

BRCA1 foci in normal S-phase nuclei are linked to interphase centromeres and replication of pericentric heterochromatin

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Breast cancer-associated protein 1 (BRCA1) forms foci at sites of induced DNA damage, but any significance of these normal S-phase foci is unknown. BRCA1 distribution does not simply mirror or overlap that of replicating DNA; however, BRCA1 foci frequently abut sites of BrdU incorporation, mostly at mid-to-late S phase. Although BRCA1 does not overlap XIST RNA across the inactive X chromosome, BRCA1 foci position overwhelmingly in heterochromatic regions, particularly the nucleolar periphery where many centromeres reside. In humans and mice, including early embryonic cells, BRCA1 commonly

associates with interphase centromere-kinetochore complexes, including pericentric heterochromatin. Proliferating cell nuclear antigen or BrdU labeling demonstrates that BRCA1 localizes adjacent to, or “paints,” major satellite blocks as chromocenters replicate, where topoisomerase is also enriched. BRCA1 loss is often associated with proliferative defects, including postmitotic bridges enriched with satellite DNA. These findings implicate BRCA1 in replication-linked maintenance of centric/pericentric heterochromatin and suggest a novel means whereby BRCA1 loss may contribute to genomic instability and cancer.

Introduction

Mutations in the tumor suppressor breast cancer-associated protein 1 (BRCA1) are associated with a high risk of breast and ovarian cancer. BRCA1 is a nuclear protein implicated in multiple processes, including genomic stability, transcription regulation, chromatin remodeling, and cell-cycle control (Starita and Parvin, 2003; for reviews see Welch and King, 2001; Deng and Wang, 2003). In normal S-phase cells, BRCA1 shows a punctate distribution with typically ~10–20 prominent accumulations (foci), but upon induced DNA damage, it relocalizes to sites of DNA repair (Scully et al., 1997b; Tashiro et al., 2000; Cantor et al., 2001). Although many studies have investigated BRCA1 foci in relation to DNA repair, little is known about the BRCA1 foci in nonirradiated cells. These have been suggested to be storage sites or, possibly, sites of endogenous damage. They are not thought to be sites of DNA replication because they distribute in a pattern distinct from that of replicating DNA (Scully et al., 1997a). However, it remains an important consideration that normal S-phase BRCA1 foci may reflect an unrecognized, but fundamental, function of BRCA1.

A key to understanding whether the BRCA1 foci in nonirradiated cells have biological significance is whether they form at specific genomic loci. The spatial association of BRCA1 at sites of DNA damage provided key evidence for its role in DNA repair. We investigate whether BRCA1 foci in normal cells form at specific nuclear or chromosomal sites, or distribute more randomly, as might be expected for storage sites or endogenous damage. BRCA1 localizes to the unpaired X and Y chromosomes in spermatocytes, implicating BRCA1 in recombination and meiotic silencing (Scully et al., 1997b; Turner et al., 2004). However, in normal somatic nuclei there is no evidence that BRCA1 spots associate with specific sites of chromatin, other than a reported association of BRCA1 with XIST RNA on the inactive X chromosome (Xi; Ganesan et al., 2002). Findings in this study demonstrate that BRCA1 foci form at particular classes of heterochromatin, linked to their replication, and suggest a novel role of BRCA1 with implications in the maintenance of genomic stability.

Results and discussion

In a fraction of cells, BRCA1 foci abut, but do not coat, the Xi

The report that BRCA1 colocalizes with XIST RNA on the inactive X chromosome (Xi) in a subset (5–10%) of cells

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Abbreviations used in this paper: BRCA, breast cancer-associated protein; CENP, centromere protein; hnRNA, heterogeneous nuclear RNA; MEF, mouse embryo fibroblast; PCH, pericentric heterochromatin; PCNA, proliferating cell nuclear antigen.

The online version of this article contains supplemental material.

(Ganesan et al., 2002) led us to further investigate the spatial relationship between XIST RNA and BRCA1. In extensive investigation of multiple cell lines, using several BRCA1 antibodies (see Materials and methods), we did not find that BRCA1 substantially overlaps XIST RNA on Xi (Pageau et al., 2006; this study). However, using methods optimized for simultaneous detection of nuclear RNA and protein (see Materials and methods), BRCA1 partially overlapped or closely abutted XIST RNA in 3–5% of hundreds of cells viewed in 2D. 3D analysis of deconvolved optical sections (Fig. 1, A and B) shows that even in cases where BRCA1 and XIST RNA appear to overlap in 2D, they largely occupy distinct spatial territories, typically with BRCA1 tightly abutting the XIST signal (Video 1, available at <http://www.jcb.org/cgi/content/full/jcb.200602055/DC1>). BRCA1 also did not colocalize substantially with other hallmarks of Xi-facultative heterochromatin (H3mK27 or ubiquitin; Fig. S1 A), which colocalize throughout the XIST RNA territory (Chaumeil et al., 2002; Smith et al., 2004). We also recorded a fraction of cells (~13% in TIG1 fibroblasts, with similar results for multiple cell lines) in which a BRCA1 spot was directly adjacent to, but clearly not overlapping (even by 2D analysis), XIST RNA (Fig. 2 D and not depicted; see Materials and methods for definition of scoring terms). The significance of these more limited associations is investigated in this study. However, overall, these findings are consistent with other evidence that BRCA1 does not have a direct role in localizing XIST RNA (Pageau et al., 2006); if BRCA1 has a spatial relationship to the Xi, it is not via an association with XIST RNA.

