibility that RAD51 maintains replication forks, a defect in RAD51 ATP binding/hydrolysis caused a phenotype suggestive of faulty replication that included reduced replication fork restart [14]. Furthermore, BRCA2 protected the nascent replication strand at stalled forks from MRE11-mediated degradation [9]. Thus, BRCA2 and RAD51 likely have multiple functions at replication forks that include replication fork maintenance and DSB repair.

To facilitate RAD51 filament formation, BRCA2 associates with RAD51 through two separate regions: the BRC motifs encoded by exon 11 [15, 16] and a conserved domain encoded by exon 27, (for this paper called Ex27) [17, 18]. The RAD51-BRC repeat interactions nucleate RAD51 onto single strand DNA to replace RPA [19]. The RAD51-Ex27 interaction stabilizes the filament by binding to an interface created by two adjacent RAD51 proteins [20, 21]. In addition Ex27 disassembles the nucleoprotein filament at the G₂-M transition after CDK phosphorylation on serine S3291 in human or S3215 in mouse [22]. Furthermore, the Ex27 domain was needed to block MRE11-mediated degradation at stalled replication forks [9]. Thus, these RAD51-BRCA2 interactions are nonequivalent and essential for RAD51 function but not fully understood.

Here we observe the importance of Ex27 for replication fork maintenance in *brca2^{lex1/lex2}* cells after exposure to varying doses of hydroxyurea (HU). HU inhibits the rate-limiting enzyme of DNA synthesis, ribonucleotide reductase to deplete nucleotides [23]. Mild exposure to HU transiently stalls replication forks; however, more severe exposure persistently stalls forks that can lead to one-ended breaks after fork collapse [11]. We show

that *brca2*^{lex1/lex2} cells exhibited defects in replication fork maintenance and chromosomal stability in an HU dose-dependent manner. These observations are consistent with Ex27's role in preserving chromosomal integrity at both stalled and collapsed replication forks.

2. Materials and methods

2.1. Cell culture conditions

Mouse ES cells were cultured in Minimal Essential Media- α (Invitrogen/Gibco, Carlsbad, California) with 15% fetal bovine serum (Invitrogen/Gibco, Carlsbad, California), 2mM glutamine, 3 g penicillin/ml, 5 g streptomycin/ml, 10^{-4} M β -mercaptoethanol and $1 \times$ leukocyte inhibiting factor (Chemicon) and cultured on plastic plates coated with 0.1% gelatin for about one hour.

2.2. Isolation of proteins on nascent DNA (iPOND)

iPOND was performed as described [13, 14] with minor modifications. In brief: mouse ES cells were incubated for 15minutes with $10\mu\text{M}$ 5'-ethynyl-2'-deoxyuridine (EdU, Invitrogen). EdU labeled cells were washed once with EdU free media and then treated with HU: 0.5mM for 1.5hours or 4mM for 1-5hours. Cells were cross-linked with 1% formaldehyde/PBS for 20minutes at room temperature, quenched with 0.125M glycine and washed three times with PBS. Cell pellets were collected and resuspended in 0.25% triton-X/PBS solution and then incubated at room temperature for 30min. for permeabilization. Permeabilized cells were washed once with 0.5% BSA/PBS and once with PBS. Cells were incubated in click reaction buffer [$10\mu\text{M}$ Biotin-azide (Jena bioscience), 10mM Na ascorbate (Sigma), $2\mu\text{M}$ copper (II) sulfate} for 1hour at room temperature. DMSO was used as a negative "no click" control. Cells were washed once with 0.5% BSA/PBS and once with PBS. Cells were resuspended in lysis buffer [1%SDS, 50mM Tris-HCl (pH8.0), protease inhibitor cocktail (Roche)] and then sonicated. Cells were centrifuged for 10minutes at 14000rpm at room temperature. Supernatants were collected and diluted with 1:1 PBS (v/v) containing protease inhibitors [protease inhibitor cocktail (Roche)]. Streptavidin-agarose beads (Novagen) were washed twice in lysis buffer and once in PBS containing protease inhibitors. Beads were washed and incubated with samples for 16-20hours at 4°C. Beads were washed and incubated 5 times as follows: once with lysis buffer, once with 1M NaCl, and then thrice with lysis buffer. Captured proteins were eluted by boiling beads in SDS sample buffer for 25minutes at 95°C. Eluted proteins were detected by western blotting with rabbit polyclonal anti-HsRAD51 (H92, Santa Cruz Biotechnology; 1:1000), rabbit polyclonal anti-H3 (C-16, Santa Cruz Biotechnology; 1:3000), rabbit polyclonal anti-Mre11 (Cell Signaling Technology; 1:500), mouse monoclonal anti- γ H2AX (JBW301; 1:4000), rabbit polyclonal anti-phospho RPA32-S4/S8 (Bethyl Laboratories; 1:3000), and rabbit polyclonal anti-phospho RPA32-S33 (Bethyl Laboratories; 1:3000).

