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Loss of Ubr2, an E3 ubiquitin ligase, leads to chromosome fragility and impaired homologous recombinational repair

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Abstract

The N-end rule pathway of protein degradation targets proteins with destabilizing N-terminal residues. Ubr2 is one of the E3 ubiquitin ligases of the mouse N-end rule pathway. We have previously shown that *Ubr*2$^{-/-}$ male mice are infertile, owing to the arrest of spermatocytes between the leptotene/zygotene and pachytene of meiosis I, the failure of chromosome pairing, and subsequent apoptosis. Here, we report that mouse fibroblast cells derived from *Ubr*2$^{-/-}$ embryos display genome instability. The frequency of chromosomal bridges and micronuclei were much higher in *Ubr*2$^{-/-}$ fibroblasts than in +/+ controls. Metaphase chromosome spreads from *Ubr*2$^{-/-}$ cells revealed a high incidence of spontaneous chromosomal gaps, indicating chromosomal fragility. These fragile sites were generally replicated late in S phase. *Ubr*2$^{-/-}$ cells were hypersensitive to mitomycin C, a DNA cross-linking agent, but displayed normal sensitivity to gamma-irradiation. A reporter assay showed that *Ubr*2$^{-/-}$ cells are significantly impaired in the homologous recombination repair of a double strand break. In contrast, *Ubr*2$^{-/-}$ cells appeared normal in an assay for non-homologous end joining. Our results therefore unveil the role of the ubiquitin ligase Ubr2 in maintaining genome integrity and in homologous recombination repair.

Keywords: Ubr2 ubiquitin ligase; N-end rule proteolytic pathway; Genome instability; Fragile sites; Homologous recombination repair; Mitomycin C sensitivity

1. Introduction

The mouse *Ubr*2 gene encodes one of several E3 ubiquitin ligases of the N-end rule pathway for ubiquitin-mediated protein degradation [1–3]. E3 ubiquitin ligases covalently transfer a 76 amino acid ubiquitin polypeptide from an enzyme called E2 to a target protein with the specificity mainly determined by the E3 ligase [4–6]. In the N-end rule pathway, the N-terminal amino acid determines the half life of the target protein [7] and a particular internal lysine is polyubiquitinated [8,9]. The only known in vivo substrates for *Ubr*2 were identified very recently. They are RGS4, RGS5, and RGS16, which function as down-regulators of signaling by specific G proteins [10,11].

Homozygous null mutation of *Ubr*2 is embryonic lethal in an inbred 129 background [3], whereas in a mixed 129/B6 background, only female *Ubr*2$^{-/-}$ mice display embryonic lethality while *Ubr*2$^{-/-}$ males sur-

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vive but are sterile [3]. The overall architecture of *Ubr2*$^{-/-}$ testes is normal and spermatogonia are intact [3]. During meiosis I in +/+ mice, the homologous chromosomes align during leptotene and zygotene stages and chromosomal synapsis is complete by the pachytene stage where the homologous chromosomes are joined by synaptonemal complexes. Male *Ubr2*$^{-/-}$ sterility is characterized by a defect in homologous pairing during meiosis I and in the death of pachytene stage spermatocytes by apoptosis [3].

Several proteins that are important for meiosis I also play surprisingly diverse roles in the DNA damage response in somatic cells [12], raising the question of whether Ubr2 is also involved in the DNA damage response. Mice that are deficient in the DNA base mismatch repair (MMR) proteins Pms2 [13] or Mlh1 [12] display defects in chromosome pairing during spermatogenesis. Mice lacking the double strand break (DSB) checkpoint protein kinase ataxia telangiectasia mutated (ATM) are also infertile with spermatocytes arresting at the zygotene/pachytene stage of meiosis I and exhibiting aberrant synapsis with unpaired axial cores [14–17]. Mutations in DNA-PK, a kinase that is essential for non-homologous end joining (NHEJ), cause elevated levels of apoptosis during spermatogenesis [18]. Several proteins that are involved homologous recombination (HR) repair are also implicated in meiosis I [12] including Rad51, the Rad51 paralogs (Rad51B, Rad51C, Rad51D, XRCC2, and XRCC3), and the cohesin complex (Smc1, Smc3, Scc1, Sa1, and Sa2) [19–21]. Finally, the repair of DNA cross-links involves not only HR repair proteins, but also the Fanconi anemia (FA) genes A, B, C, D2, E, F, G, and L [22,23], BRCA1 [22], BRCA2 [22], and ATR [24]. Male mouse knockouts for FA proteins D2 [25], A [26,27], C [26,28], and G [29] display impaired fertility with defects in axial pairing, synapsis, and an abundance of apoptotic pachytene spermatocytes. BRCA1 [12,30], BRCA2 [12], and ATR [12] are also implicated in male meiosis. A requirement for *Ubr2* in the aforementioned processes of the DNA damage response can be surveyed by assaying *Ubr2*$^{-/-}$ cells for sensitivity to particular genotoxic agents, and by direct assays that test the efficacy of specific DNA repair mechanisms.