Most BRCA1 foci localize to heterochromatic nuclear regions

To address whether the ~10–20 prominent BRCA1 foci associate with a particular category of chromatin, we investigated whether

they preferentially localize to the euchromatic or heterochromatic compartments. To delineate these compartments, we used hybridization to heterogeneous nuclear RNA (hnRNA) and labeling of splicing-factor-rich domains. Hybridization to hnRNA with a Cot-1 DNA probe delineates the inactive X chromosome (Hall et al., 2002) and heterochromatin abutting the nuclear envelope and nucleolus (Tam et al., 2004). Analysis, in two different cell lines, revealed a strong propensity for BRCA1 foci to localize in hnRNA-depleted regions; only ~19% overlapped the Cot-1 RNA signal, which fills most of the nucleoplasm (Fig. 2 A). A surprisingly large fraction of BRCA1 foci (~32%) localized to the Cot-1-depleted region abutting or within the nucleolus (Fig. 2 A). Another 14% localized to the peripheral heterochromatin, and 35% precisely colocalized with small discrete “holes” in the hnRNA signal (Fig. 2 A). Although not our focus in this study, an association with the centrosome (Starita et al., 2004) was not noted with cells and antibodies used here. The association of BRCA1 with the nucleolus is interesting because many centromeres localize there.

The preference for heterochromatic regions contrasted to the paucity of BRCA1 foci with SC-35 and SRM300, which are splicing components that label 20–30 large domains linked to RNA metabolism. These regions are surrounded by active genes in the euchromatic compartment (Shopland et al., 2003). These BRCA1 foci only rarely overlap (<1%) or contact (3%) SC-35 (Fig. 2 B) or SRM300 in mouse cells, suggesting they are largely excluded from these euchromatic “neighborhoods.”

BRCA1 has a substantial, but complex, relationship to mid-to-late replicating DNA
BRCA1 in normal S phase has not been thought to reflect routine DNA replication because BRCA1 distribution does not mirror that of replicating DNA (Scully et al., 1997a). We reexamined the relationship of BRCA1 foci to mid-to-late replicating DNA,

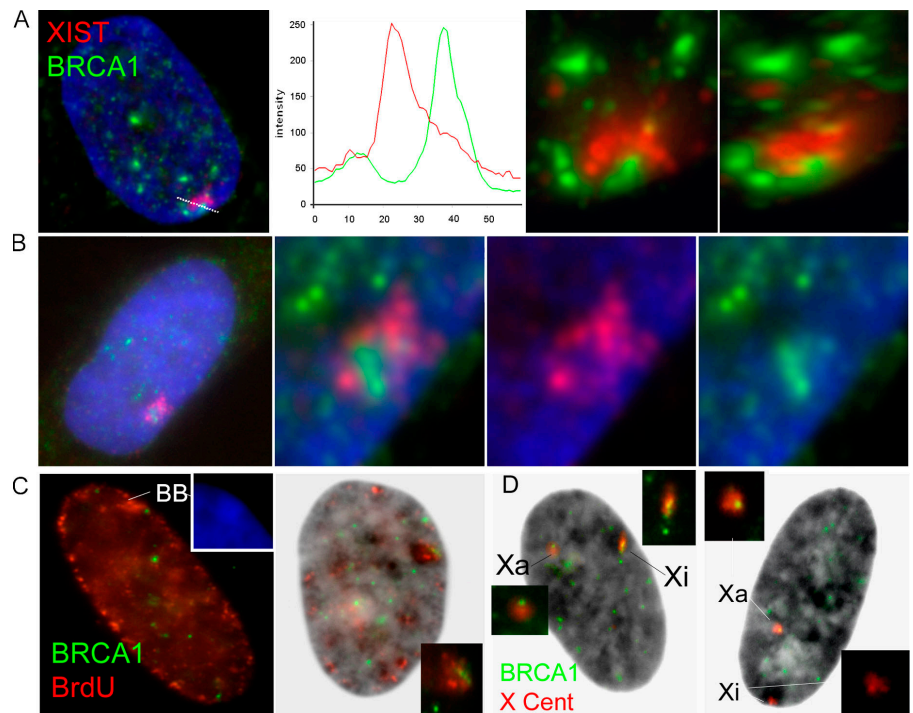


Figure 1. BRCA1 and XIST RNA/Xi relationship in human female fibroblasts. (A and B, TIG1; C, WI38; D, IMR90). (A) An optical section from a deconvolved stack, followed by a line scan of the fluorescence intensities. At right, 3D rendering with two views rotated ~20 degrees is shown (Video 1). (B) Unprocessed micrograph followed by a magnified view of a deconvolved optical slice showing XIST RNA (red) and BRCA1 (green). (C) BRCA1 (green) and a replicating Barr body (BrdU, red; DAPI, blue). (D) X centromere (red) and BRCA1 (green) with DAPI (gray). The DAPI-dense Barr differentiates Xa from Xi. Video 1 is available at <http://www.jcb.org/cgi/content/full/jcb.200602055>.

which comprises largely heterochromatic DNA. Unlike the dispersed particulate pattern of early replication, the mid-to-late pattern comprises a smaller number of larger spots (Nakayasu and Berezney, 1989; Quivy et al., 2004). Examination of whether BRCA1 foci overlapped BrdU spots confirmed the earlier conclusion that, in general, the two patterns are not the same (Scully et al., 1997a). However, close scrutiny suggested a substantial, but incomplete, relationship. Approximately 3% of the discrete BRCA1 foci overlapped a BrdU spot, but an additional 18% were abutting or adjacent to (contacting) BrdU spots. Although these mid-to-late S-phase BrdU spots occupy a much smaller area of the nucleus than SC-35 domains (Fig. 2, B and C), BRCA1 shows greater spatial association with them. Many BRCA1 spots (an additional ~27%) seemed to position consistently close (~0.3 μm) to a BrdU spot, the significance of which was initially unclear.