2.3. Cell fractionation and immunoblotting

The chromatin-bound protein fraction was isolated as described [24-26] with minor modifications. Western blots were performed with mouse monoclonal anti- γ H2AX (JBW301; 1:4000), rabbit polyclonal anti-HsRAD51 (H92, Santa Cruz Biotechnology;

1:1000), rabbit polyclonal anti-Mre11 (Cell Signaling Technology; 1:500) and rabbit polyclonal anti-H3 (C-16, Santa Cruz Biotechnology; 1:3000).

2.4. Fiber analysis

We performed DNA fiber analysis as described with minor modifications [11, 14, 24]. For replication fork restart, mouse ES cells were pulse-labeled with 25 μ M IdU for 20min, washed twice with medium, and then incubated with 0.5mM hydroxyurea (HU) for 1.5hours. HU was removed and the cells were washed twice in media and then pulse-label with 250 μ M CldU for 20min. Labeled cells were harvested to prepare DNA fiber spreads. Fibers were fixed in methanol and acetic acid (3:1) and then air-dried. DNA fibers were denatured by treating slides with 2.5M HCl for 75-80minutes. Slides were then washed with PBS two times before blocking with 1% BSA (Bovine Serum Albumin) + 0.1% Tween 20 for 1hours. Slides were then incubated with primary antibodies against CldU (rat anti-BrdU BU1/75[ICR1], Abcam, 1:500) and IdU (mouse anti-BrdU B44, 1:200) for 1.5hours. Slides were fixed with 4% PFA and wash thrice with PBS. Then AlexaFluor 555-conjugated goat anti-rat IgG (Molecular Probes, 1:500) and AlexaFluor 488-conjugated goat anti-mouse IgG (Molecular Probes, 1:400) were applied to slides and incubated for 2hours. Slides were washed and mounted in Fluroshield (sigma). Fibers were examined with a Zeiss fluorescent microscope (Axioplan2).

To determine nascent strand degradation, mouse ES cells were pulse-labeled with 25 μ M IdU for 30minutes, washed twice with medium, and then incubated with or without 4mM HU for 5hours at the indicated dose [10]. Labeled cells were processed to capture DNA fiber images as described above. The nascent DNA tract length was measured for over 500 tracts using ImageJ software (<http://rsb.info.nih.gov/ij>). The t-test was used to calculate P-values for mean fiber length.

2.5. Two-color FISH (Fluorescence in situ hybridization)

Two-color FISH was performed as described [14].

2.6. Dose response to HU

This experiment was performed as described with modifications [27]. Cells (2000) were seeded onto the wells of a 24 well plate (day 0). On day 1, cells were treated with HU (0.5mM HU for 1.5hours or 4mM HU for 5hours). Then cells were washed with PBS and incubated in fresh media. Cells were counted on day 6 with a hemacytometer.

