Absence of Brca2 causes genome instability by chromosome breakage and loss associated with centrosome amplification

Andrew Tutt*, Anastasia Gabriel*, David Bertwistle*, Frances Connor*, Hugh Paterson†, John Peacock‡, Gillian Ross‡ and Alan Ashworth*

Women heterozygous for mutations in the breast-cancer susceptibility genes *BRCA1* and *BRCA2* have a highly elevated risk of developing breast cancer [1]. *BRCA1* and *BRCA2* encode large proteins with no sequence similarity to one another. Although involvement in DNA repair and transcription has been suggested, it is still not understood how loss of function of these genes leads to breast cancer [2]. Embryonic fibroblasts (MEFs) derived from mice homozygous for a hypomorphic mutation (*Brca2*^{Tr2014}) within the 3′ region of exon 11 in *Brca2* [3], or a similar mutation (*Brca2*^{Tr}) [4], proliferate poorly in culture and overexpress the tumour suppressor p53 and the cyclin-dependent kinase inhibitor p21$^{Waf1/Cip1}$. These MEFs have intact p53-dependent DNA damage G_1–S [3,4] and G_2–M checkpoints [4], but are impaired in DNA double-strand break repair [3] and develop chromosome aberrations [4]. Here, we report that *Brca2*$^{Tr2014/Tr2014}$ MEFs frequently develop micronuclei. These abnormal DNA-containing bodies were formed through both loss of acentric chromosome fragments and by chromosome missegregation, which resulted in aneuploidy. Absence of *Brca2* also led to centrosome amplification, which we found associated with the formation of micronuclei. These data suggest a potential mechanism whereby loss of *BRCA2* may, within subclones, drive the loss of cell-cycle regulation genes, enabling proliferation and tumourigenesis.

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Results and discussion

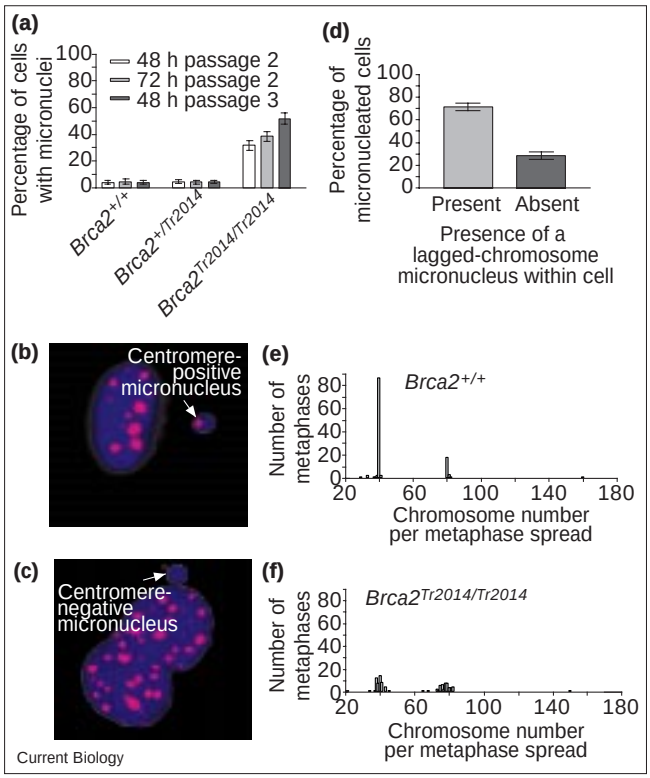
As shown in Figure 1a, MEFs derived from day 13.5 *Brca2*$^{Tr2014/Tr2014}$ embryos had a high frequency of spontaneous micronucleus formation (32% in passage 2), increasing with passage number (52% in passage 3, χ^2 for trend = 53.2, $p < 0.0001$). In contrast, micronuclei were rarely observed in MEFs derived from wild-type or heterozygous littermates, and did not increase in frequency with passage number (3–4% in passage 2 and 3).

