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A Human BRCA2 Complex Containing a Structural DNA Binding Component Influences Cell Cycle Progression

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Summary

Germline mutations of the human *BRCA2* gene confer susceptibility to breast cancer. Although the function of the BRCA2 protein remains to be determined, murine cells homozygous for BRCA2 inactivation display chromosomal aberrations. We have isolated a 2 MDa BRCA2-containing complex and identified a structural DNA binding component, designated as *BR*CA2-*A*ssociated *F*actor *35* (BRAF35). BRAF35 contains a nonspecific DNA binding HMG domain and a kinesin-like coiled coil domain. Similar to BRCA2, BRAF35 mRNA expression levels in mouse embryos are highest in proliferating tissues with high mitotic index. Strikingly, nuclear staining revealed a close association of BRAF35/BRCA2 complex with condensed chromatin coincident with histone H3 phosphorylation. Importantly, antibody microinjection experiments suggest a role for BRCA2/BRAF35 complex in modulation of cell cycle progression.

Introduction

Mutations of one copy of the *BRCA2* gene predisposes humans to breast cancer (Wooster et al., 1995; Tavtigian et al., 1996). Breast tumors from predisposed individuals often display mutations in both alleles, suggesting that BRCA2 serves as a tumor suppressor (Collins et al., 1995; Gudmundsson et al., 1995). In addition, deleterious alleles of BRCA1 and BRCA2 are responsible for almost all familial ovarian cancer, and deleterious alleles of BRCA2 are also involved in hereditary male breast cancer (Easton et al., 1993; Miki et al., 1994; Wooster et al., 1995; Tavtigian et al., 1996). BRCA2 encodes a large protein of about 390 kDa that does not possess any obvious homology with sequences available in the public database (Tavtigian et al., 1996).

Recently, a report using in vitro transactivation assays has suggested a role for the amino-terminal domain of BRCA2 in transcriptional regulation (Milner et al., 1997). However, most of the current data point to a role for the BRCA2 protein in DNA repair. A number of laboratories have reported that the BRCA2 protein interacts with RAD51 (Mizuta et al., 1997; Wong et al., 1997; Chen et al., 1998; Marmorstein et al., 1998), the human homolog of *Escherichia coli* recA (Shinohara et al., 1992). RAD51 is the hallmark of homologous recombination, suggesting a function for BRCA2 in recombination or double-strand break DNA repair. These results were strengthened by a report showing that murine embryos with a targeted disruption of BRCA2 displayed sensitivity to ionizing radiation (Sharan et al., 1997). Moreover, mouse embryo fibroblasts (MEFs) with a targeted disruption of BRCA2 exon 11 displayed increased sensitivity to ultraviolet light and methyl methanesulfonate (MMS) (Patel et al., 1998).

To decipher the role for the BRCA2 protein in cancer predisposition, attempts have been made to analyze the induction of tumors in mice with a targeted deletion in BRCA2. However, contrary to expectation, mouse strains heterozygous for mutations in BRCA2 failed to show a predisposition to tumor formation (Connor et al., 1997; Ludwig et al., 1997; Sharan et al., 1997; Suzuki et al., 1997). Homozygosity caused early embryonic lethality at day 7.5–8.5 and was accompanied by retarded embryonic growth in vivo and in vitro (Ludwig et al., 1997; Sharan et al., 1997; Suzuki et al., 1997). Interestingly, embryonic survival was prolonged to day 10.5 when the analysis was performed with BRCA2 homozygous mouse with a p53 null background (Ludwig et al., 1997). Most recently, it was shown that some animals with a homozygous deletion of exon 11 survive to maturity and succumb to thymic lymphomas (Friedman et al., 1998). Human BRCA2 exon 11 is composed of eight internal repeats known as the BRC repeats that are conserved in all mammalian BRCA2 proteins that have been sequenced (Bork et al., 1996; Koonin et al., 1996).

To elucidate the molecular mechanism by which BRCA2 induces its functional effects, we isolated the predominant BRCA2-containing complex from HeLa cells and identified its components by mass spectrometry. Here we show that the BRCA2 complex contains BRAF35 (*BR*CA2-*a*ssociated *f*actor *35*), a structural DNA binding protein with specificity for cruciform DNA. Moreover, we provide evidence for association of the BRCA2/BRAF35 complex with condensed chromatin during mitosis. Taken together, our studies suggest a role for the BRCA2/BRAF35 complex in the timely progression through mitosis.

Results

Affinity-Purification of a BRCA2-Containing Complex

To determine the polypeptide composition of BRCA2-containing complex(es), HeLa nuclear or S100 (a side fraction of nuclear extract preparation) extracts were chromatographed sequentially to enrich for BRCA2-

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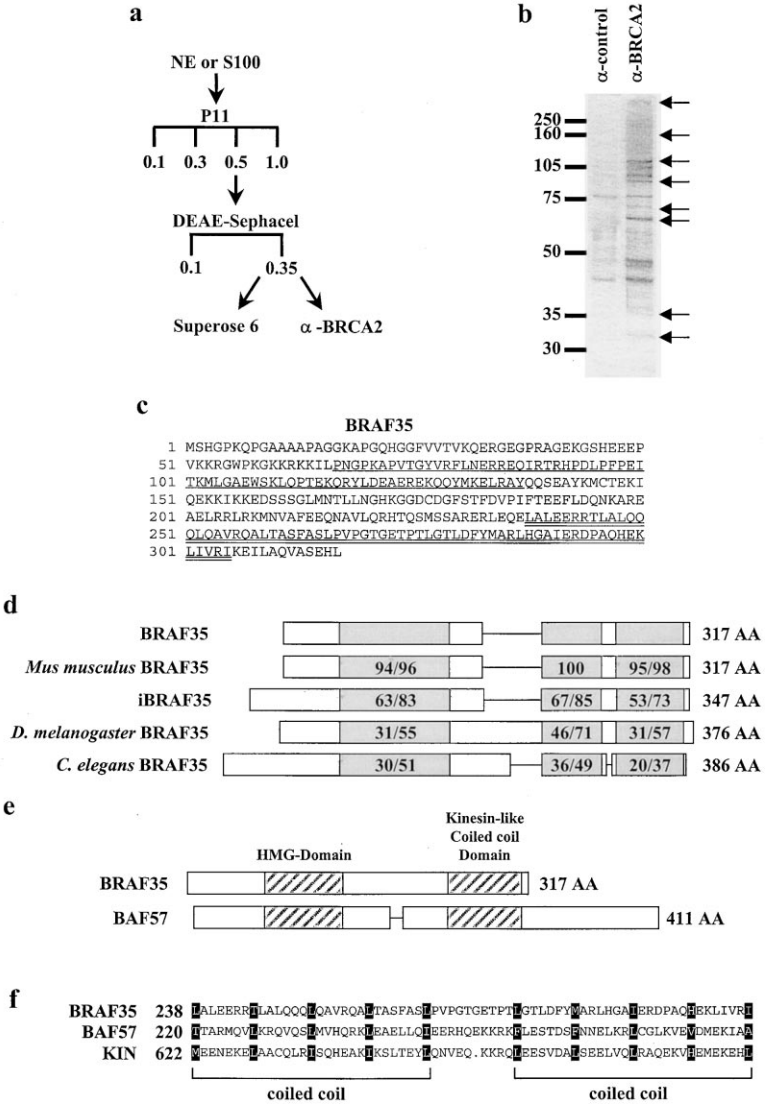