2.6. Cell cycle analysis

For the cell cycle analysis, cells were seed 1×10^6 onto a gelatinized 10cm plate and allowed to attach overnight. Cells were treated without or with HU (0.5mM, 1.5hours or 4mM, 5hours), and then cultured for 12hours or 36hours. At each time point, cells were trypsinized and fixed in 70% ethanol for at least 1hour. Fixed cells were refrigerated at 4°C until used. Cells were washed twice with PBS and resuspend in 1ml PI solution with 1 μ l of propidium iodide (PI) from a stock solution (10mg/ml), 81 μ l of RNase A from a stock solution (25mg/ml) and 0.1% Triton X-100. Cells were incubated at 37°C for 20minutes and analyzed for DNA content using a BD Facscliber flow cytometer.

3. Results

3.1. HU conditions that stall and collapse replication forks

AB2.2 mouse ES cells deleted for *Brca2* exon 27 (*brca2^{lex1/lex2}*) [18] [28] were observed to study Ex27's role during replication fork maintenance and double strand break repair. These cells produce normal levels of a C-terminally truncated BRCA2 that is deleted for less than 5% of the protein. This truncated protein interacts with RAD51 in the nucleus presumably through the BRC motifs [29]. Ex27-deletion reduced homology-directed repair [29], increased chromosomal instability [28], increased hypersensitivity to clastogens and interstrand crosslinkers [30] and decreased protection against MRE11-mediated degradation of the nascent replication strand [9]. Ex27-deleted mice exhibited a moderately reduced life span with an early onset of cancer [28]. Thus, Ex27-deletion caused chromosomal instability that enhanced cancer risk.

The goal of this study is to observe the impact Ex27-deletion has on replication maintenance and chromosomal stability when replication forks are stalled or collapsed. RPA32 phosphorylation was observed to differentiate between stalled and collapsed replication forks. RPA is a trimeric protein that binds to single strand DNA at replication forks upon uncoupling of DNA polymerases and helicases contributing to activation of a checkpoint [31]. ATR phosphorylates the 32kDa RPA subunit on serine 33 in response to stalled forks while DNA-PK_{CS} phosphorylates RPA32 S4/S8 in response to collapsed forks with DSBs [32]. Isolation of proteins on nascent DNA (iPOND) was used to purify proteins on or adjacent to the nascent replication strand [13, 14] then Western was used to observe the level of phosphorylated RPA32. Histone 3 was used as a loading control. As shown in figure 1A, mild HU conditions stall replication forks (0.5mM, 1.5hours and 4mM, 1hour) while severe HU conditions collapse replication forks (4mM, 5hours). An intermediate condition was also observed (4mM, 3hours). RPA32 phosphorylation did not obviously vary between control and *brca2^{lex1/lex2}* cells suggesting that Ex27 did not impact the timing or severity of replication fork stalling or collapse (Fig. 1B). These mild and severe HU conditions will be used for the remainder of this study.

3.2. Ex27 influences the localization of RAD51 and MRE11 to the nascent replication strand

iPOND was used to observe γ -H2AX, RAD51 and MRE11 bound to or adjacent to the nascent replication strand [13, 14]. Histone 3 was used as a loading control. Mild HU conditions (4mM, 1hour) were compared to severe HU conditions (4mM, 5hours).

Gamma-H2AX was observed since it recognizes single strand DNA at stalled replication forks [13] and DNA DSBs [33], enables sister chromatid recombination [34] and assists recruitment of other DNA repair proteins to DNA damage [35]. In addition, γ -*h2ax*^{-/-} ES cells were hypersensitive to ionizing radiation and exhibited genomic instability [36]. Mild and severe HU conditions increased γ -H2AX with no apparent difference between control and *brca2^{lex1/lex2}* cells (Fig. 2). The input shows global levels of γ -H2AX increase with severity even though levels at the nascent strand were saturated. By contrast the input levels of RAD51, MRE11, and histone 3 were the same and did not change due to HU exposure or

genotype. These observations could be due to γ -H2AX spreading beyond the stalled fork as previously suggested [13].

RAD51 was observed since it is essential for restarting stalled forks [14] and repairing DSBs at collapsed forks [11]. Previously, MRE11-independent and MRE11-dependent RAD51 loading was shown at stalled and collapsed replication forks, respectively [13]. For control and *brca2^{lex1/lex2}* cells, HU exposure increased RAD51 levels on the nascent strand (Fig. 2). However, Ex27-deletion noticeably reduced these levels consistent with Ex27's role in stabilizing the RAD51 filament [20, 21].

