Dysfunctional BRCA1 is only indirectly linked to multiple centrosomes
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A remarkable and yet unexplained phenomenon in cancer cells is the presence of multiple centrosomes, organelles required for normal cell division. Previously it was demonstrated that the tumor suppressor BRCA1 is a component of centrosomes. This observation led to the hypothesis that defective BRCA1 results in malfunctioning centrosomes and faulty centrosomes are a possible cause of cancer. Using EGFP-tagged fusion proteins and BRCA1⁻/⁻ cells we show that although some BRCA1 antibodies do label centrosomes under certain fixation conditions, BRCA1 is not a centrosomal protein. Therefore, it is unlikely that a mutation in BRCA1 directly alters centrosome structure and function. BRCA1 plays an established role in DNA damage repair and in G₂/M checkpoint regulation. We present evidence that multiple centrosomes can arise in any cell when G₂/M checkpoint fails and entrance into mitosis occurs in the presence of DNA damage.

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The great majority of human tumors contain too many centrosomes, organelles required for accurate cell division (12; 13; 16; 23). Exactly how and when numerous centrosomes arise in cancerous cells, is largely unknown, although, a link has been suggested between a mutation in the tumor suppressor protein BRCA1 and impaired centrosome function. Experimental approaches using antibodies specific for BRCA1, demonstrated centrosomal localization of BRCA1 by immunofluorescence (9) and showed that the BRCA1-BF3 domain interacted with the centrosomal protein γ-tubulin in vitro (8; 19). This suggested a centrosomal function of BRCA1 and pointed to a plausible hypothesis: mutations in BRCA1 cause centrosome abnormalities, leading to division errors, genetic instability and finally to cancer (3; 5; 6). However, it remains unclear whether a mutation in BRCA1 directly affects centrosome structure and function.

In order to investigate a possible centrosomal role of BRCA1 in detail, an EGFP-BRCA1 fusion construct was generated. MCF-7 cells were transiently transfected with this construct, and expression of the full-length fusion protein was confirmed by Western blotting (supplementary Figure 1). Transfected cells were fixed and stained with an antibody specifically recognizing γ-tubulin to visualize centrosomes. Interphase cells revealed the presence of characteristic nuclear foci (17) as demonstrated by the presence of EGFP-BRCA1 (Figure 1A). The EGFP-BRCA1 nuclear foci colocalized with nuclear endogenous BRCA1 as revealed by labeling of the transfected cells with the anti-BRCA1 antibody D9 (supplementary Figure 2). The transfection efficiency was 10% as calculated by the percentage of interphase cells showing EGFP-BRCA1 expression. Cells containing EGFP-BRCA1 progress through mitosis normally since in the population of fixed cells often daughter cells were observed that have gone through a mitotic division (Figure 1A). Since the transfection efficiency was 10%, 10% of all mitotic cells should also express EGFP-BRCA1. However, over 600 mitotic cells were examined and none of the mitotic cells did show an EGFP-BRCA1 signal localized to the centrosomes (an example is shown in Figure 1B). In addition, transiently transfected EGFP-BRCA1-positive cells were followed through mitosis by time-lapse microscopy and whereas nuclear foci were clearly visible during interphase (Figure 1D, 0'), in none of the recorded dividing cells (n = 4) centrosomal localization of EGFP-BRCA1 was observed during mitosis (Figure 1D and http://coo.med.rug.nl/sscb/lapse.htm Figure 1). In addition, MCF-7 cells were transfected with a fusion construct containing the putative γ-tubulin binding domain of BRCA1 “BF3” (8) and GFP but also this BF3-GFP fusion protein did not localize to centrosomes during mitosis (Figure 1C). Since it cannot be excluded that the EGFP-tag interfered with centrosomal localization we re-investigated the localization of endogenous BRCA1 using immunocytochemistry. Consistent to previous data (9), after paraformaldehyde/methanol fixation and staining with the anti-BRCA1 antibodies D9 and C20, a centrosomal labeling was observed in mitotic MCF-7 cells (Figure 2). Centrosomal staining of D9 (Figure 2A-D) is more pronounced and the D9 antibody showed less background staining compared to the C20 antibody (Figure 2E-H). Also the characteristic and specific nuclear BRCA1-containing foci (17) were clearly visible (see supplementary Table 1). When cells were fixed with 4 % formaldehyde, nuclear foci were visible after using the D9 and the C20 antibody. However, centrosomal labeling was not observed as was clearly demonstrated by using the D9 antibody (see supplementary Table 1). These results showed that centrosomal labeling of BRCA1-antibodies is fixative dependent. In combination with the fact that EGFP-BRCA1 and BF3-GFP do not localize to centrosomes, this suggests that BRCA1 antibodies nonspecifically bind to centrosomes after methanol fixation. To test this further we used L56Br-C1 cells that do not express endogenous BRCA1 (11). Indeed, after paraformaldehyde/methanol fixation a pronounced D9 and C20 antibody labeling was present at centrosomes of mitotic L56Br-C1 cells (Figure 2I-P). We thus demonstrate that D9 and C20 bind to centrosomes irrespective of BRCA1 presence and that BRCA1 itself is not linked to centrosomes. Our data using EGFP-BRCA1 and BF3-GFP show that BRCA1 is not localized at centrosomes. Previously, it has been demonstrated that BRCA1 and its BF3 domain bind γ-tubulin in vitro (8; 19). These data are not in agreement with our data, but it
Dysfunctional BRCA1 is only indirectly linked to multiple centrosomes. It may be possible that an interaction between γ-tubulin and BRCA1 only occurs in vitro and not in vivo, or that BRCA1 binds to γ-tubulin outside centrosomal areas.