Micronuclei — which are a recognised consequence of genome instability [5] — occur in cells that have completed at least one mitosis and contain either an acentric chromosomal fragment formed by an unrepaired double-strand break, or a lagged chromosome (that is, a chromosome that fails to segregate into the daughter macronuclei at mitosis) [6,7]. To differentiate between these two types of micronuclei, we used a pan-centromeric probe that hybridises to the major (γ) satellite region of mouse centromeric DNA. The centromere is the point of attachment of the kinetochore and mitotic spindle to the chromosome [8]. The detection of the pan-centromeric probe within a micronucleus indicates a lagged chromosome rather than an acentric chromosome fragment [6,7] (Figure 1b,c). MEFs from three *Brca2*$^{Tr2014/Tr2014}$ embryos were scored for the presence or absence of centromeres in micronuclei; 71.4% of micronucleus-containing cells had a lagged chromosome (Figure 1d). If a micronucleus containing a lagged chromosome is inherited after cytokinesis by the incorrect daughter cell or is lost at cytokinesis, the daughter cells become aneuploid. The remaining micronucleus-containing cells (28.6%) had micronuclei formed by acentric fragments. This occurs when a cell enters mitosis with an unrepaired or mis-repaired chromosomal double-strand break. The loss of these acentric fragments at cytokinesis will also result in loss or gain of genetic material by daughter cells.

As some *Brca2*$^{Tr2014/Tr2014}$ MEFs contained more than one micronucleus, we examined the relative frequency of loss of whole chromosomes or acentric fragments. In MEFs (passage 2) derived from two *Brca2*$^{Tr2014/Tr2014}$ embryos, we found a mean of 1.5 micronuclei for each micronucleus-containing cell; 60.2% (95% confidence interval (CI) 56.4–64) of micronuclei were centromere-positive and 39.8% (95% confidence interval 36–43.6) were centromere-negative, indicating that the absence of wild-type *Brca2* leads to both the presence of chromosome breaks and to chromosome missegregation at mitosis.

The centromeric major (γ) satellite represents 5–10% of the mouse genome. A persistent DNA double-strand break within this region may lead to the presence of the major satellite signal within a micronucleus. If DNA double-strand breaks or their misrepair are randomly

Figure 1



Spontaneous micronucleus formation, chromosome missegregation and the presence of chromosome breaks at mitosis in *Brca2*$^{Tr2014/Tr2014}$ MEFs. (a) Percentage of *Brca2*$^{+/+}$, *Brca2*$^{+/Tr2014}$ and *Brca2*$^{Tr2014/Tr2014}$ MEFs at passage 2 and 3 that contain micronuclei. Error bars represent CIs of the proportion. (b) *Brca2*$^{Tr2014/Tr2014}$ MEF containing a single centromere-positive micronucleus. (c) Binucleate *Brca2*$^{Tr2014/Tr2014}$ MEF with a centromere-negative micronucleus. In (b,c), the cells were hybridised with a Cy3-labelled pan-centromeric DNA probe (red); DNA was stained with 4,6-diamidino-2-phenylindole (DAPI, blue). (d) Proportion of micronuclei-containing MEFs with a lagged chromosome. Error bars represent CIs of the proportion. (e,f) Frequency histogram of metaphase chromosome number counted on 140 metaphase spreads from (e) *Brca2*$^{+/+}$ and (f) *Brca2*$^{Tr2014/Tr2014}$ MEFs, both at passage 2.

sited, the major satellite signal should be seen in only 5–10% of micronuclei [9]. The frequency of centromere-positive micronuclei in *Brca2*$^{Tr2014/Tr2014}$ MEFs is, however, more consistent with failure of the mitotic spindle/kinetochore mechanism to correctly segregate chromosomes at mitosis; an alternative hypothesis might be the preferential induction or misrepair of double-strand DNA breaks within regions of centromeric satellite repeats.