MRE11 was observed since it stabilizes the replisome [37], enables replication fork restart [38] and facilitates DSB repair [7, 8]. MRE11 is a 3'-5' exonuclease that enables 5' end resection [39]. This activity facilitates RAD51-mediated DSB repair [40]; however, is dispensable for replisome stabilization [37]. Ex27 prevents MRE11-mediated degradation of the nascent strand at stalled replication forks [9]. Exposure to mild HU conditions slightly decreased MRE11 levels; yet, Ex27 deletion intensified this reduction (Fig. 2). By contrast, exposure to severe HU conditions did not change MRE11 levels in control cells and elevated MRE11 levels in *brca2^{lex1/lex2}* cells as compared to no exposure (Fig. 2). Thus, Ex27 deletion caused a reduction of MRE11 at stalled forks but no change or perhaps an increase of MRE11 at collapsed forks in response to HU.

3.3. Ex27 influences the positioning of RAD51 and MRE11 on chromatin after release from HU

Western analysis was used to measure the chromatin bound fraction of γ -H2AX, RAD51 and MRE11 after release from mild (0.5mM, 1.5hour) and severe (4mM, 5hours) HU exposure in control and *brca2^{lex1/lex2}* cells. For γ -H2AX in control cells, mild HU exposure increased levels that return to base line within 2hours after HU release (Fig. 3A) while severe HU exposure increased levels that were maintained for 8hours after HU release (Fig. 3B). Ex27 deletion did not impact γ -H2AX levels. For RAD51 in control cells, mild HU exposure initially increased levels that fell below baseline within 2hours after HU release (Fig. 3A, C) while severe HU exposure increased levels that were maintained for 8hours after HU release (Fig. 3B, C). Ex27 deletion reduced RAD51 levels after mild and severe HU exposure, though levels began to increase 8hours after release from severe exposure (Fig. 3A-C). For MRE11 in control cells, mild HU exposure initially had no impact on MRE11 levels but was followed with a mild reduction after HU release while severe exposure caused a greater MRE11 reduction. Ex27 deletion exacerbated the reduction for chromatin bound MRE11 (Fig. 3A, B, D). Thus, Ex27-deletion reduced the levels of chromatin bound RAD51 and MRE11 after HU release. This study of chromatin bound proteins is different from iPOND for MRE11 levels in the *brca2^{lex1/lex2}* cells since the severe HU condition decreased chromatin bound MRE11 but increased iPOND purified MRE11 suggesting an enhancement of MRE11 to collapsed replication forks.

3.4. Ex27 protects nascent replication strands, restarts stalled forks and inhibits new origin firing

Ex27 was shown to protect the nascent replication strand from MRE11-mediated degradation [9]. To corroborate and expand this finding, DNA fibers were observed for cells exposed to varying HU concentrations. Cells were cultured for 30minutes in IdU (iododeoxyuridine) to label the nascent replication strand. IdU was removed and cells were exposed to 1-4mM HU for 5hours. The IdU-labeled fiber length was measured to assess nascent strand protection. For control cells, exposure to the highest HU concentration (4mM, 5hours) only modestly shortened IdU-labeled fibers (Fig. 4A, upper panel, t-test, $p=0.0544$). However, for *brca2^{lex1/lex2}* cells, exposure to HU shortened the IdU-labeled strand in a dose-dependent manner even at the lowest concentration (Fig. 4A, lower panel, $p<0.0001$). Thus, Ex27 protected the nascent strand in a graded response to increasing levels of nucleotide depletion.