Figure 1. Identification of BRAF35

(a) HeLa nuclear or S100 extract was fractionated using phosphocellulose (P11) chromatography. The 0.5 M KCl elution was concentrated on a DEAE-Sephacel column and further fractionated by Superose 6 gel filtration or affinity chromatography using BRCA2 antibodies.

(b) The affinity-purified BRCA2 complex or control eluate (protein A beads) was separated in an SDS-polyacrylamide gel (4%–15%), and proteins were visualized by silver staining. Molecular masses of marker proteins are indicated on the left and proteins judged to be specific for the BRCA2 affinity eluate are indicated by arrows.

(c) The primary amino acid sequence of BRAF35. The conserved HMG domain is single underlined and kinesin-like domain is double underlined.

(d) Diagrammatic alignment of BRAF35 (AF331191), mouse BRAF (Smarce1-related, Wattler et al., 1999; AAD15897), iBRAF (AAF66706), *D. melanogaster* BRAF (AAF46682), and *C. elegans* BRAF (T26108). The numbers in the shaded boxes represent percent identity and similarity of each protein to human BRAF35, respectively.

(e) Diagrammatic alignment of BRAF35 and BAF57.

(f) Alignment of kinesin-like domains with corresponding sequences of related proteins. Hydrophobic residues of the heptad repeats of the kinesin-like domain are shaded.

containing complex(es) (Figure 1a). Analysis of these fractions by gel filtration chromatography revealed that BRCA2 was a component of a 2 MDa complex (data not shown). To purify this complex, DEAE-Sephacel fractions were subjected to affinity-purification using mono- and polyclonal anti-BRCA2 antibodies (Marmorstein et al., 1998). Western blot analysis of the affinity-eluates confirmed the presence of BRCA2 protein (data not shown). However, the yields for the BRCA2 protein were variable in different preparations, as it could not be efficiently eluted from the affinity matrix. Polypeptides specifically eluted from the BRCA2 affinity matrix (Figure 1b) were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. This analysis identified, among a number of proteins that were specifically enriched by the BRCA2 antibodies, a 35 kDa species, designated BRAF35 (*BR*CA2-*a*ssociated *f*actor), as the predicted product of the R31109 cosmid open reading frame.

BRAF35 Is an Evolutionary Conserved Protein

Based on the above information, expressed sequence tags containing the full-length BRAF35 protein were obtained (Figure 1c). Blast analysis using BRAF35 as query identified a second human open reading frame with extensive homology to BRAF35, designated iBRAF35 (imitation BRAF35). This analysis also revealed *Mus musculus Drosophila melanogaster* and *Caenorhabditis elegans* homolog of BRAF35 (Figure 1d).

Analysis of the predicted sequence of human BRAF35 indicated that it encodes a protein containing a single sequence nonspecific HMG domain and a region with two heptad repeats similar to the coiled-coil region of kinesin. Interestingly, the only other protein with similar structural domains to BRAF35 is BAF57, a subunit of the human SWI/SNF complex (Wang et al., 1998). Both proteins contain the nonspecific HMG box and the region similar to the coiled-coil domain of kinesin (Figures 1e and 1f). It is noteworthy that both BRAF35 and BAF57 outside their HMG domains lack significant homology to any ORFs in *Sacchromyces cerevisiae*.

BRAF35 Is Associated with a 2 MDa BRCA2 Complex

To confirm the association of BRAF35 and BRCA2, we employed several approaches. We purified the BRCA2