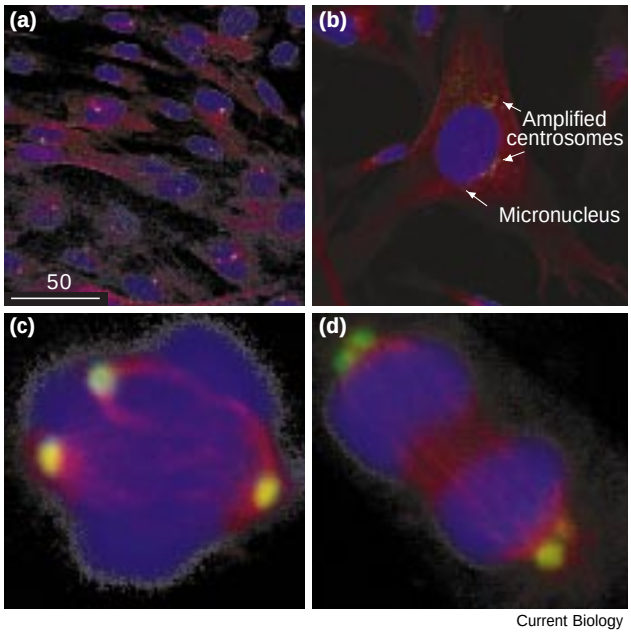
The formation of micronuclei containing lagged chromosomes should lead to aneuploidy. By counting chromosome numbers in metaphase spreads, we found that 86% (CI 79–92) of *Brca2*$^{Tr2014/Tr2014}$ MEFs (passage 2) were aneuploid compared with 20% (CI 15–27) for control MEFs derived from *Brca2*$^{+/+}$ littermates (Figure 1e,f). This confirms that absence of *Brca2* leads to aneuploidy.

The spindle pole mediates the correct segregation of chromosomes into daughter nuclei through attachment of the kinetochores of chromatids to spindle microtubules [10]. The centrosome, which nucleates the spindle pole and determines the axes of mitosis, normally duplicates once per cell cycle, at the G_1–S boundary [11]. The two daughter centrosomes migrate to opposite poles before segregating a diploid chromosome set to that pole. To determine whether the chromosome missegregation in *Brca2*$^{Tr2014/Tr2014}$ MEFs might be associated with abnormalities of centrosome number, we counted the number of centrosomes per cell in MEFs (passage 2 and 3) derived from *Brca2*$^{Tr2014/Tr2014}$ embryos and from their wild-type and heterozygous littermates. MEFs were derived from three sets of littermates, and centrosomes visualised by immunofluorescent labelling of γ tubulin, which is highly specific to the centromere, in conjunction with β-tubulin labelling of microtubules. The absence of the wild-type *Brca2* gene product was associated with abnormalities in centrosome number (Figure 2); the centrosome number increased with time in culture and passage number (Figure 3a). The median centrosome number of *Brca2*$^{Tr2014/Tr2014}$ cells at late second passage and at third passage was 3 and 4, respectively, with interquartile ranges (IQRs) of 2–5 and 2–6, respectively. MEFs derived from wild-type and heterozygous littermates had a median centrosome number of 2 (IQR 1–2) at both second and third passage The differences in frequency of centrosomes per cell between *Brca2*$^{Tr2014/Tr2014}$ and *Brca2*$^{+/+}$ or *Brca2*$^{+/Tr2014}$ MEFs were statistically significant at all time points (Kruskal–Wallis test, $p < 0.0001$).

The proportion of *Brca2*$^{Tr2014/Tr2014}$ MEFs that showed centrosome amplification rose from 44% in passage 2 to 65% in passage 3 (χ^2 for trend = 49.4, $p < 0.0001$). In contrast, only 10% of *Brca2*$^{+/+}$ MEFs exhibited centrosome amplification at both passage 2 and 3 (Figure 3b). Centrosome amplification was associated with micronucleus formation in *Brca2*$^{Tr2014/Tr2014}$ MEFs (relative risk 3.3, Fisher's exact test, $p < 0.0001$; Table 1). Despite this association, centrosome amplification was present in the absence of a micronucleus and vice versa; following an abnormal mitosis and cell division, only one daughter might inherit an abnormal centrosome number or a micronucleus. An amplified centrosome number might also result in an aberrant but bipolar mitosis (Figure 2d) with missegregation of chromosomes into the two daughter macronuclei but without the formation of a micronucleus. In an interphase population of cells, we cannot exclude the possibility that some of the γ-tubulin bodies are non-functional centrosome fragments.