**Figure 1: EGFP-BRCA1 and BF3-GFP do not localize to centrosomes.**

An EGFP-BRCA1 fusion construct was generated by inserting full length BRCA1 cDNA (21) in frame with an EGFP-containing vector (pEGFP-C3, Clontech Laboratories, Inc). Expression of full length EGFP-BRCA1 fusion protein was tested and confirmed by transfection of EGFP-BRCA1 in CHO cells and in MCF-7 cells and analyzing cell lysates by Western blotting using antibodies against BRCA1 (D9, Santa Cruz, Biotechnology, Inc, California) or against GFP (Molecular Probes, Inc) (supplementary Figure 1A-E). The putative γ-tubulin binding fragment of BRCA1 “BF3” was amplified by PCR from the EGFP-BRCA1 construct (with primers gatcggagctcagggtagaaagagacatcagcc and gaattcgaagcttactcaccatcattggtttcgt) and inserted into pBAD A (Invitrogen) at Xhol/HindIII sites resulting in pBAD BF3. GFP was cloned in frame resulting in pBAD BF3-GFP. After BF3-GFP was amplified by PCR from pBAD BF3-GFP, BF3-GFP was ligated into a pcDNA3 vector to allow expression in mammalian cells. Expression of BF3-GFP was confirmed by Western blotting using antibodies against GF (Molecular Probes, Inc) (supplementary Figure 1F). A,B: MCF-7 cells were transfected with EGFP-BRCA1 using calcium phosphate co-precipitation, paraformaldehyde/methanol fixed and labeled with an anti-γ-tubulin antibody as previously described (9). EGFP-BRCA1 signal is visible in green and the γ-tubulin antibody localization is visible in red. EGFP-BRCA1 localizes to nuclear foci in interphase cells (A), but EGFP-BRCA1 does not localize to centrosomes in interphase cells (A), nor in mitotic cells (B). C: Methanol/acetonefixed mitotic MCF-7 cell expressing BF3-GFP (green) was labeled with anti-γ-tubulin antibody (red) and the DNA was stained with DAPI (blue). BF3-GFP does not localize to the centrosomes. D: MCF-7 cells were transfected with EGFP-BRCA1 using Fugene (Roche Diagnostics) and time-lapse analysis was performed as previously described (10). In interphase cells EGFP-BRCA1 is present at nuclear foci (0’-90’) and after nuclear envelope breakdown (100’) a diffuse EGFP-BRCA1 signal is visible during mitosis. Until cytokinesis occurs (140’ to 270’), EGFP-BRCA1 remains diffusely present throughout the cell and no centrosomal localization of EGFP-BRCA1 is observed. Bar, 10 µm. For movies see http://coo.med.rug.nl/sscb/lapse.htm Figure 1.

