

SHORT COMMUNICATION

Inhibition of BRCA1 in breast cell lines causes the centrosome duplication cycle to be disconnected from the cell cycleMJ Ko¹, K Murata^{2,3}, D-S Hwang¹ and JD Parvin²¹Institute of Molecular Biology and Genetics, Seoul National University, Seoul, Korea and ²Department of Pathology, Brigham and Women's Hospital and Harvard Medical School, Boston, MA, USA

BRCA1-dependent ubiquitination activity regulates centrosome number in several tissue culture cell lines derived from breast cells. In these experiments, we asked how BRCA1 inhibits centrosome amplification. In general, supernumerary centrosomes can accumulate by three mechanisms: (1) failed cytokinesis and the accumulation of centrosomes by duplication in a repeated S-phase of the cell cycle, (2) disruption of the licensing of centrosome doubling such that they duplicate at inappropriate times in the cell cycle, or (3) fragmentation of the centrosomes. In this study, we found that inhibition of BRCA1 caused premature separation of centrioles and reduplication. By blocking cells in early S-phase before centrosome amplification secondary to BRCA1 inhibition could occur and then releasing, we found that inhibition of BRCA1 caused centrosome amplification between late S-phase and G2/M before the cell divided. These results suggest that normal BRCA1 function is critical in these cell lines to prevent centriole separation and centrosome reduplication before mitosis.

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The centrosome is the major microtubule organizing center in the cell. Centrosomes thus control cell shape and polarity during interphase, and centrosomes assemble the mitotic spindle for the orderly segregation of chromosomes during cell division. In mammalian cells, centrosome duplication is tightly linked to the cell cycle, thus insuring that the single centrosome in G1 duplicates only once in S-phase. Just as cyclin E and the transcription factor E2F triggers the initiation of DNA synthesis in S-phase, centrosome duplication in mammalian cell lines requires the E2F transcription factor and cyclin A activity (Meraldi *et al.*, 1999). In early

S-phase, in the presence of these two factors, the two centrioles of the single centrosome split, and on each centriole a daughter centriole assembles, making two centrosomes, each containing a pair of centrioles. During late S-phase and in G2, the two centrosomes move to opposite poles of the nucleus, and in mitosis nucleate the spindle.

When DNA replication is blocked in early S-phase by the treatment of cells with hydroxyurea (HU), centrosome duplication should also be blocked. In some cell lines, prolonged (40 h) incubation with HU leads to centrosome amplification (Balczon *et al.*, 1995; Meraldi *et al.*, 1999) since the centrosomes appear to continue doubling as if the cells continued to cycle. Typically, cells with mutant p53 have abnormal centrosome accumulation in HU-treated cells, whereas cells with wild-type p53 arrest centrosome duplication under these conditions (D'Assoro *et al.*, 2004).

The breast and ovarian cancer-specific tumor suppressor, BRCA1, has been shown to regulate centrosome number. Murine embryonic fibroblasts that are deficient in full-length BRCA1 were found to have extra centrosomes (Xu *et al.*, 1999). In addition, BRCA1 binds to γ -tubulin, a major centrosomal protein (Hsu *et al.*, 2001). BRCA1 phosphorylation by the Aurora A kinase at the centrosome has been linked to the regulation of the G2–M transition (Ouchi *et al.*, 2004).

Breast cancer cells frequently display an excess number of centrosomes (>2) (D'Assoro *et al.*, 2002; Lingle *et al.*, 2002; Pihan *et al.*, 2003), and these supernumerary centrosomes cause abnormal mitoses, leading to aneuploidy. We recently found that inhibition of BRCA1 led to a rapid amplification of centrosome number in several cell lines derived from breast tissue. We found that BRCA1, together with BARD1, ubiquitinated several centrosome proteins including γ -tubulin, and this ubiquitination activity of BRCA1 was required to maintain centrosome number. In those experiments, we found that inhibition of BRCA1 caused both a fragmentation of centrosomes into individual centrioles and also an increased number of centrioles (Starita *et al.*, 2004). The timing of the experiment suggested that the increase in centrosome number was unlikely to be due to accumulation of centrosomes in a cell that passes through a second S-phase after failing to divide in mitosis. However, in those experiments, we did not compare the timing of the centrosome duplication with

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landmarks in the cell cycle. In this study, we blocked cells in early S-phase when there were two centrosomes per cell, and following release we found that centrosome amplification due to inhibited BRCA1 occurred before the cell divided. These results indicated that loss of BRCA1 was permissive for licensing extra centrosome duplication at the wrong point in the cell cycle.