DNA fibers were observed to directly address Ex27's role in replication fork restart. Control and *brca2^{lex1/lex2}* cells were incubated in IdU for 20minutes to label the nascent replication strand. IdU was removed and cells were exposed to a mild HU condition (0.5mM, 1.5hours) to stall replication forks. Finally, HU was removed and cells were incubated in CldU (chlorodeoxyuridine) for 20minutes to label post-HU replication, either from restart of the stalled fork or from a new origin. If replication restarts after HU exposure, then the fiber will be green (IdU) followed by a short gap (HU-induced stall) and then red (CldU). However, with failure to restart a stalled replication fork the DNA fiber will be labeled only green while a fiber generated from a newly fired origin will be labeled only red. Without HU exposure, control and *brca2^{lex1/lex2}* cells exhibited about the same level of stalled replication forks ($p=0.98$, Yates corrected chi-square test). After HU exposure, *brca2^{lex1/lex2}* cells exhibited more stalled forks than control cells (Fig. 4B, upper panel $p<0.0001$). In addition, *brca2^{lex1/lex2}* cells exhibited more new origins (Fig. 4B, lower panel $p<0.0001$). These observations show that Ex27 is required to efficiently restart stalled replication forks and suppress new origin firing after short HU-induced blocks. These data corroborate previous reports that describe RAD51's role in replication fork restart after short HU exposure [11, 14]. Altogether, these data support a model that Ex27 enables replication fork stability by both nascent strand protection and replication fork restart after exposure to HU to suppress fork collapse and the formation of a one-sided break. This activity also suppresses new origin firing that could lead to a two-sided break.

3.5. Ex27 protects against HU-induced genomic instability in a dose-dependent manner

Since replication fork maintenance is critical for chromosomal stability, we tested the relative impact the mild and severe HU-conditions had on chromosome damage and structure using two-color fluorescence *in situ* hybridization (FISH) (Fig. 5A, B, Table 1, statistics summarized in Supplemental Table 1). Cells were stained with a telomeric probe (green), a major satellite repeat (MSR) probe in the pericentromere (red) and counterstained with DAPI (blue) [41]. Compared to control cells, *brca2^{lex1/lex2}* cells showed increased spontaneous chromatid and isochromatid breaks, suggesting an intrinsic defect in genome maintenance (Fig. 5A, B) as expected [28]. After exposure to HU, both control and *brca2^{lex1/lex2}* cells exhibited increased levels of chromatid breaks, isochromatid breaks and

radials in a dose-dependent manner. Yet, the *brca2^{lex1/lex2}* cells displayed a greater level of all HU-induced abnormalities at all HU-doses. Thus, *brca2^{lex1/lex2}* cells displayed increased levels of spontaneous breaks and increased levels of HU-induced breaks and radials. These observations show that even mild HU conditions cause a low level of breaks and radials presenting the possibility that even these mild conditions cause a low level of replication fork collapse (though undetectable with RPA p-S4/S8).

3.6. Ex27-deletion enhanced sensitivity to HU-induced replication stalling

We tested the impact HU-induced nucleotide depletion has on cell number and cell cycle distribution in control and *brca2^{lex1/lex2}* cells exposed to HU using mild (0.5mM, 1.5hours) and severe (4.0mM, 5hours) conditions. For survival, cells were seeded on day 0, HU added on day 1 and cells counted on day 6. *brca2^{lex1/lex2}* cells were more sensitive to mild (t test, $p=0.0423$) and severe ($p=0.0214$) HU conditions as compared to control cells (Fig. 5C). The cell cycle distribution was analyzed using the same HU conditions. HU exposure increased the G₁ population immediately after release (R0), then increased the G₂ population 12hours after release (R12) and finally increased the Sub-G₁ population 36hours after release (R36) (Supplemental Fig. 1). These observations were dose-dependent and more severe in the *brca2^{lex1/lex2}* cells.