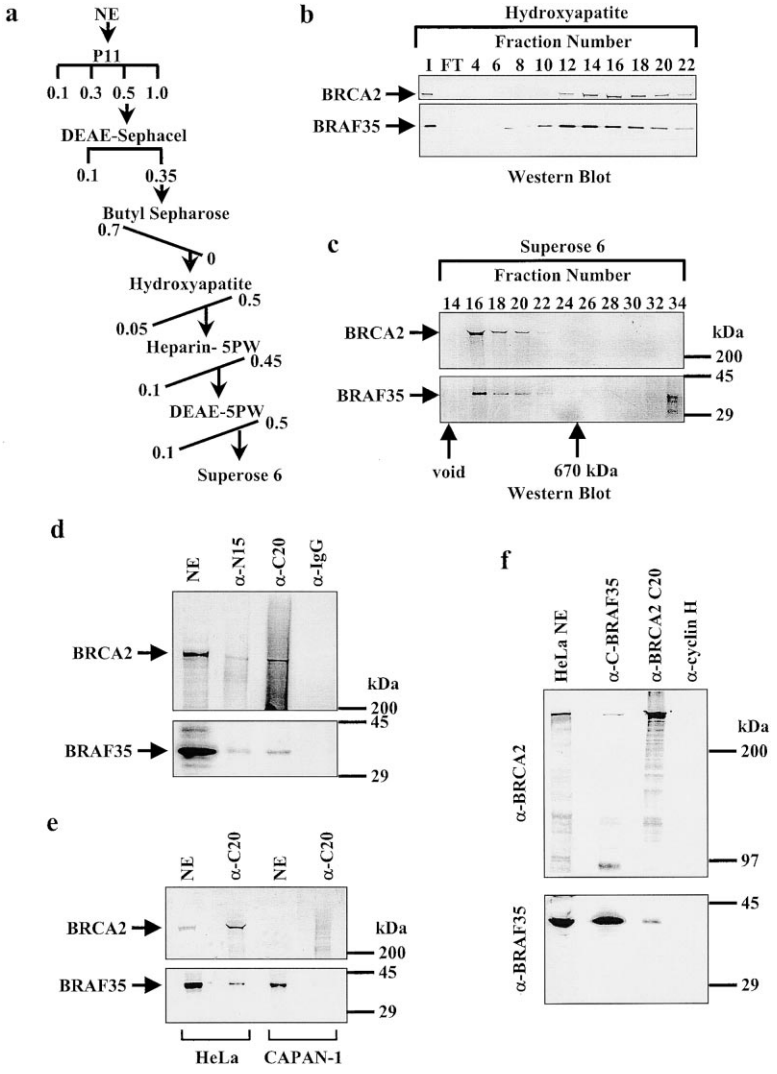

Figure 2. BRAF35 Is a Component of the BRCA2 Complex

(a) Purification Scheme. HeLa nuclear extract was fractionated by chromatography as described in Experimental Procedures. The horizontal and diagonal lines indicate stepwise and gradient elution, respectively. Concentrations are given in molars.

(b and c) Westrn blot analysis of the Hydroxyapatite and Superose 6 fractions (15 ml) using anti-BRCA2 monoclonal antibodies (5.23) and anti-BRAF35 antibodies (monoclonal mix).

(d, e, and f) Affinity eluate using C-terminal polyclonal anti-BRCA2 (C20), N-terminal polyclonal anti-BRCA2 (N15), C-terminal polyclonal anti-BRAF35 antibodies, and control polyclonal anti-cyclin H antibodies or control IgG were separated in an SDS-polyacrylamide gel and proteins were immunoblotted using anti-BRAF35 and anti-BRCA2 monoclonal antibodies. The numbers in the right denote the molecular weight markers. 500 μg of HeLa nuclear extract was used for the immunoprecipitations and HeLa NE denotes 50 μg of HeLa nuclear extract.

complex from HeLa nuclear extract using a conventional chromatographic procedure following the scheme in Figure 2a. BRAF35 coeluted with the BRCA2 protein throughout the purification scheme (Figures 2b and 2c). Analysis of the BRAF35/BRCA2 complex on the last chromatograpic step revealed the precise coelution of BRCA2 and BRAF35 as components of a large multiprotein (~2 MDa) complex (Figure 2c). We then used anti-BRCA2 antibodies to specifically precipitate BRAF35 from HeLa nuclear extract (Figure 2d). Based on the known ability of these antibodies to enrich for BRCA2 (around 5%–10% efficiency), we estimate that at least 2%–4% of endogenous BRAF35 was present in BRCA2 complex(es). Furthermore, the inability of the C-terminal anti-BRCA2 antibodies to immunoprecipitate BRAF35 from Capan-1 nuclear extract (containing a truncated BRCA2 protein, Goggins et al., 1996) further attest to the specific association of BRAF35 and BRCA2 (Figure 2e). CAPAN-1 cells harbor a truncation of BRCA2 (6174delT) on one allele, while the second allele is lost (Goggins et al., 1996). Finally, anti-BRAF35 antibodies specifically precipitate BRCA2 (Figure 2f). Taken together, these results demonstrate a specific association of BRAF35 with a multiprotein BRCA2-containing complex.

BRAF35 Is a Component of at Least Two Complexes and Can Directly Interact with BRCA2

To determine the number of BRAF35-containing complexes, we followed BRAF35 immunoreactivity following the chromatographic scheme presented in Figure 3a. Analysis of Superose 6 gel filteration revealed two peaks of immunoreactivity for BRAF35. A complex of approximately 2 MDa that contains BRCA2 eluted with a sharp peak in fraction 18 and a second smaller BRAF35 complex (approximately 500 kDa) lacking BRCA2 (Figure 3a) eluting in fractions 26–30. Therefore, BRAF35 is a component of at least two complexes displaying different molecular sizes.

To determine whether BRAF35 can directly interact with BRCA2, we performed protein–protein interaction studies using GST-BRCA2 constructs spanning the open reading frame (approximately 5 μg of each GST-construct was used, Figure 3b). This analysis revealed a specific interaction of BRAF35 with a fragment of BRCA2 spanning the amino acids 1648–2190 contained within BRC6 through 8 of BRCA2 (Figure 3b).

To address whether cancer-causing mutations in BRCA2 disrupt the BRCA2/BRAF35 complex, we analyzed the nuclear extract from CAPAN-1 cells by Su-