Two cell lines were used for the current study. MCF10A cells are analogous to normal mammary epithelium with wild-type p53. The second cell line, Hs578T, is a breast cancer cell line with mutated p53. When using the MCF10A cells, we could not use siRNA since these cells did not transfect efficiently. Instead, we infected a recombinant adenovirus expressing a fragment of RNA helicase A (RHA), called BRCA1 inhibiting peptide fragment (BIF), that binds to the carboxy-terminus of BRCA1 (Schlegel *et al.*, 2003; Starita *et al.*, 2004). Expression of BIF inhibits normal BRCA1 ubiquitination activity and causes centrosome amplification (Starita *et al.*, 2004). Transfection of siRNA specific for BRCA1 was effective in the Hs578T cell line (Figure 1D), and this technique caused centrosome amplification. Previously published data

showed that HU, which blocks the cell cycle in early S-phase, abrogated centrosome amplification in MCF10A cells expressing BIF (Starita *et al.*, 2004).

If centrosomes were accumulating in BRCA1-inhibited cells secondary to failed cytokinesis, then blocking the cell cycle at a later point would block centrosome amplification. Alternatively, if inhibition of BRCA1 caused centrosome amplification independent of cell division, then blocking the cell cycle at a later point might be permissive for finding supernumerary centrosomes. Cells were blocked using either HU or bleomycin. By flow cytometry analysis, HU-blocked cells predominantly had unreplicated DNA, consistent with early S-phase (Figure 2c, 0 time-point). Centrosomes per cell were counted by two methods. In one set of experiments, cells were stained with antibody specific for γ -tubulin and a dye-conjugated secondary antibody. Since it is a major centrosomal protein, normal asynchronous cells had one or two γ -tubulin-positive spots (Figure 1Ba). Alternatively, cells were transfected with a plasmid expressing GFP-centrin and subsequently fixed and mounted without antibody staining. Centrin localized in centrioles, and in a normal cell