We also reexamined the relationship to the replicating Xi. As shown in Fig. 2 D, the subset of cells that showed BRCA1 association (either abutting or adjacent; Fig. 1 C) during replication of Xi increased two- to threefold over asynchronous cultures, which is consistent with an increased association in late S phase (Chadwick and Lane, 2005).

Many BRCA1 foci are spatially linked to components of the human interphase centromere-kinetochore complex

The aforementioned findings led us to investigate whether BRCA1 has a relationship to heterochromatin associated

with centromeres. Using an antibody to centromere protein C (CENP-C), which is a constitutive component of the interphase centromere-kinetochore complex, the patterns of CENP-C and BRCA1 in human fibroblasts (TIG1) were again distinct, yet exhibited a substantial spatial association (Fig. 3 A). We categorized these associations into three types, as follows: 3% of BRCA1 spots were completely coincident with CENP-C spots, another 12% directly abutted or contacted (no separation visible by light microscopy), and an additional 24% were suggestive of a close pairing. Very similar observations (3% coincident, 14% abutting/adjacent, and 16% close) were made when we hybridized to α -satellite DNA (Fig. 3 B) or used CENP-B, which binds α -satellite, as a marker (Fig. 3 C).

We next asked whether BRCA1 foci that abut Xi reflect a relationship to centromeres. The frequency with which we found BRCA1 partially overlaps (~2%) or resides adjacent to (8%) the X centromere may largely account for BRCA1-XIST RNA association (3% partial overlap and 13% adjacent). Using the Barr body to distinguish the active and inactive X (Fig. 1 D), there was not a major difference in BRCA1 association with Xi versus active X chromosomes (Xa) centromeres (10% vs. 7%, respectively). Thus, the relationship of BRCA1 to Xi primarily reflects a relationship to centromere-associated constitutive heterochromatin, rather than specifically Xi-facultative heterochromatin. However, we do not exclude the possibility that the slightly higher association with Xi is caused by its more heterochromatic nature.

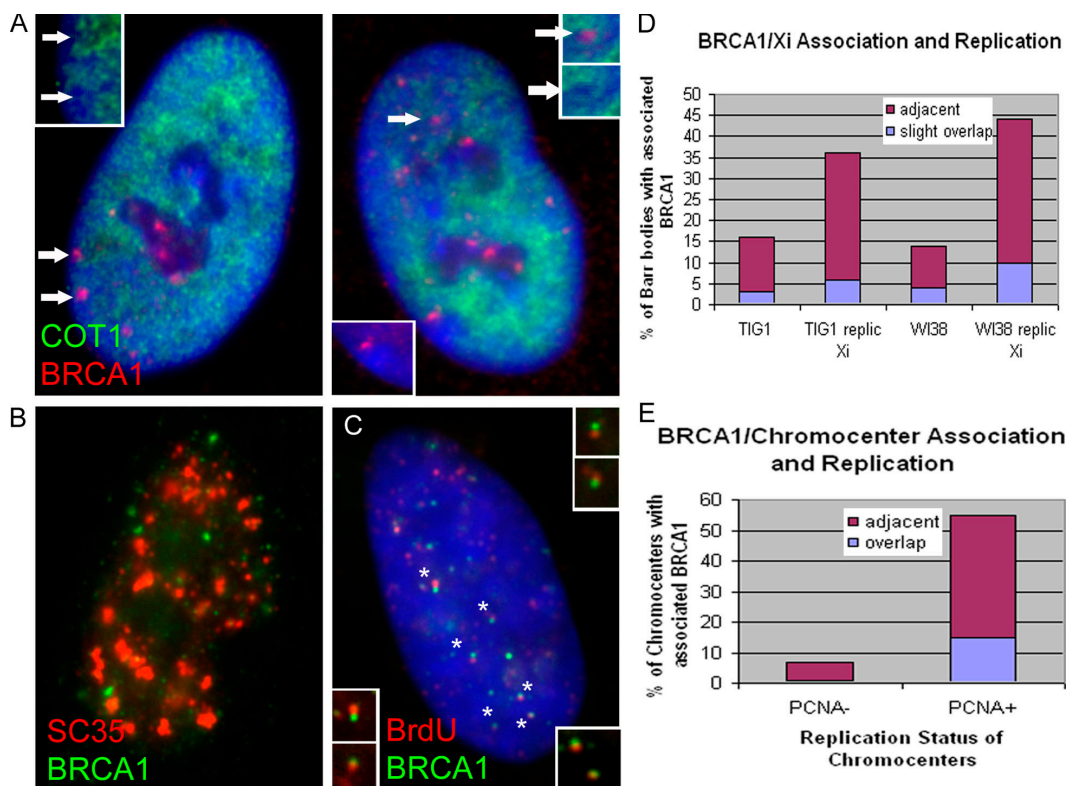


Figure 2. **BRCA1 relationship to the heterochromatic compartment and replication in human fibroblasts.** (A) BRCA1 (red) and Cot1 hybridization to hnRNA (green; DAPI, blue). (B) BRCA1 (green) and SC-35 domains (red). (C) BRCA1 (green) relative to BrdU-labeled, late-replicating DNA (red). (D and E) BRCA1 association increases during replication. (D) The replicating Xi in human cells was distinguished by BrdU and DAPI staining for the Barr body ($n = 100$). (E) Replicating mouse chromocenters were distinguished by PCNA label ($n = 1100$).

The frequency with which BRCA1 signals either overlap or directly abut interphase centromere markers indicates a substantial, albeit incomplete, association. Although the “close, but not contacting” category is less clear, this could reflect a spatial linkage to some component of the centromere–kinetochore complex (for review see Cleveland et al., 2003), which has many components that do not all completely coincide in nuclei (Sugata et al., 2000) and are not all known. Therefore, the relatively consistent gap between BRCA1 and CENP-C could contain some other component of this structure (see the following section).