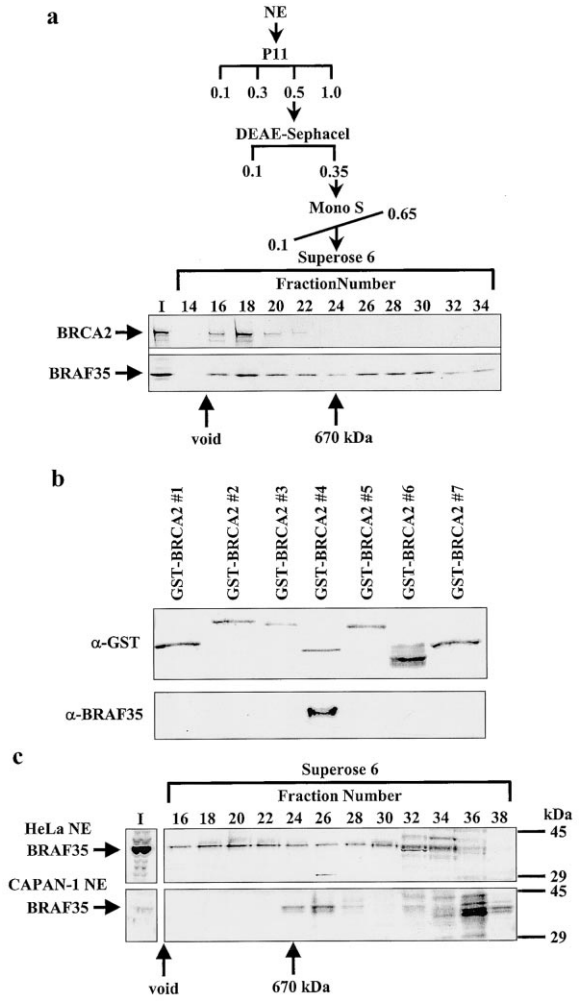

Figure 3. BRAF35 Is a Component of Two Complexes and Can Directly Interact with BRCA2

(a) HeLa nuclear extract was fractionated by chromatography as indicated. The horizontal and diagonal lines indicate stepwise and gradient elution, respectively, with KCl concentrations in molars. Fractions from the final Superose 6 column were separated in an SDS-polyacrylamide gel, and proteins were immunoblotted with BRCA2 or BRAF35 antibodies. The void and a 670 kDa standard used for column calibration are shown below.

(b) SDS-PAGE followed by Western blotting using anti-GST antibodies of purified recombinant GST-BRCA2 fusion proteins synthesized as described in Experimental Procedures. Recombinant GST-BRCA2 fusion proteins (5 μg) denoted on the top of the figure were incubated with bacterial lysate containing recombinant BRAF35 (10 μg) as outlined in Experimental Procedures. Following elution with glutathione, samples were subjected to Western blotting analysis using anti-BRAF35 antibodies.

(c) SDS-PAGE followed by Western blot analysis of BRAF35. HeLa or CAPAN-1 nuclear extract was fractionated using Superose 6 chromatography. The void and a 670 kDa standard used for column calibration are shown below. The numbers in the right denote the molecular weight markers. I denotes the input (15 μl of nuclear extract) for each column. While 15 μl was sufficient to detect BRAF35 in Superose 6 fractions derived form HeLa nuclear extract, we had to analyze 500 μl of CAPAN-1 derived fractions in order to detect BRAF35.

perose 6 gel filtration. In contrast to the 2 MDa BRAF35 complex in HeLa cells, CAPAN-1 cells contained a much smaller complex at 670 kDa (Figure 3c). Moreover, we were unable to reliably detect the truncated BRCA2 in CAPAN-1 nuclear extract, consistent with the recent report indicating that mutations in BRCA2 result in the loss of nuclear localization signal (Spain et al., 1999). These results are consistent with the disruption of 2 MDa BRCA2/BRAF35 complex in CAPAN-1 nuclear extract.

BRAF35 Displays a Pattern of Expression Similar to BRCA2

We next addressed the pattern of BRAF35 expression in tissues. Western blot analysis using anti-BRAF35 antibodies revealed the presence of BRAF35 in a wide variety of adult tissues examined with the highest levels expressed in testis and ovary (data not shown). This is consistent with the expression pattern of BRCA2 (Tavtigian et al., 1996). These results prompted us to analyze the RNA expression pattern of BRAF35 in developing mouse embryos using in situ hybridization. Marked regional differences in mouse BRCA2 expression is first seen at day E11.5 of embryonic development (Sharan et al., 1997). Thus, we compared mouse BRAF35 expression at E11.5 to that of BRCA2. As shown in Figure 4, BRAF35 displayed a nearly identical pattern of tissue expression to that of BRCA2 with highest expression observed in tissues with high mitotic index, such as the proliferating ventricular zones of the fore-, mid-, and hindbrain (Figures 4a–4d). These results are consistent with the role for the BRAF35/BRCA2 complex in cells during mitosis.

BRAF35 Decorates the Early Mitotic Chromosomes during Initiation of Mitotic Chromosome Condensation

To directly assess the role of BRAF35 in mitotic cells, we examined mitotic HeLa cells using indirect immunofluorescence. Strikingly, anti-BRAF35 antibodies stain mitotic chromosomes during early stages of chromosome condensation (Figure 5). This staining is coincident with the phosphorylation of serine 28 (Ser-28) of histone H3, known to delineate condensed mitotic chromosomes (Goto et al., 1999). Remarkably, at the onset of metaphase to anaphase transition, BRAF35 staining is no longer visible while Ser-28 phosphorylation still persists (Figure 5; early anaphase).

Since BRAF35 is a component of at least two complexes, one of which is lacking BRCA2, we analyzed the mitotic chromosomes using both polyclonal and monoclonal anti-BRAF35 and anti-BRCA2 antibodies (Figure 6). To directly analyze the chromatin-associated structures, cells were stained following extraction of the soluble material by detergent treatment (Nickerson et al., 1990). This staining was compared to staining of condensed chromosomes using antibodies against phosphorylated forms of Ser-10 (polyclonal) or Ser-28 (monoclonal) of histone H3. This analysis revealed the colocalization of BRAF35 and BRCA2 on mitotic chromosomes coinciding with histone H3 phosphorylation (Figures 6a–6d). Taken together, these results suggest a role for the BRAF35/BRCA2 complex in early phases of mitotic cell cycle progression.