Since BRCA1 is not localized to centrosomes, it is unlikely that a mutation in BRCA1 directly alters centrosome structure and function. The intriguing question however as to how numerous centrosomes arise in BRCA1Δ+ cells and in tumor cells in general still remains. These cells have a malfunctioning DNA repair machinery and faulty checkpoint functions in common (15; 23). To determine how centrosomes behave in vivo under these conditions, we performed time-lapse experiments using EGFP-α-tubulin and γ-tubuli-GFP expressing CHO cells.
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cells. We damaged the DNA by irradiation and treated the cells with UCN-01 to override the G2/M checkpoint (7), and spindle formation and centrosome abnormalities were examined. The first mitotic spindle after irradiation was bipolar, however cytokinesis often failed and tetraploid cells with multiple nuclei appeared (Figure 3A,C and http://coo.med.rug.nl/sscb/lapse.htm Figure 2,4). This multinuclear cell per definition has too many centrosomes and when such a cell reenters mitosis, multipolar spindles develop (Figure 3B,D and http://coo.med.rug.nl/sscb/lapse.htm Figure 3,5).

Previously, it has been demonstrated in fixed p53−/− cells and fixed Aurora-A overexpressing cells that multiple centrosomes most likely arise through defects in cell division (2; 14). Here, we present evidence with time-lapse microscopy that this is indeed one order of events by which multiple centrosomes can arise. Failed cytokinesis is not the only route to multiple centrosomes, since it was reported that centrosome amplification can also occur during prolonged S phase (1; 22), during a prolonged G2 phase in Rad51 depleted cells (4), or during mitosis in the presence of incompletely replicated DNA (10).

The question remains what is the biological relevance to form multiple or fragmented centrosomes. In normal cells, extra centrosomes may ensure that cells with damaged DNA do not undergo a successful mitosis and die after mitotic catastrophe. Strong evidence supporting this hypothesis was obtained in Drosophila embryos where centrosomes are inactivated in the presence of impaired DNA integrity and damaged nuclei are eliminated from the developing embryo (18; 20). Whether multiple centrosomes similarly act as a back-up mechanism to abolish cells with DNA damage in mammalian systems remains to be investigated. It also may be possible that multiple centrosomes are just a byproduct arising in cancerous cells and even lead to increased genomic instability.

Regardless of the biological relevance, we have shown that BRCA1 is not a centrosomal protein and our data are therefore incompatible with a model in which BRCA1 directly regulates centrosome number. BRCA1 plays an established role in DNA damage repair and in G2/M checkpoint regulation (15; 23). We present evidence that multiple centrosomes can arise in any cell when G2/M checkpoint fails and entrance into mitosis occurs in the presence of DNA damage. Now, it will be of interest to investigate what the fate is of cells with extra copies of centrosomes and whether this is favorable or unfavorable for multicellular organisms.

Acknowledgements

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Figure 2: Localization of BRCA1 antibodies to centrosomes.
A-H: Paraformaldehyde/methanol fixed MCF-7 cells were labeled with the anti-BRCA1 antibody D9 (Santa Cruz) (A), or with the anti-BRCA1 antibody C20 (Santa Cruz) (E) and with a γ-tubulin antibody (B,F). DNA was stained with DAPI (C,G) and an overlay is given of each labeling (D,H). I-P: Paraformaldehyde/methanol fixed L56Br-C1 cells were labeled with the anti-BRCA1 antibody D9 (I) or with the anti-BRCA1 antibody C20 (M) and with a γ-tubulin antibody (J,N). DNA was stained with DAPI (K,O) and an overlay is given of each labeling (L,P). In mitotic MCF-7 cells, the D9 (A,D) and C20 (E,H, arrows) antibodies colocalize with centrosomes, although the C20 antibody shows some background staining. Although L56Br-C1 cells do not express BRCA1 protein (11), the D9 (I,L) and the C20 (M,P, arrows) antibodies do localize to the centrosomes comparable to the centrosomal localization in MCF-7 cells. Bar, 10 μm.
Figure 3: Multiple centrosomes and multipolar spindles develop when cells continue to divide with damaged DNA.

In CHO cells expressing α-tubulin-EGFP (A,B) or GFP-γ-tubulin (C,D) (10), DNA damage was induced by irradiation with 10 Gy after which 0.3 μM UCN-01 was added to the medium to override the G0/M checkpoint. Irradiated and UCN-01 treated CHO cells enter mitosis with damaged DNA, cytokinesis fails and multinuclear cells develop (A, C). A multinuclear cell re-enters mitosis and multipolar spindles with numerous centrosomes arise (B,D). Time-lapse imaging recordings were generated as described previously using a confocal laser-scanning microscope (Leica TCS SP2, Leica Microsystems, Heidelberg, Germany or Zeiss LSM510NLO, Carl Zeiss, Jena, Germany (10)). Time is given in minutes. Arrows indicate centrosomes. For movies see http://coo.med.rug.nl/sscb/lapse.htm Figure 2, 3, 4 and 5.
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Supplementary Table 1: Labeling pattern of BRCA1 varies with different BRCA1 antibodies and fixation protocols.