BRCA1 structurally associates with mouse chromocenters and pericentric heterochromatin

Because BRCA1 most often “neighbors” (rather than overlaps) these centromere components, we next investigated BRCA1’s relationship to pericentric heterochromatin (PCH), which would also lie adjacent to centromeric DNA. Mouse cells have a more well-defined organization of centric and pericentric DNA than do human cells (Schueler et al., 2001); in mouse cells, centromeres cluster into 5–10 chromocenters that are easily visualized with DAPI stain. Fig. S1 B confirms a recent report (Guenatri et al., 2004) demonstrating that the DAPI-dense chromocenters comprise pericentric heterochromatin (mouse major satellite) and the centromeric DNA (minor satellite) is smaller and positions at the periphery of the larger blocks of PCH.

BRCA1 staining revealed a clear structural relationship with chromocenters (Fig. 4 A). Although not all chromocenters have associated BRCA1, and vice versa, in all of the several different lines examined (mouse 3T3, 3X mouse, mouse embryo fibroblasts (MEFs), and mouse ES cells), 26–38% of BRCA1 spots in an asynchronous population directly associated with a chromocenter. In addition, a subpopulation of cells showed higher association; in some cells, almost all BRCA1 spots were with a chromocenter (Fig. 4 A). Typically one or two BRCA1 foci were at the immediate periphery of each chromo-

center, but, not infrequently, several foci or elongated BRCA1 accumulations “hugged” the contour of the chromocenter (Fig. 4 A). Occasionally, a “paint” of nearly all the DAPI-bright PCH was apparent (Fig. 4 A, top middle). Interestingly, this association is present even in very early (1-d differentiated) embryonic stem cells (Fig. 4 A, bottom right). This is potentially important because BRCA1 knockout is early embryonic lethal (Deng and Wang, 2003).

We next examined the relationship between BRCA1 and the minor satellite (equivalent of human α satellite) of the centromere proper. When visualized together (Fig. 4 B), their relationship mirrored that seen (see previous section) between human centromeres and BRCA1, as follows: 6% coincident, 10% adjacent/abutting, and 27% close. However, when viewed with DAPI in three colors, it became apparent that, often, minor satellite and BRCA1 signals that had no direct contact were in fact associated with a common chromocenter. These observations bolster the significance of close/“paired” signals in the human; even when the BRCA1 foci are not coincident with a centromeric marker, they are spatially linked by their common association with the PCH of the chromocenter (Fig. 4 B and Video 2, available at <http://www.jcb.org/cgi/content/full/jcb.200602055/DC1>). The link between BRCA1 and centric DNA may be through the PCH, but, in either case, results indicate a connection between the discrete BRCA1 S-phase foci and centromeres, which are structures key to the proper segregation of chromosomes and maintenance of genomic integrity.

BRCA1 association with chromocenters is temporally linked to their replication

In both human and mouse, some cells show greater BRCA1 association with chromocenters than others, as illustrated in Fig. 4 C (middle). We addressed whether this difference might relate to replication, using proliferating cell nuclear antigen (PCNA) as a marker of the replication machinery (Bravo, 1986). Chromocenters replicate roughly synchronously in a given cell

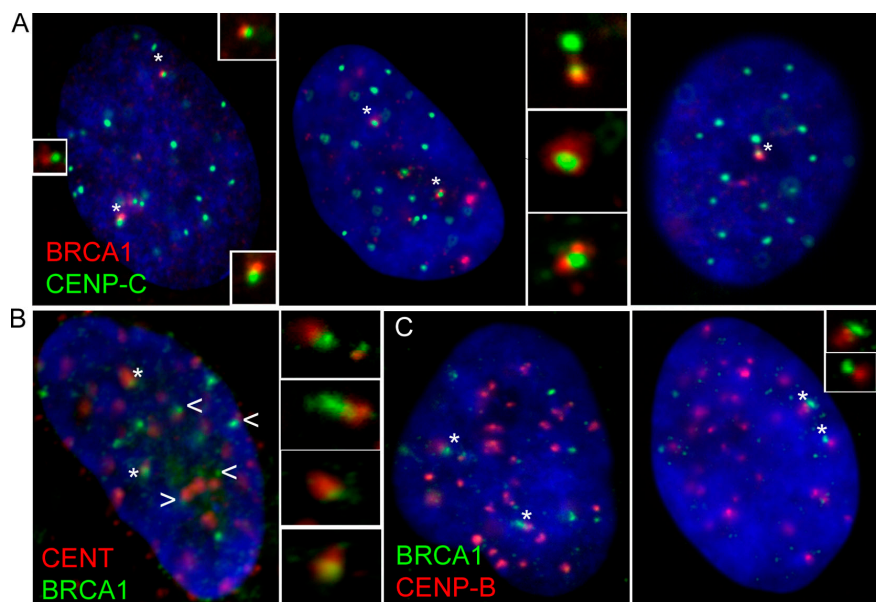


Figure 3. **BRCA1 and human centromere markers in interphase fibroblasts.** (A) BRCA1 (red) and CENP-C (green) in TIG1 cells. (B) BRCA1 (green) and centromeric DNA (red) in IMR90. (C) BRCA1 (green) and CENP-B (red) in TIG1. Asterisks mark sites of association highlighted in insets and arrowheads mark some of the other sites of association.

in mid-to-late S phase (Fig. 4 C; Guenatri et al., 2004; Quivy et al., 2004). In cells in which most chromocenters had prominent PCNA label, a higher association of BRCA1 was clearly evident. Of chromocenters that label with PCNA, 55% have BRCA1 associated (15% overlap and 40% abutting), in contrast to <7% with no PCNA label (<1% overlap and 6% abutting; Fig. 2 E). Cells with the most striking BRCA1 painting of chromocenters also labeled for replication of the chromocenter. This demonstrates a temporal relationship between widespread, largely synchronous BRCA1 association and replication of PCH.