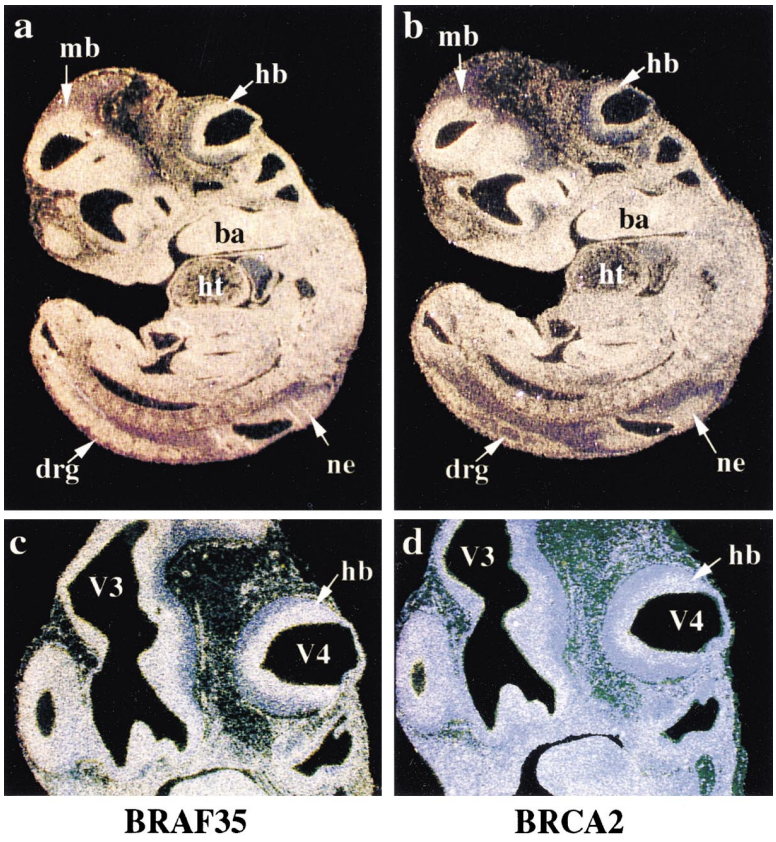

Figure 4. BRAF35 and BRCA2 Have Overlapping Expression Patterns during Development in Tissues with High Mitotic Index

In situ hybridization of sagittal sections from E11.5 mouse embryos reveals expression of both BRAF35 (a and c) and BRCA2 (b and d) in strikingly similar patterns. mRNA expression is detected by radioactive in situ hybridization and appears white. Cell nuclei are counterstained with Hoeschst 33258 nuclear dye (red). Both genes are expressed in the neuroepithelium of the neural tube (ne) and in the first and second branchial arches (ba), as well as in the ventricular zone of the neuroepithelium within the midbrain (mb) and hindbrain (hb) adjacent to the third ventricles (V3) and fourth ventricles (V4), respectively. ht = heart, drg = dorsal root ganglia.

BRAF35 Displays Specific Binding to Cruciform DNA

The condensin complex involved in chromosome condensation was shown to display high affinity for structured DNA, such as cruciform DNA (Kimura and Hirano, 1997). The localization of BRAF35 to condensed chromatin and the fact that a unique feature of HMG domain allows high affinity for DNA with sharp bends, such as four-way junction (4WJ) DNA (Bianchi et al., 1989), prompted us to examine the DNA binding activity of BRAF35 (Figure 7a). We compared the DNA binding activity of BRAF35 to that of the BAF57 HMG domain (Wang et al., 1998) (Figure 7b).

Similar to that of BAF57, recombinant BRAF35 displayed strong binding to 4WJ DNA in gel-shift assays (Figure 7c). However, in contrast to two other sequence-specific HMG domain-containing proteins, LEF1 and mTF1, BRAF35 did not display detectable DNA binding activity when either of the duplex "arms" of the 4WJ DNA were used as probes (Figure 7d, and data not shown). These results demonstrate that the DNA binding properties of BRAF35 are highly specific and similar to that of the condensin complex (Kimura and Hirano, 1997).

Microinjection of Anti-BRCA2 or Anti-BRAF35 Antibodies into Synchronized HeLa Cells Causes a G2 Cell Cycle Delay

To functionally assess the role of the BRCA2/BRAF35 complex in cell cycle progression, synchronized HeLa cell nuclei (double thymidine block) were injected with either affinity-purified anti-BRAF35 or anti-BRCA2 antibodies and injected cells were analyzed 12 or 14 hr following release from the block (Figure 8a). Cells were stained to identify the injected antibodies and counterstained with anti-human CENP-F antibodies (Liao et al., 1995). CENP-F is a reliable marker for identifying cells in G2 because it is only detected after cells have completed S phase. S phase cells that incorporate BrdU are CENP-F negative while cells that have completed S phase and do not incorporate BrdU are CENP-F positive (T. Yen personal communication). Mitotic and newly divided cells (telophase/early G1) were visually identified and counted. Following release from the block, the IgG injected cells entered mitosis after approximately 10 hr and exited after 12 hr (Figure 8b). In contrast to IgG injected cells, injection of anti-BRCA2 or anti-BRAF35 antibodies results in a pronounced delay in entry into mitosis as evident by a 3-fold increase in CENP-F (G2)-positive cells 12 hr after release from the thymidine block (Figures 8b and 8c). Analysis of anti-BRCA2 or anti-BRAF35 injected cells at 14 hr after release indicated that more of the injected cells were able to enter mitosis, although a significant number were still delayed in G2 (Figure 8b). Importantly, analysis of cells injected with antibodies against various proteins that are important for mitotic cell cycle progression including CENP-E, hBUBR1, hZW10, hROD, and CENP-F failed to display a delay in entry into mitosis attesting to the specificity of anti-BRCA2 and anti-BRAF35 antibodies (data not shown).