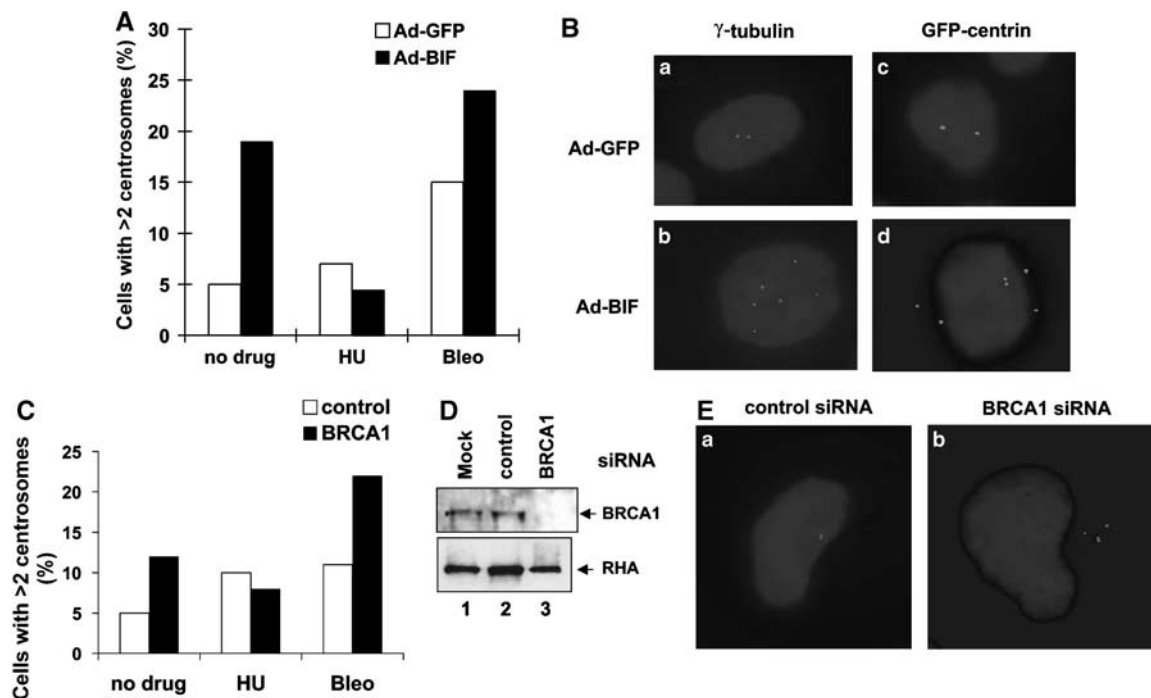


Figure 1 Inhibition of BRCA1 causes centrosome amplification in bleomycin-treated cells. (A) MCF10A cells were treated with 2.5 mM hydroxyurea (HU) or 10 μ g/ml bleomycin, 3 h after infection with recombinant adenoviruses expressing either BIF or GFP. The use of the adenovirus to inhibit BRCA1 was carried out exactly as previously described (Schlegel *et al.*, 2003; Starita *et al.*, 2004). Cells were fixed and stained for γ -tubulin 24 h postinfection. The histogram shows the percentage of cells containing more than two centrosomes. Over 100 cells were counted for each data point. (B) Examples of centrosomes visualized with γ -tubulin specific antibody and Texas-red conjugated secondary antibody (a, b) or visualized by transfection of a GFP-centrin2 expression plasmid (c, d) in a control-adenovirus-infected cell (a, c) or a BIF-expressing adenovirus-infected cell (b, d). Both adenovirus constructs express GFP diffusely in the cytoplasm. The photomicrographs were exposed under conditions in which the free GFP was not detected but the GFP focused in centrosomes was readily apparent. (C) Hs578T cells were treated with 8 mM HU or 10 μ g/ml bleomycin 19 and 17 h, respectively, post-transfection with BRCA1 siRNA or control siRNA (GL2; specific for luciferase). Cells were fixed and stained with γ -tubulin antibody 43 h post-transfection, and over 100 cells were counted for each condition. The histogram shows the percentage of cells with more than two centrosomes. (D) Lysates from mock transfected (lane 1), control siRNA, transfected (GL2; lane 2) or BRCA1-specific siRNA (lane 3) were analysed on 6% SDS-PAGE and immunoblotted for BRCA1 (top panel) and RHA as a loading control (bottom panel). (E) Hs578T cells transfected with control siRNA (a) or BRCA1 siRNA transfected cell (b) were stained with an antibody directed against γ -tubulin to visualize centrosomes.