3.7. Model for early localization of MRE11 to stalled forks

We find an interesting relationship between BRCA2 Ex27 and MRE11 localization to the nascent replication strand. The iPOND data show that compared to control, *brca2^{lex1/lex2}* cells exhibited reduced MRE11 levels coincident with an ATR-induced response to replication fork stalling (RPA32 p-S33) but enhanced MRE11 levels coincident with a DNA-PK_{CS}-response to DSBs (RPA p-S4/S8). These observations suggest that Ex27 enables MRE11 positioning to stalled, but not collapsed, replication forks (Fig. 6). Thus, Ex27 could position MRE11 to stalled forks before collapse. To support this possibility both Ex27 (Fig. 4B) and MRE11 [38] enable replication fork restart and MRE11 stabilizes the replisome independent of nuclease activity [37]. Thus, Ex27 could enable MRE11-mediated stabilization of stalled forks. In addition, it is possible that this MRE11 is positioned for a rapid response to DSBs; thereby, minimizing their deleterious consequences. The elevated MRE11 levels seen in *brca2^{lex1/lex2}* cells after severe HU exposure could be a simple consequence of reduced restart that would lead to fork collapse and DSBs. BRCA2/RAD51 functions are essential for chromosomal maintenance, but could also cause chromosomal rearrangements through rogue replication [14, 24]. Hence this early localization of MRE11 to stalled forks could suppress rearrangements.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Highlights

1. BRCA2 exon 27 (Ex27) suppresses rearrangements caused by stalled replication forks.
2. Ex27 suppresses rearrangements caused by collapsed replication forks.
3. Ex27 enables MRE11 positioning to stalled forks before collapse.

Figure 1.

iPOND analysis on RPA32 to identify conditions that stall and collapse replication forks. (A) Identification of mild and severe HU conditions in control cells. Mild conditions stall forks (RPA32 p-S33) while severe conditions collapse forks (RPA p-S4/S8). No click (NC): for iPOND, the nascent strand is labeled with EdU that has an alkyne group that allows click chemistry (copper-catalyzed cycloaddition to a biotin azide that produces a stable covalent linkage) [42, 43]. Thus, the no click control will not purify with streptavidin. (B) A comparison of RPA32 phosphorylation in control and *brca2^{lex1/lex2}* cells to mild (0.5mM HU, 1.5hours and 4mM HU for 1hour) and severe (4mM HU for 5hours) conditions that stall and collapse replication forks, respectively.

Figure 2.
iPOND purification of γ -H2AX, RAD51 and MRE11 in control and *brca2^{lex1/lex2}* cells exposed to mild (4mM, 1hour) and severe (4mM, 5hours) HU conditions.

Figure 3.

An HU release time course for chromatin bound proteins in control and *brca2lex1/lex2* cells exposed to mild and severe conditions. NT, no treatment. R2, two hours after HU release. R8, eight hours after HU release. (A) Mild HU conditions (0.5mM, 1.5hour). (B) Severe HU conditions (4mM, 5hours). (C) Quantitation of three experiments for RAD51 levels. (D) Quantitation of three experiments for MRE11 levels.

Figure 4.

Fiber analysis for control and *brca2*^{lex1/lex2} cells exposed to varying HU conditions. (A) Measurement of nascent strand protection for control (upper panel) and *brca2*^{lex1/lex2} (lower panel) cells exposed to varying HU concentrations (0-4mM) for 5hours. The length of the IdU-labeled strand is shown after 5hours of HU treatment. For control cells: number of fibers counted and mean fiber length for 0mM HU (1332, 9.01μm) and 4mM HU (1676, 9.4μm). Statistics (t-test) $p=0.0544$. For *brca2*^{lex1/lex2} cells: number of fibers counted and mean fiber length for 0mM HU (438, 9.6μm), 1mM HU (645, 7.7μm), 2mM HU, (646, 5.2μm), 4mM HU, (596, 5.4μm). Statistics is $p<0.0001$ for all comparisons (0v1μM, 0v2μM, 0v4μM, 1v2μM, 1v4μM, 2v4μM) (B) Measurement of replication fork restart (upper panel) and new origin fork firing (lower panel) for cells exposed to mild HU conditions (0.5mM HU, 1.5hours). Fibers labeled with only IdU (green) indicate stalled replication forks while fibers labeled with only red (CldU) indicate newly fired origins. Number of fibers counted for control cells exposed to 0mM HU (926) and 0.5mM HU (1809). Number of fibers counted for *brca2*^{lex1/lex2} cells exposed to 0mM HU (1957) and 0.5mM HU (2539). Statistics (Yates-Corrected Chi-Square Test) comparing control to *brca2*^{lex1/lex2} cells for stalled forks at 0mM HU ($p=0.9866$) and 0.5mM HU ($p<0.0001$). Statistics comparing control to *brca2*^{lex1/lex2} cells for new origin at 0mM HU ($p=0.0054$) and 0.5mM HU ($p<0.0001$).