Interestingly, following injection with anti-BRAF35,

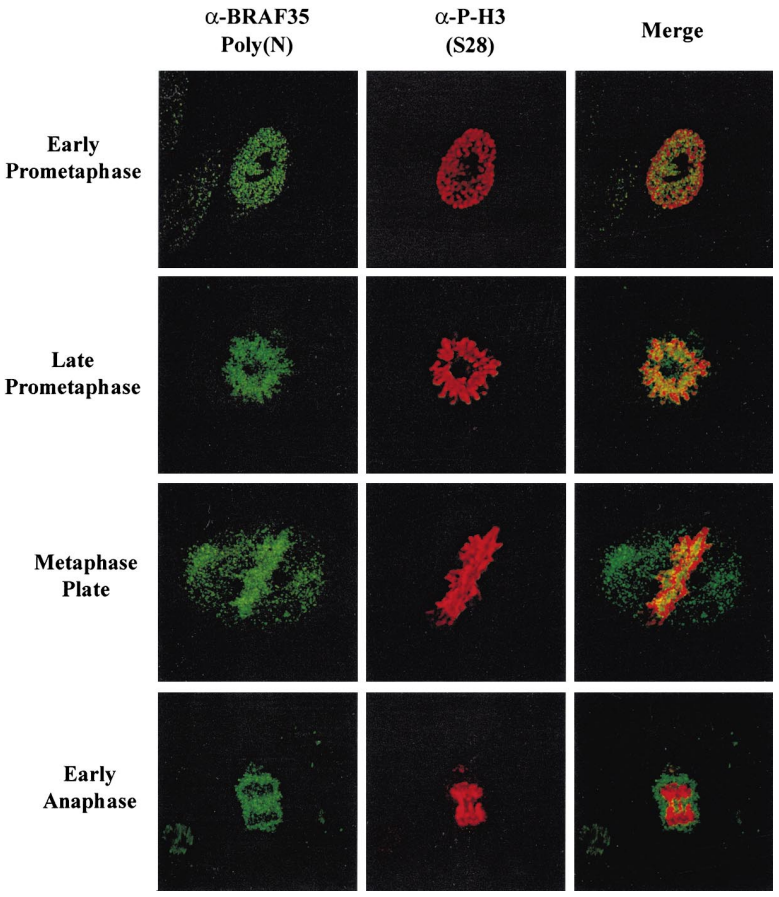

Figure 5. BRAF35 Delineates Condensed Chromosomes during Prophase to Metaphase Transition

Hela cells were stained using anti-BRAF35 polyclonal antibodies raised against the first twenty amino acids of BRAF35 (α-BRAF35 poly (N)) according to the protocol outlined in Experimental Procedures. Mitotic cells were counterstained with the antibody against the histone H3 phosphorylated at serine 28 (α-P-H3 (S28)).

anti-BRCA2, or anti-IgG antibodies, analysis of G2 cells using anti-phosphorylated histone H3 antibodies (identifies cells in initial phases of chromatin condensation) revealed a similar percentage of cells (approximately 5%) displaying phosphorylation of Ser-28 of histone H3. These results indicate that the impediment in entry into mitosis by microinjection of anti-BRCA2 and anti-BRAF35 antibodies is likely due to events that occur following Ser-28 phosphorylation. Taken together, these results suggest a role for BRCA2/BRAF35 complex in the timely progression through mitosis.

Discussion

The novelty of this work lies in the following. First, it demonstrates the presence of BRCA2 in a 2 MDa multiprotein complex. Second, through characterization of BRAF35, a DNA binding component of the complex has been identified. Third, it establishes BRAF35 as an architectural DNA binding protein capable of binding to cruciform DNA. Fourth, it reveals an intimate association of BRA35 and BRCA2 with chromatin during early phases of mitotic chromosome condensation. Finally, through antibody microinjection experiments, it suggests a role for the complex in regulation of cell cycle progression.

Chromosome condensation is a cellular process in which entangled chromatin fibers are resolved and packaged into physically separate compact structures, the mitotic chromosomes. This process is prerequisite for the subsequent segregation of chromosomes in anaphase and is essential for maintaining the integrity of genetic information throughout mitosis. Recent genetic and biochemical studies in eukaryotes have pointed to two distinct complexes, condensin and cohesin, as playing a central role in chromosome condensation and sister-chromatid cohesion, respectively (Hirano, 1999). The emerging theme from these studies is that such regulatory complexes are not only involved in chromosome segregation but also play a fundamental role in DNA repair and dosage compensation (Chuang et al., 1994; Hirano, 1999). Similar to such regulatory complexes, the BRAF35/BRCA2 complex not only confers a regulatory role in progression through mitosis but also has been reported to be involved in DNA repair (Mizuta et al., 1997; Wong et al., 1997; Chen et al., 1998; Marmorstein et al., 1998), consistent with a dual role for this complex in modulation of DNA repair and cell cycle regulation.

It is also conceivable that the BRAC2/BRAF35 complex modulates the components of the chromosome condensation/segregation machinery once recruited to the mitotic chromosome. Indeed, the fact that at the onset of metaphase to anaphase transition, the BRAF35/BRCA2 complex can no longer be detected at the mitotic chromosomes suggests a role for the complex in the early phases of chromosome condensation. Furthermore, the spontaneous accumulation of chromosomal abnormalities, including breaks and aberrant chromatid exchanges, observed in cells from BRCA2-deficient