Here, we investigate whether *Ubr2*$^{-/-}$ mouse fibroblasts display spontaneous chromosome instability and fragility. We also test the sensitivity of *Ubr2*$^{-/-}$ cells to doxorubicin, IR, and mitomycin C and ask whether the cells have normal levels of HR repair and NHEJ. We report that loss of *Ubr2* results in high incidence of chromosome breaks, a hypersensitivity to mitomycin C, and a severe deficiency in HR DSB repair.

2. Materials and methods

2.1. Establishment of mouse embryonic fibroblasts and their immortalization

Ubr2$^{-/+}$ mice of the inbred 129/SvJ background [3] were intercrossed and *Ubr2*$^{-/-}$ and +/+ mouse embryonic fibroblasts (MEFs) were prepared from E13.5 mouse embryos from the resulting litters and immortalized using SV40 T-antigen [31]. The PCR primers used for identifying the *Ubr2*$^{-}$ and + alleles were UBR2f (5′-CTA CTG CAT GCT GTT TAA TGA TGA G), UBR2r (GGA GGT AGA AAC ATG CAA ATC TCT G), and Neof (5′-CCA GCT CAT TCC TCC CAC TCA TGA TC).

2.2. Metaphase chromosome spreads

Log-phase MEFs were treated with 0.3 μg/ml colcemid (Invitrogen Inc., Carlsbad, CA) for 30 min, trypsinized and resuspended in Dulbecco's modified eagle medium (DMEM) with 10% fetal bovine serum, pelleted by centrifugation, resuspended in 1× phosphate buffered saline (PBS), pelleted by centrifugation, and resuspended in 0.4% KCl for 15 min. A half volume of 3:1 methanol:acetic acid solution was then added one drop at a time to the cell suspension. The cells were pelleted by centrifugation, resuspended in 5 ml 3:1 methanol:acetic acid solution, pelleted again by centrifugation, resuspended in a 200 μl of 3:1 methanol:acetic acid solution, and dropped onto glass slides to obtain spreads. Chromosomes were stained with GIEMSA (Invitrogen Inc.) or DAPI (Vysis, Downers Grove, IL).

2.3. Assay for late replication

BrdU (30 μM) was added to asynchronous growing MEFs at 80% confluence. It had previously been determined that 4.5 h is the appropriate duration of BrdU incorporation for the late replication timing assay for such immortalized mouse fibroblasts [32]. Subsequent to culturing fibroblasts for 4.5 h in presence of BrdU, 50 ng/ml colcemid (Life Technologies, Grand Island, NE) was added 1 h before harvest. Cell suspensions were incubated in 0.4% KCl at 37 °C for 13–15 min, followed by fixation with 3:1 methanol:acetic acid. Metaphase spreads were prepared by dropping the BrdU-treated cells onto cover slips followed by treatment in 70% formamide/2XSSC (0.3 M sodium chloride, 0.03 M sodium citrate dihydrate, pH 7.0) at 73 °C for 2 min to denature the chromosomes. Spreads were incubated in blocking buffer (1× PBS, 10% fetal bovine serum, 0.2% Tween-20) and the incorporated BrdU was detected using monoclonal anti-BrdU antibody (Sigma, St. Louis, MO) at a 1:20 dilution in blocking solution followed by treatment with a 1:150 dilution of Texas-Red anti-mouse antibody (Jackson Immunoresearch, Westgrove) in blocking buffer. Images were captured using Quips mFISH software (Vysis), transferred to NIH image (http://rsb.info.nih.gov/nih-image), and the number of pixels occupied by the chromosomes bearing the gaps or breaks (DAPI) and by fluorescently labeled

BrdU (Texas-Red) on these same chromosomes were then calculated.

2.4. MEF survival following mitomycin C, IR, and doxorubicin treatment

To determine the sensitivity of *Ubr2*$^{-/-}$ and +/+ MEFs to IR and mitomycin C, immortalized MEFs were seeded at 1000 cells/10 cm plate. Colonies were counted 7 days subsequent to gamma-irradiation (IR) and 9 days after the initiation of mitomycin C treatment at the following concentrations: 0, 0.2, 0.3, and 0.4 μM. Mitomycin C was maintained in the media throughout the experiment. For each IR or mitomycin C treatment, the post-irradiation survival is represented as the number of colonies surviving treatment divided by the number of colonies in the untreated control. For each condition, averages were calculated from three plates.

To determine the sensitivity of *Ubr2*$^{-/-}$ and +/+ MEFs to doxorubicin, 10^4 cells were plated on to 96-well plates, followed by treatment with doxorubicin (10, 50, and 100 ng/ml) (Sigma). After 3 days, cells were exposed to 0.5 mg/ml of MTT [3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide] (Sigma) for 5 h. Media was removed and 0.15 ml of DMSO was added to each well, followed by incubation at 37 °C for 5 min. The absorbance was measured at 550 nm. This assay [33] is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT and form a dark blue formazan crystal which is impermeable to cell membranes, thus resulting in its accumulation within healthy cells. The number of surviving cells is directly proportional to the level of the created formazan product.