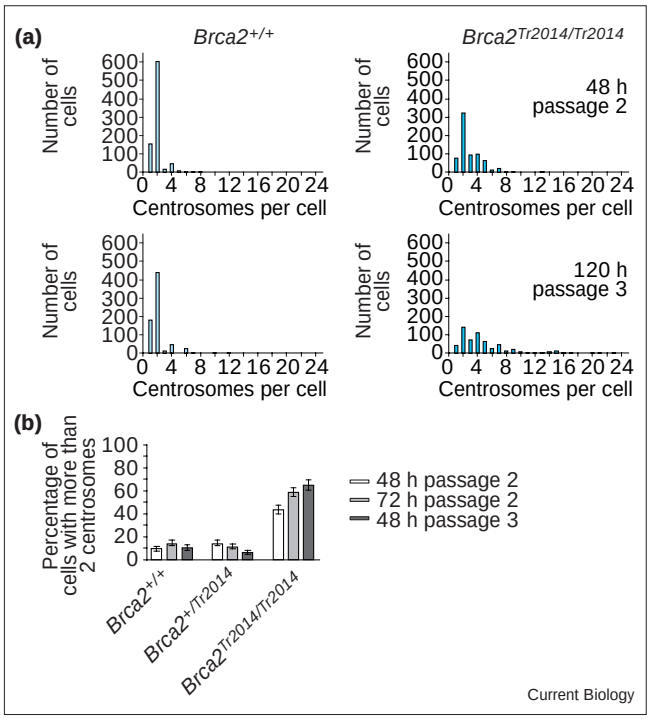
Human tumours arise through a multi-step process involving mutations in a number of oncogenes and

Figure 2



Centrosome amplification in *Brca2*$^{Tr2014/Tr2014}$ MEFs. Cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-γ-tubulin antibody (green), Cy3-conjugated anti-β-tubulin antibody (red) and the DNA dye 7-amino actinomycin D (blue). Centrosomes appear green/yellow because of colocalisation of γ tubulin and β tubulin at the centrosome. Microtubules appear red and DNA blue. (a) *Brca2*$^{+/+}$ interphase MEFs at 60 × magnification showing 1–2 centrosomes per cell. The bar represents 50 μm. (b) *Brca2*$^{Tr2014/Tr2014}$ interphase MEFs at the same magnification showing centrosome amplification and the presence of a micronucleus. (c,d) *Brca2*$^{Tr2014/Tr2014}$ mitotic MEFs. The one in (c) has three functional centrosomes and a tripolar spindle; the one in (d) has four functional centrosomes and a bipolar mitosis.

Figure 3



The frequency of centrosome amplification increases with passage number in *Brca2*$^{Tr2014/Tr2014}$ MEFs. (a) Frequency distribution histograms of centrosome number per cell in *Brca2*$^{+/+}$ and *Brca2*$^{Tr2014/Tr2014}$ MEFs at passage 2 and 3. Centrosome number was grossly increased, and increased further with passage number, in *Brca2*$^{Tr2014/Tr2014}$ MEFs. The histograms for *Brca2*$^{+/Tr2014}$ MEFs is not shown but is essentially identical to that for *Brca2*$^{+/+}$ MEFs. (b) The proportion of *Brca2*$^{Tr2014/Tr2014}$ MEFs that demonstrate centrosome amplification was significantly higher than in *Brca2*$^{+/Tr2014}$ or *Brca2*$^{+/+}$ MEFs and increased with passage number. Error bars represent CIs of the proportion.

tumour suppressor genes [12]; underlying genomic instability may accelerate this process [13]. Genomic instability at the chromosomal level is a common feature of human tumours, occurs early in tumourigenesis and commonly leads to the development of aneuploidy [12]. Tumours exhibiting chromosome instability do not show genomic instability at the nucleotide sequence level and vice versa [14]. Chromosome segregation at mitosis is tightly regulated; abnormalities of the centrosome, mitotic spindle and kinetochore apparatus can lead to chromosome missegregration and consequent aneuploidy [8,10,12,15–17]. Breast tumours in heterozygotes for *BRCA1* and *BRCA2* mutations have lost their wild-type allele [2] and are most frequently aneuploid [18,19]. Here, we report that loss of wild-type *Brca2* leads to chromosome missegregation and aneuploidy in association with amplification of centrosome number. This suggests that loss of *BRCA2* may result in aneuploidy as a consequence of abnormality in spindle-pole number. Whether *Brca2* directly regulates centrosome duplication or spindle-pole function remains to be established.