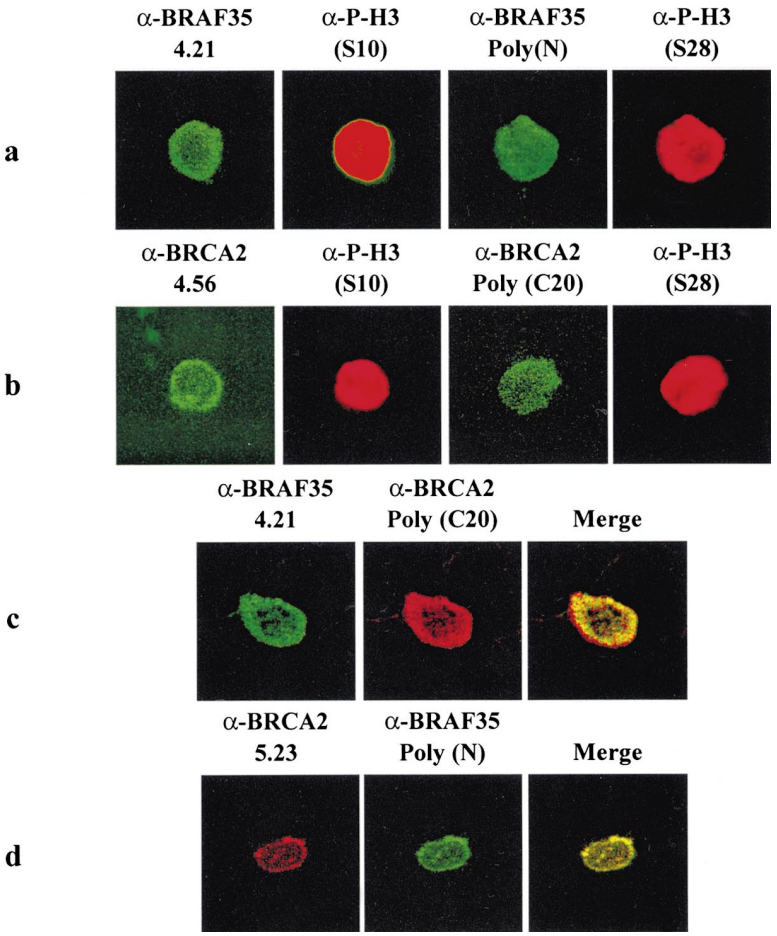

Figure 6. BRAF35 and BRCA2 Are Localized to Condensed Chromosomes

(a and b) HeLa cells were stained with monoclonal and polyclonal anti-BRCA2 and anti-BRAF35 antibodies following the protocol outlined in Experimental Procedures. Mitotic cells were counterstained with either monoclonal antibodies against phosphorylated form of Ser-28 of histone H3 or polyclonal antibodies against the phosphorylated form of Ser-10 of histone H3.

(c and d) Mitotic chromosomes were stained with monoclonal and polyclonal anti-BRAF35 or anti-BRCA2. Merge denotes the overlay of green and red signals resulting in the appearance of yellow.

mouse (Yu et al., 2000) are reminiscent of a role for the complex in chromosome segregation.

The identification of BRAF35 as a structural DNA binding component of the BRCA2 complex also suggests a role for this complex in association with sites of unusual DNA architectures such as the ones formed during DNA recombinational repair. Such recombinational events are frequently required during the cell cycle progression (Aguilera et al., 2000). Our results also extend the proposed role for BRCA2 in DNA repair and recombination by identifying BRAF35 as a component of the complex that might serve to target it to sites of DNA damage. It is noteworthy that the human BRAF35 gene maps to chromosome sub-band 19p13.3. As loss of heterozygosity at 19p13.3 has been reported in about 50% of ovarian cancers (Jenkins et al., 1993; Wang et al., 1999), BRAF35 constitutes a candidate tumor suppressor gene in such cancers.

Experimental Procedures

Affinity Purification of BRCA2 from Nuclear or S100 Extract

Affinity purification of BRCA2-associated polypeptides was performed using either mono- or polyclonal anti-BRCA2 antibodies (Marmorstein et al., 1998). Antibodies were coupled to 1 ml protein A agarose beads (Repligen) as described (Harlow and Lane, 1988). The resin was incubated overnight at 4°C with 10 mg HeLa DEAE-Sephacel precleared with 1 ml protein A beads. Binding was carried out in NEBB buffer (250 mM NaCl, 30 mM Tris 7.6, 10% glycerol, 0.1 mM EDTA, 5 mM BME, 0.2 mM PMSF, 0.1% NP-40), then the support was washed successively with NEBB buffer containing 500 mM NaCl, 0.1% NP-40; 500 mM NaCl, 0.5% NP-40; 1.0 M NaCl, 0.1% NP-40; and 20 mM NaCl, 1.0% NP-40. The washed beads were then eluted with three column volumes of 100 mM glycine, pH 3.0.

Conventional Chromatographic Purification of the BRCA2 Complex

The BRCA2 complex was purified from 2 g of HeLa nuclear extract (Figure 2a). Nuclear extract was loaded on a 250 ml column of phosphocellulose (P11, Whatman) and fractionated stepwise by the indicated KCl concentrations in buffer A (20 mM Tris.HCl, pH 7.9, 0.2 mM EDTA, 10 mM βME, 10% glycerol, 0.2 mM PMSF). The P11 0.5 M KCl fraction (250 mg) was loaded on a 45 ml DEAE-Sephacel column (Pharmacia) and eluted with 0.35 M KCl. The 0.35 M KCl elution (140 mg) was dialyzed to 700 mM NH_4SO_4 in buffer HB (20 mM Hepes, pH 7.6, 4 mM dithiothreitol, 0.5 mM EDTA, 10% glycerol, 0.5 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin) and loaded on a Butyl Superose (Pharmacia). The column was resolved using a linear 10 column volume gradient of 700 to 0 mM NH_4SO_4 in buffer HB. BRCA2-containing fractions were dialyzed to 10 mM K_xPO_4 in buffer HA (5 mM Hepes, pH 7.6, 1 mM dithiothreitol, 0.5 mM PMSF, 10 μM $CaCl_2$, 10% glycerol, 40 mM KCl, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin) and loaded on a BioScale CHT5-I column (BioRad). The column was resolved using a linear 15 column volume gradient of 10 to 600 mM K_xPO_4 in buffer HA. Fractions containing BRCA2 were dialyzed to 100 mM KCl in buffer A containing 1 μg/ml aprotinin, leupeptin, and pepstatin, and loaded on Heparine-5PW (TosoHaas). The column was resolved using a linear 20 column volume gradient of 100 to 450 mM KCl in buffer A containing 1 μg/ml aprotinin, leupeptin, and