2.5. Non-homologous end joining assay

pGreenLantern plasmid (BD Biotechnology, Franklin Lakes, NJ) was linearized by cleavage with *Eco*R1 and *Hin*dIII and the completeness (>99%) of cleavage was verified by agarose gel electrophoresis. Both enzymes cleave between the CMV promoter and GFP open reading frame (ORF). Transient transfections were carried out following the Fugene-6 protocol (Roche Inc., Alameda, CA). Purified linearized DNA (1 μg) was mixed with 3 μl of Fugene-6 and 100 μl of serum-free DMEM (Invitrogen), incubated for 15 min at room temperature, and added to 5×10^5 log-phase cells in each well of a six-well plate. Cells were trypsinized 2 days later and subjected to flow cytometry.

2.6. Homologous recombination assay

Ubr2$^{-/-}$ and +/+ cells were transfected with the plasmid p59xDR-GFP6 [34] using the Fugene-6 transfection reagent (Roche Inc.) and hygromycin-resistant stable cell lines were obtained. The intactness of this integrated reporter construct was determined using PCR. Cell lines carrying intact reporter constructs were transiently transfected with the I-*Sce*I expression construct pCBASce [34] or with the control plasmid pBluescript (Stratagene, La Jolla, CA). The plasmid pCAG-NZE [35] carrying an intact GFP expression construct was used to test transfection efficiency. Two days after transfection, cells were subjected to flow cytometry to identify GFP-expressing cells. Homologous recombination efficiency was calculated by normalizing the GFP positive cells arising in pCBASce-transfected or pBluescript-transfected cells to one control cell line, and the HR efficiency was expressed as a percentage.

2.7. Statistical analyses

P-values representing the significance of the interaction between *Ubr2* genotype and IR dose, mitomycin C concentration, or doxorubicin concentration, were obtained using the General Linear Model in SAS/STAT software (SAS Institute Inc., Cary, NC). All other statistical analyses were performed using the software package R [36] which can be downloaded from http://cran.r-project.org/. For the NHEJ and HR repair assays, differences in measurements were tested across categorical groupings using the Kruskal–Wallis test [37]. For the replication timing analysis, the *P*-value was obtained using a Binomial test available at http://www.herine.net/stat/papers/dbinom.pdf.

3. Results

3.1. Absence of hypersensitivity to IR or a defect in NHEJ in *Ubr2*$^{-/-}$ fibroblasts

Somatic cells deficient in DSB cell cycle checkpoints are hypersensitive to IR [38]. To determine whether *Ubr2*-deficiency also confers hypersensitivity to IR, we intercrossed *Ubr2*$^{-/+}$ mice of the 129Sv/J inbred background and identified *Ubr2*$^{-/-}$ and +/+ mouse embryonic fibroblasts using PCR [3] (Fig. 1A). A number of MEF lines were obtained and exposed to γ-IR. The *Ubr2*$^{-/-}$ MEFs were not significantly more sensitive to IR than the +/+ cells ($P = 0.435$, using a General Linear Model) (Fig. 1B). This suggested that Ubr2 is unlikely to be required for the DSB cell cycle checkpoint.

The normal sensitivity to γ-IR also suggested that *Ubr2* is unlikely to be required for NHEJ. To verify that this is the case, we directly tested the ability of *Ubr2*$^{-/-}$ cells to perform NHEJ of double strand breaks using a plasmid-based assay [39,40]. In this assay, a double strand break was generated by cleaving a GFP reporter plasmid (see Section 2) between the GFP ORF and its promoter, using two enzymes such that the promoter and the ORF of the reporter gene are separated by incompatible ends (Fig. 1C, top). The NHEJ path-