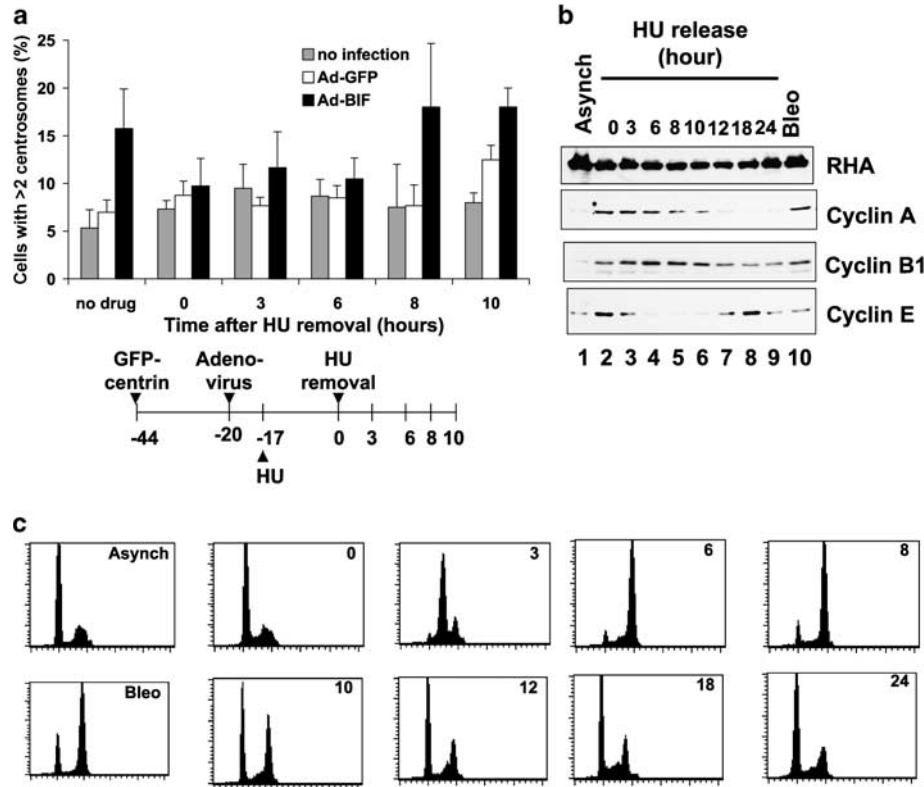


Figure 2 Centrosome amplification in BRCA1-inhibited MCF10A cells occurred in G2/M phase. (a) Results of four experiments were combined. In some experiments, the GFP-centrin2 expressing plasmid was transfected in MCF10A cells 24 h before infection with adenovirus expressing either BIF or GFP and treated with 2.5 mM HU 3 h postinfection. After 17 h, cells were released from HU arrest and fixed at 0, 3, 6, 8, 10 h after removal of HU. In other experiments, cells were infected by adenovirus and treated with HU and fixed as above, and centrosomes were stained using an antibody specific for γ -tubulin. The percentage (\pm s.e. of the mean) of cells (>100 counted) with amplified centrosomes was scored. The results among individual experiments were consistent regardless of the technique by which centrosomes were visualized. The no drug sample was fixed at time 0. (b) MCF10A cells were treated with HU for 17 h, and after HU release cells were harvested at the indicated time points. Each sample was immunoblotted using antibodies specific for cyclin A (second panel), cyclin B1 (third panel), or cyclin E (bottom panel). RNA helicase A (RHA) was used to evaluate equal loading of samples (top panel). Samples from asynchronous cells (Asynch, lane 1) and cells treated with 10 μ g/ml bleomycin for 18 h (Bleo, lane 10) were included. (c) Flow cytometry data of the same cells from (b) was done after staining fixed cells with propidium iodide. Infection of the adenoviruses into MCF10A cells caused no discernible change in the FACS tracing (data not shown).

centrin was observed as pairs of pinpoints in each centrosome (Figure 1Bc). Inhibition of BRCA1 by expressing the BIF peptide in MCF10A cells resulted in many cells with >2 γ -tubulin-positive centrosomes (Figure 1Bb) or cells with extra centrin-containing foci (Figure 1Bd). In the cell shown in Figure 1Bd, seven centrin-positive foci were apparent, five single foci and two paired. The unpaired centrin foci indicate that inhibition of BRCA1 causes inappropriate separation of centrioles. Since there were greater than four total centrioles per BRCA1-inhibited cell, BRCA1 normally blocks the accumulation of supernumerary centrioles and thus, centrosomes.

In the absence of added HU, 5% of the control MCF10A cells had more than two centrosomes, thus setting a background level. By contrast, BIF expression caused nearly 20% of the cells to have extra centrosomes. When cells were treated with HU, BIF expression did not cause centrosome amplification (Figure 1A). We interpret from this result that the cell

cycle was blocked at a stage too early for centrosome amplification secondary to BRCA1 inhibition. Treatment of cells with bleomycin causes a DNA damage response and an intra-S-phase block (Wyllie *et al.*, 1996; Suto *et al.*, 1999; D'Amours and Jackson, 2001). Flow cytometry analysis of these bleomycin-treated MCF10A cells was consistent with a late S to G2/M block since most cells had replicated the genome (Figure 2c). When treated with bleomycin, 15% of the control cells had >2 centrosomes, whereas the same cells expressing BIF had 25% with >2 centrosomes. Although bleomycin treatment caused an increase in the background number of cells with extra centrosomes, BRCA1 inhibition caused an even higher increase.

Similar results were obtained when inhibiting BRCA1 expression by siRNA transfection in the breast cancer cell line, Hs578T. Transfection of siRNA specific for BRCA1 reduced the BRCA1 protein content of the cells (Figure 1D), and consistent with earlier results (Starita *et al.*, 2004), loss of BRCA1 protein

caused centrosome amplification (Figure 1E). The effects on centrosome number after inhibiting BRCA1 in Hs578T cells treated with HU or bleomycin were distinct from the results obtained with the MCF10A cells (Figure 1C). Both HU and bleomycin caused a modest increase in the centrosome number in the cells treated with the control siRNA. Similar to the MCF10A results, HU treatment blocked an increase in the number of extra centrosomes in BRCA1-inhibited cells. By contrast, bleomycin treatment did result in an increase in the number of cells with centrosome amplification after inhibiting BRCA1 expression.