Figure 5.

Biological outcomes for control and *brca2*^{lex1/lex2} cells exposed to mild (0.5mM HU for 1.5hours and 4mM HU for 1hour) and severe (4mM HU for 4hours) conditions. **(A)** Metaphase spreads from *brca2*^{lex1/lex2} cells that show a chromatin break (top), and isochromatid break (middle) and radial (bottom). **(B)** Quantitation of chromosomal abnormalities per metaphase spread (MPS). Chromatid breaks (left panel), isochromatid breaks (middle panel) and radials (right panel). **(C)** Survival fraction after exposure to mild (0.5mM, 1.5hours) and severe (4mM, 5hours) HU conditions. Cells were seeded on day 0, HU added on day 1 for 1.5hour (0.5mM) or 5hours (4mM). Cells were counted on day 6.

Figure 6.

BRCA2 Ex27 positions MRE11 at stalled forks but not collapsed forks. We hypothesize MRE11 at stalled forks is not catalytically active and could enable fork stabilization and/or prepare for a rapid DSB repair response. MRE11 levels are robust at collapse forks independent of Ex27 possibly as a consequence of impaired fork restart that would elevate DSBs.

Table 1

Summary of metaphase spreads.

HU		No HU	No HU	0.5mM-1.5h	0.5mM-1.5h	4mM-1h	4mM-1h	4mM-5h	4mM-5h
Cell lines		AB2.2	Lx1/2	AB2.2	Lx1/2	AB2.2	Lx1/2	AB2.2	Lx1/2
Ab/MPSs		220	253	110	107	106	105	102	105
CTBs	0	218	246	108	100	97	86	101	81
CTBs	1	2 (0.9%)	6 (2.4%)	1 (0.9%)	6 (5.6%)	3 (2.8%)	16 (15.2%)	1 (1.0%)	15 (14.3%)
CTBs	2	0	1 (0.4%)	1 (0.9%)	1 (0.9%)	1 (0.9%)	2 (1.9%)	0	7 (6.7%)
CTBs	3	0	0	0	0	0	1 (0.9%)	0	0
CTBs	4	0	3 (1.2%)	0	0	0	0	0	0
CTBs	5>	0	0	0	0	0	0	0	2 (1.9%)
ICBs	0	213	226	96	82	89	85	86	58
ICBs	1	6 (2.7%)	25 (9.9%)	13 (11.8%)	22 (20.6%)	16 (15.1%)	20 (19.0%)	15 (14.7%)	34 (32.4%)
ICBs	2	1 (0.5%)	1 (0.4%)	1 (0.9%)	3 (2.8%)	1 (0.9%)	0	1 (1.0%)	12 (11.4%)
ICBs	3	0	1 (0.4%)	0	0	0	0	0	1 (0.9%)
ICBs	4	0	0	0	0	0	0	0	0
ICBs	5>	0	0	0	0	0	0	0	0
Radials	0	220	253	104	95	98	86	92	58
Radials	1	0	0	5 (4.5%)	9 (8.4%)	6 (5.6%)	8 (7.6%)	8 (7.8%)	20 (19.0%)
Radials	2	0	0	0	3 (2.8%)	2 (1.9%)	6 (5.7%)	2 (2.0%)	12 (11.4%)
Radials	3	0	0	1 (0.9%)	0	0	1 (0.9%)	0	5 (4.8%)
Radials	4>	0	0	0	0	0	4 (3.8%)	0	10 (9.5%)

Ab: abnormalities, MPSs, metaphase spreads, CTBs: chromatid breaks, ICB: isochromatid breaks