The high frequency of micronuclei in *Brca2*$^{Tr2014/Tr2014}$ MEFs, indicating the completion of aberrant mitoses, may reflect the absence of a checkpoint monitoring the number of spindle poles [20]. Nevertheless, we cannot exclude the possibility that *Brca2*$^{Tr2014/Tr2014}$ MEFs have an intrinsic defect in the mitotic spindle checkpoint. The additional presence of unrepaired chromosome breaks and intact p53-mediated G_1–S and G_2–M checkpoints may explain the proliferative failure in *Brca2*$^{Tr2014/Tr2014}$ MEFs [3,4]. We hypothesise that sporadic loss of wild-type *BRCA2* within the breast epithelial cells of *BRCA2* heterozygotes will, in sub-clones, drive the loss of cell-cycle checkpoint-regulation genes, enabling proliferation. Indeed, lack of p53 rescues proliferative failure in *Brca2*$^{Tr2014/Tr2014}$ MEFs (F.C. and D.B., unpublished observations). The mitotic spindle checkpoint is also regulated by p53 [21], and absence of p53 leads to centrosome amplification and chromosome missegregation [22]. The combined loss of function of p53 and *BRCA2* would be expected to cause accelerated genomic instability and

Table 1

Centrosome amplification is associated with micronucleus formation in *Brca2*$^{Tr2014/Tr2014}$ MEFs.

Number of centrosomes per cell	Number of cells with micronuclei	Number of cells without micronuclei	Total number of cells
> 2	181	92	273
1–2	29	116	145
Total number of cells	210	208	418

tumourigenesis. While this manuscript was in preparation, it was reported that a homozygous *Brca1* mutation also leads to centrosome amplification and chromosome missegregation [23]. As BRCA2 is known to complex with BRCA1 in mitotic cells [24], *BRCA1* and *BRCA2* may function within a common pathway that maintains accurate chromosome segregation at mitosis, and thus loss of function of either gene causes chromosome instability and leads to tumourigenesis.

In summary, the absence of wild-type *Brca2* led to the development of spontaneous micronuclei, a marker of chromosome instability, and the proportion of cells containing micronuclei increased with passage number. We have shown that chromosome missegregation is the major mechanism leading to formation of these micronuclei and have confirmed that aneuploidy is the consequence. Absence of *Brca2* also led to increased centrosome number in these cells. Centrosomes nucleate mitotic spindles and form the poles of division at mitosis. The observed abnormality of centrosome number suggests a mechanism for the disordered chromosome segregation observed in *Brca2*$^{Tr2014/Tr2014}$ MEFs and thus a potential mechanism by which absence of wild-type *BRCA2* may lead to tumourigenesis as a result of loss of chromosome stability in epithelial cells.

Supplementary material

Additional methodological details are available at http://current-biology.com/supmat/supmatin.htm.

Acknowledgements

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Supplementary material

Absence of Brca2 causes genome instability by chromosome breakage and loss associated with centrosome amplification

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Supplementary materials and methods

Cell culture

Generation of MEFs from *Brca2*$^{+/Tr2014}$ intercrosses was performed as described [S1]. Passage 1 MEFs from genotyped littermate quarter-embryo aliquots, stored in 0.5 ml 10% DMSO in DMEM supplemented with 10% FCS, 2 mM L-glutamine and penicillin under liquid nitrogen, were thawed into medium as above and plated into T-175 culture flasks overnight. For the assessment of micronucleus frequency and for immunofluorescence experiments, 2×10^5 cells were re-plated into 6 cm tissue culture dishes containing 18 mm glass coverslips and were cultured at 37°C in a 10% CO_2 atmosphere; 72 h after thawing and start of culture, cells were washed, trypsinised, split 1:2 and replated in 6 cm dishes with coverslips for passage 3 time points. For fluorescence in situ hybridisation (FISH) experiments, 4×10^4 MEFs were plated in 4 ml medium in slide chamber flasks (Nunc) and cultured as above.