One interpretation of the results of Figure 1 is that the treatment of cells with HU halts the cell cycle upstream of the point at which BRCA1 function is important to inhibit centrosome reduplication. Since bleomycin causes a later block in the cell cycle, after the DNA has replicated, then it is possible that amplification of centrosomes secondary to inhibition of BRCA1 occurs between early S-phase and late G2. Thus, the centrosome amplification observed in the presence of bleomycin is inconsistent with supernumerary centrosomes accumulating after a failed cytokinesis and repeated S-phase. Alternatively, bleomycin causes double-stranded DNA breaks (Wyllie *et al.*, 1996) and since DNA damage can cause centrosome fragmentation and amplification under certain conditions (Hut *et al.*, 2003), it is possible that the supernumerary centrosome production due to BRCA1 inhibition was due to the DNA damage.

To distinguish between effects of being later in the cell cycle versus DNA damage, the cell cycle was blocked with HU, released, and centrosome number was evaluated at time points. Progress through the MCF10A cell cycle after HU treatment was analysed by flow cytometry and by tracking the changes in concentration of the various cyclins (Figure 2b and c). At the time of HU block, cyclin E levels were high and rapidly declined 3 h after HU washout. Cyclin E levels returned 12–18 h after HU release, indicating the timing of the next S-phase. Since cyclin A is associated with a later stage of S-phase, it persisted 8–10 h after HU washout, and cyclin A had not increased by 24 h, when the time course was halted. The mitotic cyclin B1 peaked at 6–10 h after removal of HU (Figure 2b). Consistent results were observed by FACS analysis. At time 0, in the presence of HU, most of the cells had unreplicated DNA. At 3 h after removal of HU, a prominent S-phase peak was observed, and at 6–8 h almost all of the cells had fully replicated DNA. At 10 h the G1 peak appeared, indicating that mitosis occurred between 8 and 10 h after HU removal. A new S-phase was apparent at the 18 h time point (Figure 2c). If inhibition of BRCA1 in MCF10A cells caused a failure of cytokinesis, causing the cell to reduplicate its centrosomes at a second S-phase, then centrosome amplification should become apparent at 18 h at the very earliest. Alternatively, if centrosome amplification occurs at an earlier point, then BRCA1 inhibition renders centrosome duplication to be no longer dependent upon cyclin A.

In Figure 2a, results of four experiments were combined in which MCF10A cells were transfected with a vector to express GFP-centrin, or cells were not transfected with a marker, but rather were stained for γ -tubulin. In each case, adenovirus expressing a control protein (GFP) or BIF were infected into the MCF10A cells, 3 h postinfection 2.5 mM HU was added to the medium, 20 h postinfection (defined as time 0) the HU was removed, and cells were cultured for differing lengths of time. For both methods of labeling centrosomes the results were consistent. In the absence of HU and at the 20 hpi time point (labeled as 'no drug') about 5% of the control cells had >2 centrosomes. By contrast, in cells expressing BIF about 15% of the cells had centrosome amplification. Consistent with Figure 1A, 7–10% of cells at time 0 had >2 centrosomes under each condition. After removing HU for 3–6 h there was little change in the number of cells with supernumerary centrosomes. By contrast, at 8–10 h after removal of HU, BIF expressing cells had centrosome amplification. At 10 h after HU release, 18% of BIF-expressing cells had >2 centrosomes as compared to 8% of uninfected controls or 14% of GFP-expressing cells (Figure 2a). Reproducibly, after treatment of cells with HU, the control adenovirus caused a modest amount of centrosome amplification that was not observed without the HU treatment. Certainly, the BIF expression caused a significantly larger increase in cells with >2 centrosomes than did the control adenovirus. Based upon the cell cycle analysis after HU release (Figure 2b and c), centrosome amplification secondary to BRCA1 inhibition occurred in G2- or M-phase of the MCF10A cell cycle. Clearly, overduplication of centrosomes was not occurring at a second S-phase, as would be predicted if the centrosome amplification was due to a failure of cytokinesis.

Results with inhibiting BRCA1 in Hs578T cells also showed that centrosome amplification occurred at an inappropriate late stage of the cell cycle. From the cyclin concentrations (Figure 3b) and flow cytometry data (Figure 3c) at time points after removal of HU, it could be concluded that S-phase persisted for 8 h after HU release. Mitosis occurred at 12–16 h after HU removal. At times 0 and 4 h, there was no centrosome amplification in either the control siRNA sample or in the BRCA1-specific siRNA sample. At 8 h after the removal of the HU, centrosome amplification was evident in the samples that were transfected with the BRCA1 siRNA. As with the MCF10A cells, after treatment and release of HU, the controls had a modestly increased level of cells with >2 centrosomes, but the BRCA1-specific siRNA had a significantly higher percentage of cells with extra centrosomes at 8 h post-HU release (Figure 3a). In the Hs578T cells BRCA1 inhibition caused centrosome amplification at about the time S-phase was completed.