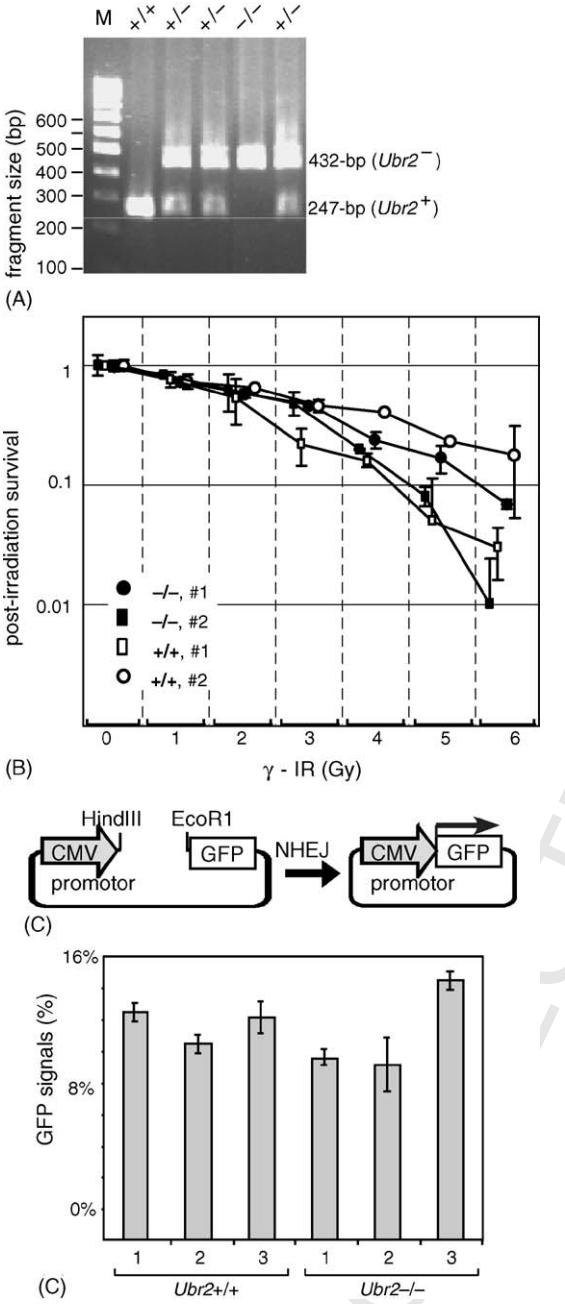


Fig. 1. $Ubr2^{-/-}$ cells are not hypersensitive to IR or defective in NHEJ. (A) Identification of $Ubr2^{-/-}$ and +/+ MEFs using PCR. M, DNA size markers. (B) Survival of $Ubr2^{-/-}$ (filled symbols) and +/+ (open symbols) MEFs following treatment with γ-IR. Each treatment was done in triplicate and averages and standard deviations are shown. To reduce overlap of the symbols in the charts, the symbols representing different cell lines at the same treatment dosage were slightly horizontally offset. (C) Assay for NHEJ reveals no significant difference between $Ubr2^{-/-}$ and +/+ cells. Repair of a break with non-homologous DNA ends by NHEJ results in GFP expression. Three $Ubr2^{-/-}$ and three +/+ cell lines were each subjected to this assay three times and averages and standard deviations are shown.

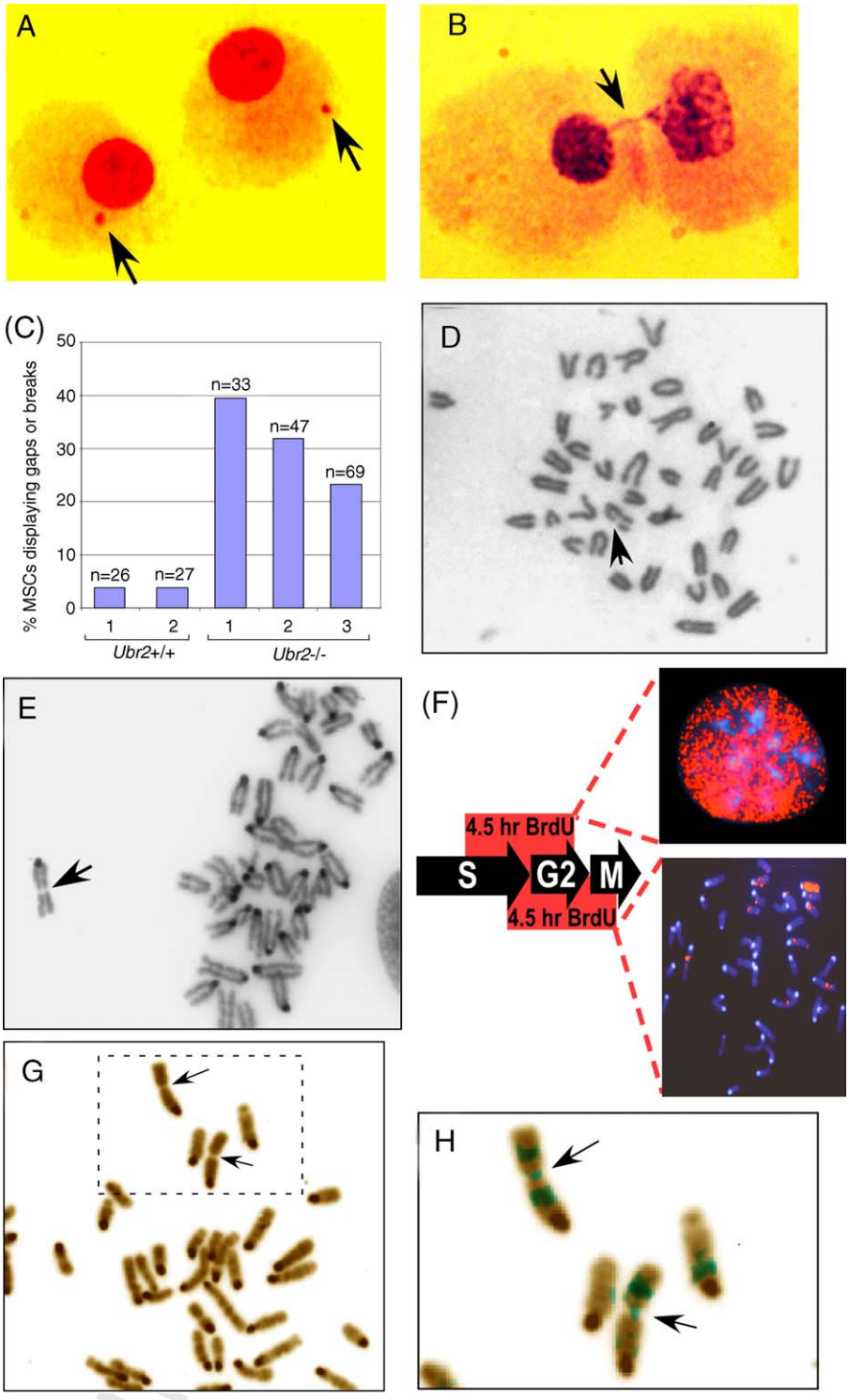
way would seal the broken ends and allow the reporter construct to express GFP. The +/+ and $Ubr2^{-/-}$ MEF lines displayed similar levels of GFP expression (Fig. 1C, bottom) that were not significantly different ($P = 0.398$ using the Kruskal–Wallis test [37]). This indicated that the NHEJ pathway is indeed functional in $Ubr2^{-/-}$ cells.