<table>
<thead>
<tr>
<th>Antibody</th>
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<th>Mitotic centrosomes</th>
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<tr>
<td></td>
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<td>++ (Supp. Figure 2A)</td>
<td>++</td>
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<tr>
<td></td>
<td>Formaldehyde</td>
<td>++</td>
<td>ND</td>
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<tr>
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<td>++</td>
<td>++ (Figure 2A)</td>
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MCF-7 cells were fixed using different fixation protocols. Cells were fixed with methanol/acetone (3:1) (10), with 4% formaldehyde or with paraformaldehyde/methanol as previously described (9). Cells were labeled with antibodies specifically recognizing γ-tubulin to visualize centrosomes and cells were labeled using anti-BRCA1 antibodies. The labeling pattern was analyzed for three different anti-BRCA1 antibodies: C20 (Santa Cruz), D9 (Santa Cruz) and MS110 (Calbiochem). It was analyzed whether or not label was observed at nuclear foci in interphase cells and at centrosomes in interphase cells or in mitotic cells. When a positive and pronounced signal was observed, this is indicated as + or ++. A negative signal is indicated as -. Bg indicates that under the given conditions background staining was observed. γ-Tubulin antibodies do not give a signal when 4% formaldehyde fixation was used. Therefore centrosomes in the formaldehyde fixed interphase cells cannot be visualized. In case the D9 antibody was used, it is still possible to determine whether there is colocalization at centrosomes during mitosis, since this antibody does not give a background signal, however in case of the C20 or MS110 antibody, due to background staining centrosomal staining after formaldehyde fixation can not be investigated and these conditions are marked with ND (not determined).
**Supplementary Figure 1: Expression of EGFP-BRCA1 and BF3-GFP.**

MCF-7 cells (human cells in which BRCA1 antibodies do recognize endogenous BRCA1) and CHO cells (hamster cells in which BRCA1 antibodies do not recognize endogenous BRCA1) were transfected with EGFP-BRCA1 or with BF3-GFP. Cell lysates were analyzed by Western blotting using antibodies against BRCA1 (D9, Santa Cruz, Biotechnology, Inc.) or by using antibodies against GFP (Molecular probes, Inc). Lane A: control MCF-7 cells, a specific band representing endogenous BRCA1 is visible after using the D9 antibody. Lane B: control CHO cells, no specific band is visible after using the D9 antibody. Lane C: CHO cells transfected with EGFP-BRCA1, a specific band representing EGFP-BRCA1 is visible after using the D9 antibody. Lane D: control MCF-7 cells, no specific band is visible after using the anti-GFP antibody. Lane E: MCF-7 cells transfected with EGFP-BRCA1, a specific band is visible representing EGFP-BRCA1 after using the anti-GFP antibody. Lane F: MCF-7 cells transfected with BF3-GFP, a specific band is visible representing BF3-GFP after using the anti-GFP antibody.
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Supplementary Figure 2: EGFP-BRCA1 does localize to nuclear foci and not to centrosomes, whereas anti-BRCA1 antibody D9 does localize to centrosomes and nuclear foci.

MCF-7 cells were transfected with EGFP-BRCA1 using calcium phosphate co-precipitation, methanol/acetone fixed as previously described (10) and labeled with the anti-BRCA1 antibody D9 (A-C), or cells were double labeled with anti-BRCA1 antibody D9 and γ-tubulin antibodies to visualize centrosomes (D-F). The BRCA1 antibody localization is visible in red (A,D), the EGFP-BRCA1 signal is visible in green (B) and the γ-tubulin signal, marking centrosomes is visible in green (E). The D9 antibody localizes to centrosomes (arrows, A,D) and to nuclear foci (A,D), whereas EGFP-BRCA1 is exclusively localized at nuclear foci. Overlapping signal of green (EGFP-BRCA1) and red (BRCA1 antibody) is present in foci (C). The signal of D9 antibody, localized outside the nucleus, colocalizes with centrosomes because these foci colocalize with γ-tubulin (D-F).