Previous work has shown that mouse chromocenters have a defined architecture such that DNA replication (and likely chro-

matin assembly) occurs at the periphery of the large major satellite block, and the newly replicated DNA then moves into the central region of the chromocenter (Quivy et al., 2004). This fits well with the distribution of BRCA1, which is mostly concentrated at the chromocenter periphery. Because BRCA1 did not always localize to PCNA-labeled chromocenters, it may transiently associate close to the time of replication. The fact that BRCA1 is more juxtaposed to PCNA than overlapping it is consistent with other evidence that BRCA1 may have a post-replicative role. Similar observations were made with a 15-min terminal pulse of BrdU (Fig. S1 C).

Because one recent study reports that BRCA1 regulates topoisomerase II α (topoII α) during routine DNA replication

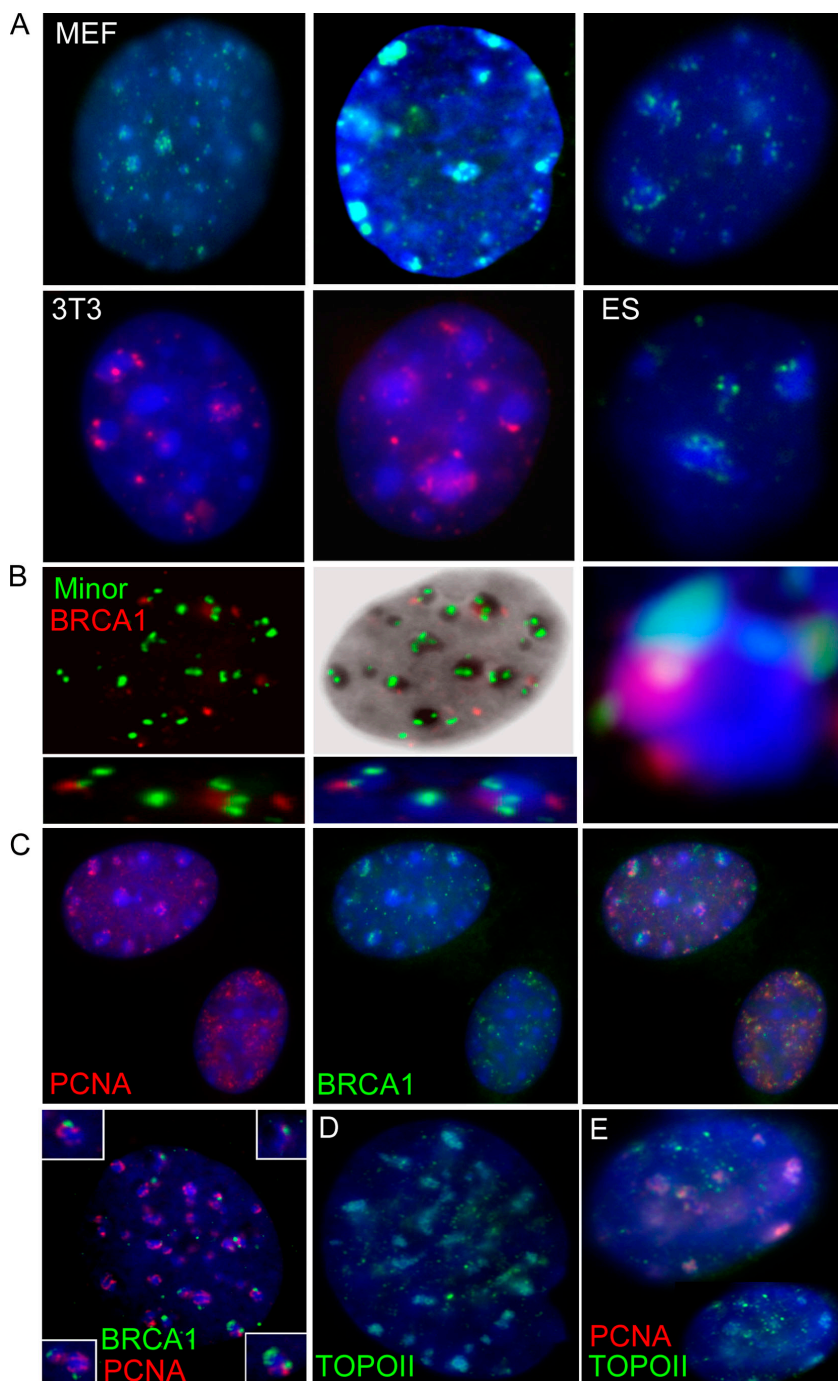


Figure 4. **BRCA1, mouse chromocenters, and the relationship to replication.** (A) Mouse cell lines included MEFs (BRCA1, green), 3T3 (BRCA1, red), and mouse ES cells (BRCA1, green). (B) BRCA1 (red) and minor satellite DNA (green). Far right shows 3D-rendered image of BRCA1 and minor satellite on a chromocenter (blue). Middle photo shows chromocenters in black. (C) Cells were labeled for replication with PCNA (red) and BRCA1 (green). In top panel, the upper nucleus has more BRCA1 and PCNA on chromocenters. (D and E) topoll- α (green) on mouse chromocenters (blue) is seen in many S-phase cells (PCNA, red).