3.2. Genomic instability in $Ubr2^{-/-}$ cells

Micronuclei are features reported in somatic cells deficient in several DNA damage response proteins including FA [41], BRCA1 [42], ATR [43], and ATM [44]. Nucleoplasmic bridges have been reported in cells from FA patients [41] and in *ATM*-deficient cells [45,46]. Primary $Ubr2^{-/-}$ and +/+ fibroblasts from $Ubr2^{+/-}$ 129/SvJ intercrosses were examined for micronuclei and nucleoplasmic bridges. The scale of these analyses was curtailed by an under-representation of E13.5 $Ubr2^{-/-}$ embryos in pregnant females and by difficulties in growing primary $Ubr2^{-/-}$ MEFs. Nevertheless, micronuclei and nucleoplasmic bridges were scored without prior knowledge of which slides were experimental and which slides were the controls. In the absence of exogenous reagents, primary $Ubr2^{-/-}$ MEFs displayed structures resembling micronuclei in 5 out of 100 cells scored (Fig. 2A), while no unambiguous micronuclei were seen in 100 primary +/+ MEFs examined (not shown). Structures resembling nucleoplasmic bridges were observed in 6 out of 100 untreated primary $Ubr2^{-/-}$ MEFs (Fig. 2B) and in 0 out of 100 untreated primary +/+ MEFs examined (not shown).

Micronuclei can arise from chromosomes breaks [44]. Nucleoplasmic bridges can be the product of

Fig. 2. Evidence of chromosomal fragility in $Ubr2^{-/-}$ MEFs. (A) Evidence of spontaneous micronuclei (arrows) in primary $Ubr2^{-/-}$ MEFs. (B) Primary $Ubr2^{-/-}$ MEF displaying a spontaneous nucleoplasmic bridge possibly consisting of chromosomal material joining two nuclei (arrow). (C) Proportion of metaphase chromosome spreads displaying chromosomal breaks or gaps in three $Ubr2^{-/-}$ and two +/+ cell lines. (D) Break or gap in one sister chromatid in a metaphase chromosome spread from a $Ubr2^{-/-}$ MEF (arrow). (E) Breaks or gaps in both sister chromatids in a spread from a $Ubr2^{-/-}$ MEF (arrow). (F) Diagram of the assay employed to identify late replicating regions on chromosomes: BrdU, red; DAPI, blue. (G and H) Late replication detected at gaps or breaks in an $Ubr2^{-/-}$ spread from a cell that had been exposed to BrdU while in late S phase. (G) Negative of DAPI stained chromosome reveals gaps or breaks (arrows): DAPI, brown. (H) The chromosomes displaying gaps or breaks shown at higher magnification and displaying fluorescent immunostaining for BrdU (green) that had been incorporated while the cells were in late S phase. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)



translocations that give rise to dicentric chromosomes [47,48]. The presence of elevated numbers of micronuclei and nucleoplasmic bridges in *Ubr2*$^{-/-}$ cells raised the possibility that *Ubr2*-deficiency may cause genome fragility in the mouse genome that are a source of these defects. Expressed fragile sites can be visualized as gaps or breaks in metaphase chromosome spreads. The frequency of spontaneous gaps and breaks was deter-

mined in spreads from two +/+ and three *Ubr2*$^{-/-}$ cell lines. *Ubr2*$^{-/-}$ spreads revealed a substantially higher incidence of gaps and breaks than +/+ spreads ($P = 2.2 \times 10^{-16}$ using a Chi-square test) (Fig. 2C). Gaps and breaks involving one sister chromatid (Fig. 2D) or both sister chromatids at corresponding sites (Fig. 2E) were observed, indicating fragility in the *Ubr2*$^{-/-}$ genome.