Immunofluorescence microscopy and micronucleus assay

Subconfluent MEFs of genotype *Brca2*$^{+/+}$, *Brca2*$^{+/Tr2014}$ and *Brca2*$^{Tr2014/Tr2014}$ derived from three separate litters were washed in PBS and fixed in 100% methanol at –20°C for 10 min and then rehydrated in PBS. Coverslips were blocked in PBS with 10% FCS for 30 min, then incubated with anti-γ-tubulin monoclonal antibody (Sigma GTU-88) at 1:200 dilution in PBS with 4% FCS for 1 h, washed three times with PBS, incubated with donkey anti-mouse IgG antibody (Jackson Immuno Research Laboratories Inc, 1:200 dilution) in PBS for 1 h, washed three times in PBS and incubated with Cy3-conjugated anti-β-tubulin monoclonal antibody (Sigma) for 1 h. DNA was stained with either 7-amino-actinomycin D (2 μg/ml), then washed and mounted on Mowiol (Calbiochem), or mounted in Mowiol with DAPI (1.5 μg/ml).

Determination of micronucleus frequency

Cells were viewed using a Zeiss Axioskop fluorescence microscope with standard epifluorescent filters for DAPI, Cy3/Texas Red, and FITC, and a 63 or 100 × Plan Neofluor objective. At least 200 MEFs were sampled from each of three embryos per genotype for each culture time point in three independent experiments. The presence or absence of a micronucleus in each cell was recorded.

Determination of centrosome number per cell

Cells were viewed using a Nikon Eclipse 600 fluorescence microscope with standard epifluorescence filters for Cy3/Texas Red and FITC. The number of FITC-labelled γ-tubulin foci were counted through the entire thickness of each cell. At least 200 MEFs were sampled from each of three embryos per genotype for each culture time point in at least three independent experiments. The number of centrosomes in each cell was recorded. The microscope was linked to an MRC 1024 confocal imaging system equipped with a krypton argon laser and Laser Sharp software. Images for figures were made using a full thickness image reconstructed by superimposition of 1 μm interval sections through the cell.

FISH with pan-centromeric (γ) major satellite probe

Subconfluent MEFs of *Brca2*$^{Tr2014/Tr2014}$ genotype from two separate litters in two independent experiments were grown in slide flasks as described above and cultured for the indicated time, washed in PBS before fixation in fresh 3:1 methanol:glacial acetic acid at 4°C for 30 min and air dried overnight. Slides were dehydrated through an ethanol series and were denatured and hybridised with a Cy3-conjugated mouse major satellite pan-centromeric probe according to the manufacturer's protocol (Cambio Ltd). After hybridisation, slides were mounted with Vectashield containing 1.5 μg/ml DAPI, coverslipped and viewed using a Zeiss Axioskop with standard epifluorescence filters for DAPI and Texas Red/Cy3 and 63 × and 100 × Plan Neofluor objective linked to a Photometrics Series 200 CCD camera and IPLab Spectrum software. At least 200 micronucleus-containing MEFs derived from each of three *Brca2*$^{Tr2014/Tr2014}$ embryos were scored for the presence of either a Cy3 signal-positive or a signal-negative micronucleus or both. The total number of all individual signal-positive or signal-negative micronuclei from at least 200 micronucleus-containing MEFs derived from each of two *Brca2*$^{Tr2014/Tr2014}$ embryos was also recorded.

Metaphase chromosome counts

Passage 2 MEFs from *Brca2*$^{Tr2014/Tr2014}$ and *Brca2*$^{+/+}$ littermates were colcemid-blocked for 6 h (Gibco BRL, final concentration 0.1 μg/ml), harvested and resuspended in 0.8% trisodium citrate at 37°C for 30 min and then fixed in three changes of ice cold 3:1 methanol:acetic acid and dropped on acid-cleaned cold slides. Slides were dried overnight and stained with 3% Giemsa in Gurr buffer pH 6.8 and mounted in DPX. Metaphase spreads were photographed, and chromosome number was counted on at least 140 metaphase spreads per genotype from two litter sets by a blinded observer.

Supplementary references

S1. Connor F, Bertwistle D, Mee PJ, Ross GM, Swift S, Grigorieva E, *et al.*: Tumorigenesis and a DNA repair defect in mice with a truncating ***Brca2*** mutation. *Nat Genet* 1997, 17:423-430.