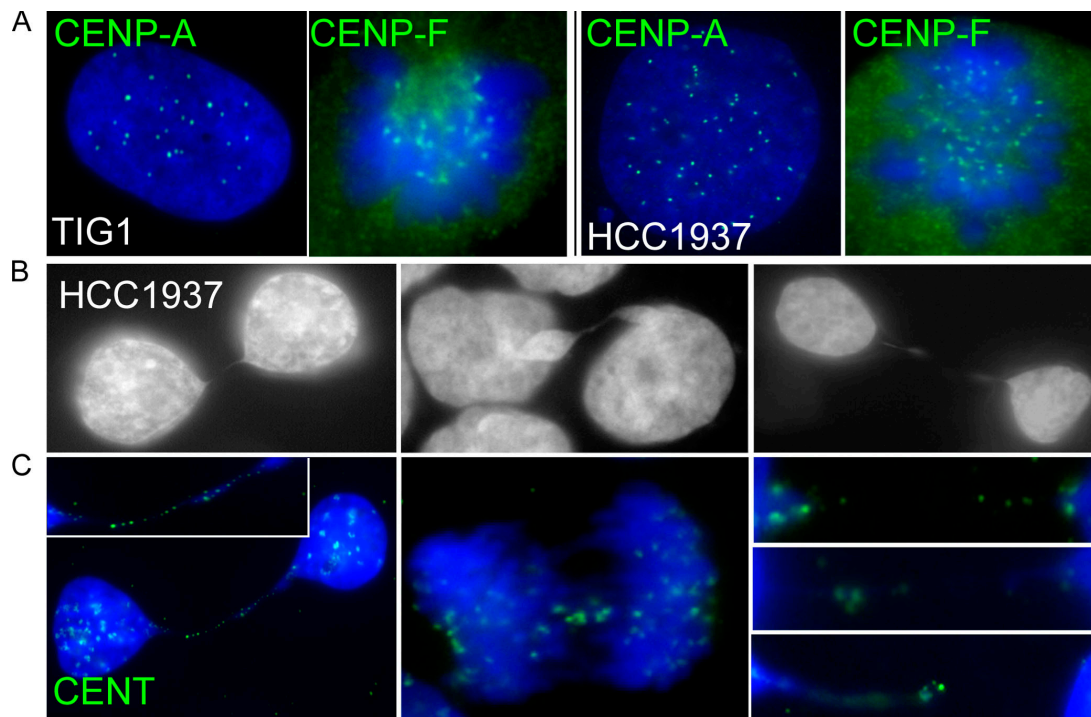


Figure 5. **Centromeric markers and DNA bridges in BRCA1^{-/-} HCC1937 cells.** (A) TIG1 (left) and HCC1937 cells (right) stained for CENP-A and -F. (B) In HCC1937 early G1 daughter cells, DAPI staining shows thin DNA bridges. (C) Centromeric DNA (green) in DNA bridges (left and right) and lagging chromosomes (middle) in HCC1937 cells.

(Lou et al., 2005), we briefly addressed whether topoII α associates with mouse chromocenters. Although topoII α is enriched at mitotic centromeres, and there is one report of its association with late S-phase BrdU (Agostinho et al., 2004), it is not known to be enriched at chromocenters/centromeres during S phase. As shown in Fig. 4 D, we found topoII α commonly enriched on mouse chromocenters; in $\sim 40\%$ of interphase cells, topoII α concentrates on essentially all chromocenters. Many of these cells are in S phase, with PCNA on their chromocenters (Fig. 4 E).

Initial characterization of BRCA1 mutant cells is suggestive of mitotic defects

Our findings suggest that BRCA1 may have a role in replication-linked maintenance of peri/centromeric heterochromatin. As the study of X inactivation has demonstrated, the epigenetic state of heterochromatin is controlled at numerous levels that work synergistically and provide redundancy; for example, heterochromatic features of the Xi are compromised only very slightly over the long term if XIST RNA is lost from somatic cells (Csankovszki, 2001). Similarly, reintroduction of XIST RNA in somatic cells would not simply correct a deficit in heterochromatin (Wutz and Jaenisch, 2000; Hall et al., 2002). Thus, short-term loss or gain of BRCA1 could have no immediate effect on heterochromatin but still be important for its long-term maintenance and stability in an organism. We found that short-term acute loss of BRCA1 by RNAi in HeLa cells impacts proliferation and reduces mitotic figures by $>60\%$ (Fig. S2, available at <http://www.jcb.org/cgi/content/full/jcb.200602055/DC1>),

which is consistent with other reports. Although this could reflect an impact on the complex epigenetic state or repair of pericentric heterochromatin, it could also reflect other short-term effects of BRCA1 loss on centrosome function (Starita et al., 2004), DNA decatenation (Lou et al., 2005), or cell-cycle checkpoints (Cao et al., 2003; Deng and Wang, 2003).

Breast tumor cells such as HCC1937 are exposed to longer-term BRCA1 loss. As an initial effort to investigate some properties of centric heterochromatin, we examined CENP-A, a constitutive interphase kinetochore component directly linked to specifying a centromeric property, and CENP-F, the first transient kinetochore protein bound in G2 (Maiato et al., 2004). Localization of these centromeric components appeared normal in these BRCA1 mutant cells (Fig. 5 A). However, given the essential role of CENP-A in kinetochore assembly, this may not be surprising.