One property associated with fragile sites is late replication in S phase [49–52]. To determine whether the gaps or breaks in *Ubr2*$^{-/-}$ spreads are associated with late replicating regions, fibroblasts were subjected to an assay for late-replication timing. Unsynchronized exponentially growing *Ubr2*$^{-/-}$ and +/+ cells were exposed to BrdU for a 4.5 h interval and then spreads were prepared and subjected to fluorescent immunostaining for BrdU that had been incorporated into chromosomal DNA. Cells that were in late S phase at the time of BrdU addition incorporated BrdU in late S phase and this was identified on the condensed metaphase chromosomes using fluorescent immunostaining (Fig. 2F, bottom). Cells that were in early- or mid-S phase during the exposure to BrdU failed to reach M phase in the 4.5 h interval and consequently were not represented among the spreads (Fig. 2F, top). Zero percent ($n = 25$) of BrdU-labeled +/+ spreads displayed gaps or breaks. This frequency is similar to the percent spreads displaying gaps or breaks from cells without BrdU treatment (Fig. 2C) indicating that the presence of BrdU had little or no effect the frequency of chromosome gaps or breaks. Inspection of *Ubr2*$^{-/-}$ metaphase chromosome spreads displaying BrdU incorporation revealed that 24/30 chromosomal gaps or breaks examined were associated with late replicating chromosomal regions (Fig. 2G and H). The proportion of BrdU was determined for each chromosome using NIH image (http://rsb.info.nih.gov/nih-image). On an average, 26.4% of the chromosomes displayed BrdU signal. A Binomial test was used to determine that the co-localization of chromosome breaks and BrdU signal was statistically significant ($P = 2.9 \times 10^{-9}$).

3.3. *Ubr2*$^{-/-}$ cells do not display resistance to doxorubicin but are hypersensitive to mitomycin C

Deficiencies in the MMR genes *PMS*2 and *MLH*1 not only lead to a predisposition to fragile sites after treatment with the replication inhibitor aphidicolin [53] but also to a hypersensitivity to mitomycin C [54]. Cells deficient in HR repair genes have been reported to show spontaneous chromosome gaps and breaks and also a sensitivity to mitomycin C [35,55–64]. FA cells also display fragility (after aphidicolin treatment) [65] and a pronounced mitomycin C sensitivity [66]. *Ubr2*$^{-/-}$ cells were much more sensitive to mitomycin C than +/+ cells; this was particularly evident when cells were exposed to 0.3 μM mitomycin C and only +/+ cells survived (Fig. 3A). The General Linear Model revealed that this effect was highly significant ($P < 0.0001$). The Ubr2 protein may thus be involved in MMR, DSB HR repair, and/or DNA cross-link repair.

Loss or reduction of expression of *MLH*1, *PMS*2, and other MMR genes have been linked to an

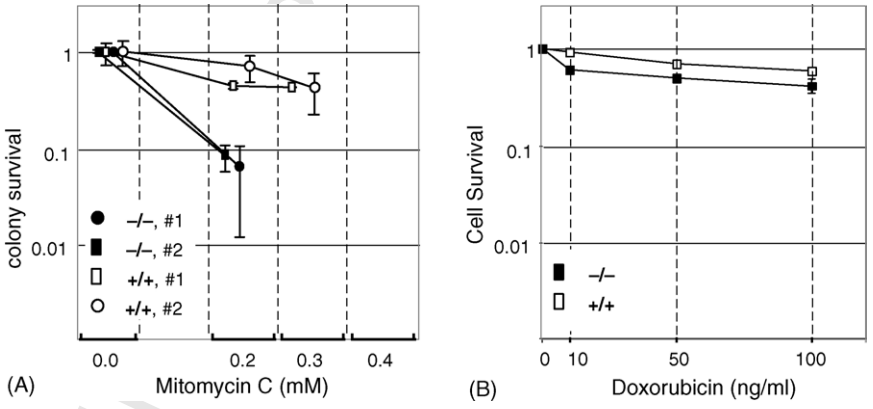


Fig. 3. Survival of *Ubr2*$^{-/-}$ and +/+ MEFs following treatment with mitomycin C or doxorubicin. (A) Plates were treated with mitomycin C at the indicated concentrations. Each treatment was done in triplicate and averages and standard deviations are shown. To reduce overlap of the symbols in the charts, the symbols representing different cell lines at the same treatment dosage were slightly horizontally offset. No *Ubr2*$^{-/-}$ or +/+ MEFs survived exposure to 0.4 μM mitomycin C. (B) *Ubr2*$^{-/-}$ and +/+ MEFs were treated with doxorubicin at indicated concentrations, and the survival of cells was determined by MTT assay [33] (see Section 2). Percent survival of each treatment was calculated by dividing the absorbance value of untreated cells. All treatments were triplicated.