In both MCF10A and Hs578T cells inhibition of BRCA1, by either expressing the BIF peptide or transfecting a specific siRNA, caused the centrosomes to over-duplicate at a late point of the cell cycle. The centrosome normally duplicates in S-phase after cyclin A induction (Meraldi *et al.*, 1999). The data from these

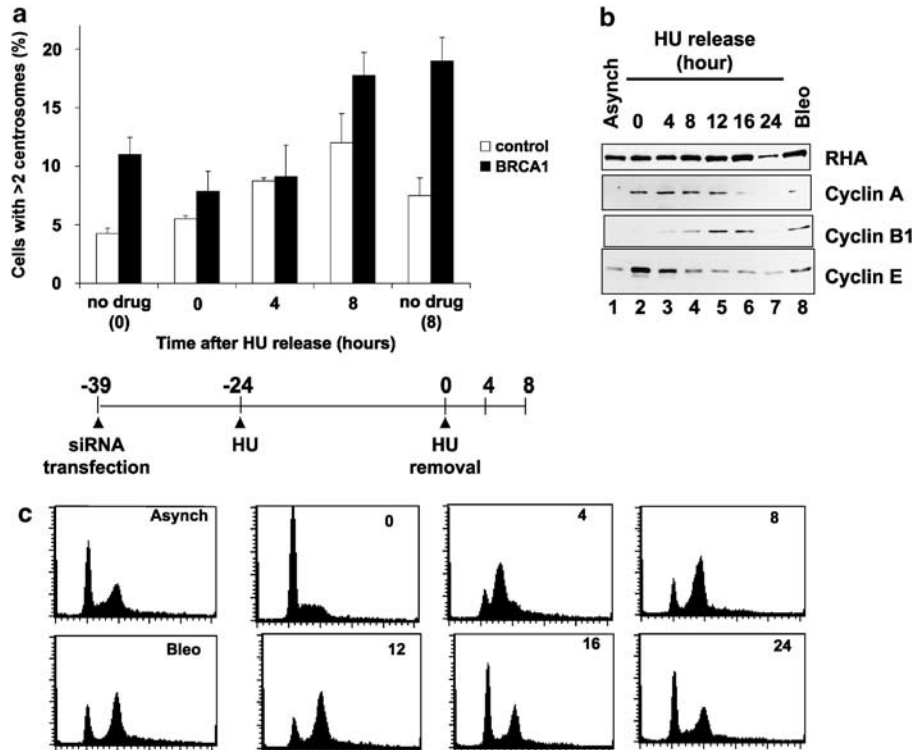


Figure 3 RNAi specific for BRCA1 in Hs578T cells caused centrosome amplification in S/G2 phase. (a) Hs578T cells were transfected with a BRCA1 specific siRNA or a control siRNA (GL2) and were treated with 8 mM HU or untreated (no drug) at 19 h post transfection. After 24 h incubation in the presence of HU, cells were released from HU, fixed at 0, 4, or 8 h and stained for γ -tubulin. No drug (0) refers to cells fixed at time 0, and no drug (8) refers to control cells at the 8 h time point after HU removal. Results from four experiments were combined, and the average percentage of cells with extra centrosomes (plus the s.e.m.) is shown. (b) Hs578T cells were treated with HU for 24 h followed by release at the indicated time points. Bleomycin (Bleo)-treated samples were in the presence of the drug for 16 h. Each sample was used for immunoblotting with antibodies specific for cyclin A (second panel), cyclin B1 (third panel), and cyclin E (bottom panel). RHA was analysed as a loading control (top panel). (c) Flow cytometry results from the same cells as used in panel b. Transfection of siRNA specific to BRCA1 or control caused no discernible change in the FACS tracing (data not shown).

experiments are consistent with a model that BRCA1 functions as a licensing factor for centrosome duplication, and loss of BRCA1 function then results in reduplication of the centrosome without passing through mitosis and into a new S-phase. In published studies, centrosomes from G2-phase cells are intrinsically resistant to reduplication even if exposed to G1-phase cytoplasm (Wong and Stearns, 2003). Based on our data, we suggest that in these two breast cell lines BRCA1-dependent ubiquitination activity marks the centro-

somes, and this covalent modification by BRCA1 then inhibits reduplication.

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