Lou et al. (2005) reported that a high fraction ($\sim 10\%$) of HCC1937 cells had lagging chromosomes or DNA bridges after mitosis. We attempted to confirm these findings, but extended our analysis to MCF7 (BRCA1⁺) breast cancer cells and normal diploid fibroblasts. It was obvious in DAPI-stained slides of HCC1937 that many early G1 daughter pairs contain a “bridge” of DNA extending between them (Fig. 5 B); in contrast, this was almost never seen in BRCA1⁺ MCF7 or in normal fibroblasts. Many mitotic figures showed lagging chromosomes, and early G1 pairs showed thin bridges of DNA. For example, in 100 G1 daughter pairs, visible DNA bridges were seen 31 times in HCC1937 cells, in contrast to 3 times in diploid fibroblasts (TIG1) and 4 times in MCF7 cells. Most mitotic figures with

Table 1. Association of BRCA1 foci with various nuclear compartments

Nuclear compartment/ structure	Association (contact) of BRCA1 foci with compartment/structure
XIST RNA	Little overlap, but up to 16% contacting
Replicating Xi (BrdU-labeled Barr body)	Frequency of association increases over asynchronous population
Heterochromatic, hnRNA(Cot1)-depleted regions	Overwhelmingly associated (81%)
SC-35 domains within euchromatin	Overwhelmingly separate (~95% apart)
Late-replicating DNA	Mostly abutting or adjacent, 21%
Human interphase centromere markers similar for CENP-B, -C, and α -satellite	Mostly abutting or adjacent, 15–17%
Mouse chromocenters (DAPI) corresponding to major satellite	Mean association (26–38%) with some cells much higher (>75%) association
Replicating chromocenter	Much higher association (mean 55%)
Xi and Xa centromere	Association with both Xi (10%) and Xa (7%)

The frequency of discrete accumulations (foci) of BRCA1 was scored relative to the nuclear marker, typically in hundreds of cells. Most analysis was in 2D directly through the microscope, but certain results were confirmed by 3D analysis as described in the text. Only accumulations of BRCA1 clearly above background were scored; we do not exclude that low levels of dispersed BRCA1 could be present in the nucleoplasm.

lagging chromosomes showed a normal bipolar configuration; thus, in most cases, a multipolar spindle (i.e., centrosome) defect was not apparent. Although we did not observe an appreciable difference in mitotic defects in a BRCA1-reconstituted HCC1937 cell line (Lou et al., 2005), as noted earlier, once any defects in PCH or aneuploid cells are generated, reversion to normal mitotic figures would be difficult. Finally, we addressed whether the thin bridges connecting G1 daughters contained satellite DNA. Although many DNA bridges were just thin threads, a large fraction (35/40) contained α -satellite DNA (Fig. 5 C). These results are consistent with the possibility that a defect in centric/pericentric heterochromatin is present.

Conclusions

Although BRCA1 nuclear distribution has been studied for some time, this is the first study to identify a preferential relationship with centric and pericentric heterochromatin, and link this temporally to replication of these structures (summarized in Table 1). This may have escaped earlier detection because BRCA1 distribution does not simply mirror that of replicating DNA, but we show there is indeed a meaningful relationship suggesting a novel biological role for BRCA1. Because most (~80%) of the bright BRCA1 foci localize to hnRNA-depleted sites, those not with peri/centric DNA may be mostly with some other heterochromatin (e.g., telomeres, etc). The widespread, largely synchronous localization of BRCA1 foci to mouse chromocenters suggests a link to routine replication rather than just repair, but the highly repetitive (or condensed) nature of this DNA poses specific requirements that may involve repair-related or other BRCA1 functions (e.g., chromatin remodeling/assembly, transcriptional regulation, or topoII-mediated roles).

This work points to a new direction of BRCA1 research involving routine replication and maintenance of peri/centric heterochromatin. Although a specific role of BRCA1 requires

further investigation, any involvement of BRCA1 in maintaining centric/pericentric heterochromatin would have profound significance for understanding how BRCA1 mutations contribute to genomic instability and cancer. This study also clarifies an earlier report that BRCA1 appeared to have an extensive and specific relationship to XIST RNA and Xi facultative heterochromatin (Ganesan et al., 2002). Recent studies from the Livingston laboratory, and other laboratories, indicate that mitotic loss of the Xi and gain of an Xa is the most common means whereby X chromosome dosage is increased in certain types of breast cancers (Sirchia et al., 2005; Richardson et al., 2006). Therefore, the demonstration in this study that BRCA1 associates with constitutive more than facultative heterochromatin fits well with recent evidence that the most prevalent mechanism of Xi loss in BRCA-/- cancer may involve increased errors in chromosome segregation. Additionally, loss of BRCA1 may contribute to a generalized failure of heterochromatin maintenance.

Materials and methods

Cells and cell culture

Human diploid fibroblast lines WI38 (CCL-75) and IMR-90 (CCL-186) were obtained from the American Type Culture Collection, and TIG-1 (AG06173) were obtained from Coriell Cell Repositories. In addition, 3X mouse cells (Smith et al., 2004) and MCF7 cells were used. WI38, TIG-1, MCF7, and 3X mouse cells were grown in MEM supplemented with 10% FBS, 2 mM L-glutamine, 1% penicillin/streptomycin, NIH 3T3 cells, and MEFs and were grown in DME supplemented with 10% FBS, 2 mM L-glutamine, and 1% penicillin/streptomycin. HCC1937 cells were obtained from American Type Culture Collection (CRL-2336) and were grown in RPMI with Hepes medium supplemented with 10% FBS, 2 mM L-glutamine, and 1% penicillin/streptomycin. For BrdU labeling, cells were plated for 48 h, followed by a 15-min treatment with 30 μ M BrdU just before fixation. HCC1937 cells reconstituted with BRCA1 were obtained from J. Chen (Mayo Clinic, Rochester, MN; Lou et al., 2005).