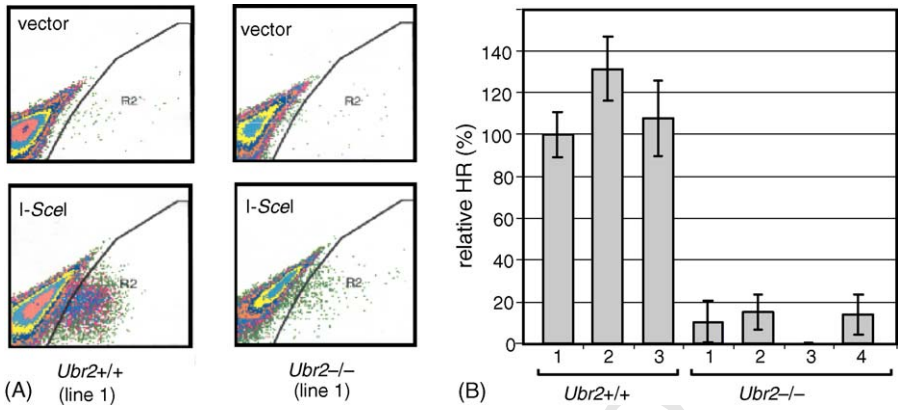


Fig. 4. Homologous recombination mediated double strand break repair is deficient in *Ubr*2$^{-/-}$ cells according to an I-*Sce*I-based assay [34]. (A) Identification of *Ubr*2$^{-/-}$ and +/+ MEF cells that reconstituted GFP expression using flow cytometry. For each plot, the cells represented to the right of each diagonal line expressed GFP, while cells represented to the left of this line were considered negative for GFP expression. (B) Multiple independently derived *Ubr*2$^{-/-}$ cell lines display reduced HR DSB repair. Flow cytometry results were obtained for seven independently derived cell lines. Averages and standard deviations of three repetitions of the procedure are shown. The GFP expression obtained for the +/+ reporter cell line 1 was arbitrarily assigned a value of 100 and the values obtained for the other six reporter lines are in relation to this value.

increased resistance to doxorubicin [67–69]. To determine whether *Ubr*2$^{-/-}$ cells display resistance to doxorubicin, *Ubr*2$^{-/-}$ and +/+ cells were treated with increasing concentrations of doxorubicin. In contrast to the reports for MMR mutants, the *Ubr*2$^{-/-}$ cells were not less sensitive to doxorubicin than +/+ cells (Fig. 3B). In fact, the *Ubr*2$^{-/-}$ cells were significantly *more* sensitive to doxorubicin ($P > 0.0001$ using a General Linear Model).

3.4. *Ubr*2$^{-/-}$ cells are defective in homologous recombination mediated double strand break repair

Direct measurements of DSB HR repair using I-*Sce*I nuclease-based assays revealed that deficiencies in the HR genes *Xrcc*2 and *Xrcc*3 produce severe (>25-fold) reductions in HR repair [35,58] while loss of FA gene function has been associated with mild (<3.5-fold) reductions in the efficiency of HR repair [70–72]. To determine whether *Ubr*2 is involved in HR repair, we subjected *Ubr*2$^{-/-}$ cells to an assay that generates a functional GFP gene via the HR repair of a DSB generated using I-*Sce*I [34]. In wild type (+/+) cells with a normal capacity for HR repair, the double strand breaks were repaired, resulting in GFP expression that was readily identifiable using flow cytometry. In contrast, the introduction of vector plasmid resulted in much lower levels of GFP-expressing cells (Fig. 4A). The number of GFP-expressing cells was, on average, approximately 12 times lower in *Ubr*2$^{-/-}$ MEFs upon I-*Sce*I expression compared to +/+ cells (Fig. 4A and B), a highly significant difference according to the Kruskal–Wallis test ($P = 1.92 \times 10^{-6}$) [37]. This indicated that DSB HR repair is compromised in *Ubr*2$^{-/-}$ cells. It should be pointed out that this assay did not distinguish single strand anneal (SSA) from the classical HR repair.

4. Discussion

Various properties of *Ubr*2$^{-/-}$ MEFs were surveyed in order to determine whether the mouse *Ubr*2 ubiquitin ligase plays a role in maintaining genome stability and/or DNA repair. Primary *Ubr*2$^{-/-}$ MEFs displayed an elevated incidence of micronuclei and nucleoplasmic bridges in the absence of exogenous agents. *Ubr*2$^{-/-}$ MEFs also exhibited high frequencies of spontaneous gaps or breaks in metaphase chromosomes, indicating chromosomal fragility. Similar to fragile sites in human cells [49–52], the chromosomal breaks and gaps in mouse *Ubr*2$^{-/-}$ chromosomes correlated with late replication in S phase. *Ubr*2$^{-/-}$ MEFs failed to display hypersensitivity to IR, appeared normal for NHEJ, and did not display resistance to doxorubicin, a trait associated with defects in MMR genes [67–69,73]. However, *Ubr*2$^{-/-}$ cells were hypersensitive to the DNA cross-linking agent mitomycin C and displayed a pronounced defect in HR repair.

The *Ubr*2$^{-/-}$ phenotype resembled the phenotypes brought on by defects in the five Rad51 paralogs, but appeared to be somewhat milder. Like Ubr2, the Rad51 paralogs are not essential for cell viability [21], but mouse knockouts of paralogs (*Xrcc*2 [74], *Rad51B* [75], and *Rad51D* [76]) display embryonic lethality. All par-

alogs appear to be very important for HR repair [21]. The, on average, 12-fold reduction in HR repair of I-*Sce*I sites in *Ubr*2$^{-/-}$ MEFs was less severe than the >100-fold and 25-fold reductions in HR repair of I-*Sce*I sites reported for hamster cells defective in *Xrcc*2 [58] and *Xrcc*3 [35], respectively. BCCIP, like the paralogs, interacts with Rad51 and also causes >20-fold reductions in HR repair [77]. It is possible that the loss of one or more of the six other Ubr proteins will produce a HR repair defect that is as severe as in paralog-deficient cells. Similar to *Ubr*2$^{-/-}$ MEFs, paralog-deficient cells exhibit spontaneous gaps or breaks [56,57,60,62–64], are hypersensitive to mitomycin C [21], and intact for NHEJ [21]. Paralog-deficient cells are only slightly sensitive to IR [21]. The precise roles of the paralogs in HR and HR repair have not been thoroughly determined [21].

The *Ubr*2$^{-/-}$ phenotype also bears some resemblance to mutations in the FA-ATR-BRCA1 pathway that responds to DNA cross-links. I-*Sce*I-based assays reveal mild reductions in DSB HR-repair in FA cells (<3.5-fold) [70–72] and *BRCA*1-deficient cells (five- to six-fold) [78]. However, *BRCA*2-deficient cells exhibited a 6–100-fold decrease, depending on the cell line [34]. Similar to *Ubr*2 mutants, FA, *BRCA*2-deficient, *BRCA*1-deficient, and ATR-deficient cells are all sensitive to mitomycin C [22,79,80] but competent in NHEJ [22,34,71,78,80]. Finally, elevated frequencies of micronuclei, nucleoplasmic bridges, and gaps or breaks in spreads are associated with deficiencies in the FA-ATR-BRCA1 pathway [41,65,81–83]. It has been proposed that fragile sites are regions of the genome where DNA replication forks stall or collapse leading to late replication [51,84,85]. It was further proposed that a subset of these unreplicated single stranded regions escape checkpoint controls and proceed into mitosis where they break or stretch during the preparation of spreads [65,81,82]. The late replication at the spontaneous gaps or breaks in *Ubr*2$^{-/-}$ chromosomes and the associated HR repair defect are consistent with this hypothesis.

Ubr2 joins a list of ubiquitin and SUMO E3 ligases that have been implicated in HR repair. Monoubiquiylation of FANC-D2 is considered a central event in the response to DNA cross-links and requires several proteins in the FA-ATR-BRCA1 pathway [22,86,87] including the ubiquitin ligase FANC-L [23]. Ubiquiylated FANC-D2 co-localizes [88] with the ubiquitin ligase BRCA1 [89–93] which ubiquitinylates FANC-D2 in vitro [94]. Another example is the ubiquitin-mediated proteolysis of Rad51 via an unidentified ligase that is regulated by RAD51C [95]. Rad51 also interacts with unconjugated SUMO and over-expression of wild type SUMO or an unconjugatable mutant SUMO inhibits HR repair [96]. Finally, the DNA replication protein PCNA is modified by SUMO [97] and this suppresses HR during S phase, perhaps to prevent unwanted HR repair [98].

The mechanisms that underlie the Ubr2-dependent maintenance of genome integrity and HR repair are unknown. Although we recently found that Ubr2 (and a related protein Ubr1) ubiquitylate RGS4, RGS5, and RGS16, failure to degrade these proteins is unlikely to be responsible for the genome stability and DNA repair defects of *Ubr*2$^{-/-}$ cells, given that the function of the RGS proteins is to control extracellular stimulus-mediated signaling pathways [10,11]. Proteins that are more likely to mediate Ubr2's role in genome stability and HR repair include Recql4 and Scc1. We have recently found that human Ubr2 and Ubr1 form a complex with Recql4 [99], a putative helicase that is mutated in both Rothmund–Thomson syndrome (RTS) [100,101] and RAPADILINO syndrome [102]. Cells from RTS patients display chromosomal instability [103,104], suggesting that a disrupted *Ubr*1–*Ubr*2–*Recql*4 circuit may contribute to the genome instability of *Ubr*2$^{-/-}$ mouse cells. Scc1 is a subunit of the cohesin complex that is recruited to DSBs during HR repair [19]. Conditional cleavage of fission yeast SCC1 by a protease called separase has been shown to be required for DNA repair [105]. In budding yeast, one of the SCC1 cleavage products has been shown to be degraded by yeast UBR1 [106], a homolog of mouse Ubr2 [1]. Disruption of this degradation results in chromosome loss that is likely due to the perturbation of cohesin function by the SCC1 fragment [106]. In mice, Ubr2-deficiency may therefore alter cohesin function resulting in genome fragility and a HR repair defect. Future work will address whether Ubr2 is involved in both SSA and classical HR repair, whether other Ubr ligases are involved, and whether the role of Ubr2 in HR repair and the prevention of chromosomal fragility are linked to interactions with Rad51, Rad51 paralogs, Recql4, and cohesin.

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