Antibodies

Monoclonal antibodies to mouse (GH118) and human (MS110) BRCA1 were a gift from D. Livingston and S. Ganesan (The Dana Farber Cancer Institute, Boston, MA; Ganesan et al., 2002). A monoclonal antibody to polyubiquitinated proteins (UbFk2) was obtained from Affinity BioReagents. Polyclonal antibodies to BRCA1 (KAPST0201) were obtained from Assay Designs and C. Deng (National Institutes of Health, Bethesda, MD). Antibodies to BrdU (rat monoclonal) were obtained from Harlan. Polyclonal antibodies to CENP-C (rabbit) were obtained from W. Earnshaw (University of Edinburgh, Edinburgh, UK), and CENP-B antibodies (rabbit; H-65) were obtained from Santa Cruz Biotechnology, Inc. An antibody to CENP-A was obtained from M. Valdivia (Universidad de Cadiz, Cadiz, Spain), and an antibody to CENP-F was obtained from D. Cleveland (University of California, San Diego, La Jolla, CA). Antibodies to PCNA were obtained from Immunovision (Human). An antibody to topoll α was obtained from Lab Vision (rabbit).

Immunofluorescence

Initial studies involved testing two fixation methods; paraformaldehyde fixation followed by Triton X-100 extraction, as described by Ganesan et al. (2002), or brief Triton X-100 extraction before fixation, as described previously (Clemson et al., 1996; Tam et al., 2004). For extraction, cells were extracted in CSK buffer with 0.5% Triton X-100 and 2 mM vanadyl adenosine for 5 min. Cells were then fixed in 4% paraformaldehyde in 1 \times PBS for 10 min, incubated with primary antibodies in 1 \times PBS/1% BSA for 1 h at 37°C, and rinsed successively in 1 \times PBS, 1 \times PBS + 0.1% Triton X-100, and 1 \times PBS for 10 min. Detection was carried out with secondary antibodies tagged with fluorescein, rhodamine, or Texas red (Jackson Immuno-Research Laboratories).

DNA and RNA FISH

RNA FISH used previously established protocols (Clemson et al., 1996; for review see Tam et al., 2004). XIST RNA was detected with a 10-kb

plasmid (pG1A) spanning intron 4 to the 3' end of *XIST*, or with plasmid (pXISTHb-B) containing intron 1 (Clemson et al., 1996). Probes were nick translated using biotin-11-dUTP or digoxigenin-6-dUTP (Boehringer Mannheim). Hybridization was detected with either antidigoxigenin antibody (Boehringer Mannheim) coupled with rhodamine or fluorescein or, for biotin detection, avidin conjugated to Alexa Fluor-streptavidin 594 (red) or fluorescein (Boehringer Mannheim). RNAsin was added for simultaneous RNA FISH and antibody staining. After detection and washing, cells were re-fixed in 4% paraformaldehyde in PBS for 10 min at 25°C and processed for RNA FISH as described in this section.

For combined BRCA1 staining and DNA FISH, cells were stained first for BRCA1 and fixed in 4% paraformaldehyde for 10 min. Cells were then denatured in 0.2 N NaOH for 5 min at room temperature (rather than 70% formamide with heat) because this better preserved BRCA1 staining. A probe directed against all human centromeres (Open Biosystems Human Pancentromere Paint BIOTIN) was obtained from Cambio and hybridized overnight at 37°C as directed by the manufacturer and detected with Alexa red streptavidin diluted 1:500 in 4×SSC/1% BSA. Oligos (5'-GAACAGTGTATATCAATGAGTTAC-3' and 5'-CCACACTGTAGAACATATTAGATG-3') to the mouse minor satellite were used at 5 pmol in 10% formamide. The X centromere was labeled with a Spectrum Orange X centromere paint (Vysis) according to the manufacturer's directions and rinsed as described in the previous paragraph.

Microscopy and image analysis

Digital imaging analysis was performed using an Axiovert 200 or an Axiopt microscope (Carl Zeiss MicroImaging, Inc.) equipped with a 100× PlanApo objective (NA 1.4; Carl Zeiss MicroImaging, Inc.) and 83,000 multibandpass dichroic and emission filter sets (Chroma Technology Corp.) set up in a wheel to prevent optical shift. Images were captured with a camera (Orca-ER; Hamamatsu) or a cooled charge-coupled device camera (200 series; Photometrics). Where rhodamine was used for detection in red, a narrow band-pass fluorescein filter was inserted to correct for any bleed-through of rhodamine fluorescence into the fluorescein channel. Optical sections and 3D images were created using Axiovision 4.4 (Carl Zeiss MicroImaging, Inc.). Images were captured at 0.1-μm intervals, and stacks were deconvolved with a constrained iterative algorithm. Rendered images are maximum value projections.

Definition of molecular cytology scoring terms

For scoring purposes, the following definitions are used for scoring terms: association indicates any relationship that appears to involve "contact" (no physical separation visible by 2D light microscopy). These were further divided into three categories of association. (1) Painting, which typically indicates almost complete overlap of two signals, but in this analysis any overlap >50% would have been included. (2) Abutting/partial overlap, which indicates a signal which very closely pressed against another, such that as viewed in two dimensions there appears a slight overlap of the two signals. 3D analysis may show the two signals are actually not overlapping. (3) Adjacent, which indicates two signals that are juxtaposed and appear in contact, but for which even 2D analysis indicates no overlap. "Closely paired" signals are distinct in that this category indicates two signals that do not contact, but are separated by ~0.2–0.4 μm.

Online supplemental material

Fig. S1 shows hallmarks of X inactivation on the Xi, localization of major and minor satellite DNA relative to chromocenters, and localization of BRCA1 and BrdU to mouse chromocenters. Fig. S2 shows decreased proliferation in BRCA1 siRNA-treated versus control siRNA-treated cells and contains RNAi methods. Video 1 is a 3D movie of BRCA1 and XIST. Video 2 is a 3D movie of BRCA1 and a mouse chromocenter. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200602055/DC1>.

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