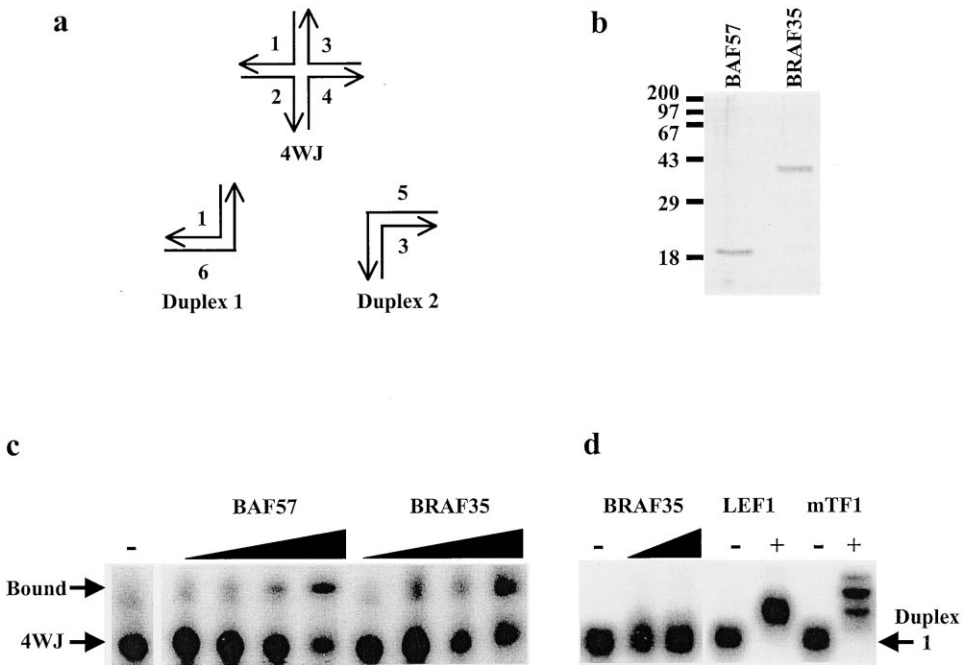

Figure 7. BRAF35 Can Specifically Bind to Cruciform DNA

(a) Diagram of the structure of the 4WJ DNA and the two duplex DNA that correspond to its "arms."

(b) The full-length BRAF35 and truncated BAF57 proteins were purified from *E. coli* and analyzed on a *Coomassie* blue-stained SDS-gel (10%).

(c) The DNA binding activities of BRAF35 and truncated BAF57 were analyzed by gel mobility assays using 4WJ DNA as the probe. The protein concentrations were 0.1, 0.3, 1.0, and 1.5 μg, respectively.

(d) The DNA binding activity of BRAF35 was compared to that of LEF1 and mTF1 proteins using the duplex "arms" of 4WJ DNA as probes. The protein concentration was 0.3 and 1.0 μg for BRAF35 and 1.0 μg for LEF1 and mTF1.

pepstatin. Fractions containing BRCA2 were dialyzed to 100 mM KCl in buffer A containing 1 μg/ml aprotinin, leupeptin, and pepstatin, and loaded on DEAE-5PW (TosoHaas). The column was resolved using a linear 15 column volume gradient of 100 to 500 mM KCl in buffer A containing 1 μg/ml aprotinin, leupeptin, and pepstatin. BRCA2-containing fractions were fractionated on a Superose 6 HR 10/30 (Pharmacia) equilibrated in 0.7 M KCl in buffer A containing 0.1% NP-40, and 1 μg/ml aprotinin, leupeptin, and pepstatin.

For analysis of multiple complexes, BRCA2 was purified from 1.2 g of HeLa nuclear extract as illustrated in Figure 3a. Nuclear extract was loaded on a 200 ml column of phosphocellulose (P11, Whatman) and fractionated stepwise by the indicated KCl concentrations in buffer A (20 mM Tris.HCl, pH 7.9, 0.2 mM EDTA, 10 mM βME, 10% glycerol, 0.2 mM PMSF). The P11 0.5 M KCl fraction (200 mg) was loaded on a 20 ml DEAE-Sephacel column (Pharmacia) and eluted with 0.35 M KCl. A portion of the 0.35 M KCl elution (10 mg) was dialyzed to 100 mM KCl in buffer A and loaded on a MonoS 5/5 column (Pharmacia). The column was resolved using a linear 10 column volume gradient of 100 to 650 mM KCl. Fractions containing BRCA2 (500 mM KCl, 0.5 mg) were dialyzed to 700 mM KCl, 0.1% NP-40 in buffer A and loaded on a Superose 6 HR 10/30 (Pharmacia) equilibrated in the same buffer containing 1 μg/ml aprotinin, leupeptin, and pepstatin.

Gel-Shift Assays

The 4WJ DNA and its duplex DNA "arms" were prepared according to Bianchi (Bianchi et al., 1989). Binding reactions were performed as described (West and Austin, 1999). Full-length BRAF35 was prepared similar to that described for BAF57 (Wang et al., 1998).

In Situ Hybridization

Radioactive in situ hybridization was performed as described (Lutz et al., 1994) using antisense probes prepared from EST AA007769 (BRAF35) and AA571559 (BRCA2) and 8 μM sections of paraffin-embedded embryos after fixation in 4% paraformaldehyde and dehydration. Slides were coated with photographic emulsion, exposed for 5–10 days, counterstained with Hoechst 33258 nuclear dye and photographed using darkfield and fluorescent illumination. Figures were processed using Adobe Photoshop software.

Immunoprecipitation and Immunofluorescence

Immunoprecipitation experiments were performed as described (Bochar et al., 2000a, 2000b). Briefly, HeLa or Capan-1 nuclear extract (500 μg) was incubated with different antibodies (2–5 μg) bound to protein A beads (10 μl of packed beads, Repligen). The beads were then washed three times with a buffer containing 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.05% NP-40. Following centrifugation, beads were resuspended in 20 μl of protein sample buffer and boiled for 5 min. Eluates were separated by SDS-PAGE and transferred onto a polycvinylidence difluoride membrane (Millipore) for Western blotting.

Immunofluorescence experiments were performed using monoclonal and polyclonal antibodies against BRCA2 and BRAF35 as described (Yarden and Brody, 1999). For experiments shown in Figure 6, cells grown on coverslips were washed with ice-cold PBS twice, then treated with ice-cold cytoskeleton buffer containing: 10 mM PIPES pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM EGTA, 0.5% triton X-100. After 5 min on ice, cells were aspirated and were treated with ice-cold stripping buffer containing: 10 mM Tris-HCl pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 1% Tween 40 (v/v), 0.5% sodium deoxycholate (v/v) for 5 min. Cells were then washed three times with ice-cold PBS and fixed in modified Streck tissue fixative (150 mM 2-bromo-2-nitro-1,3-propanediol (Sigma), 108 mM diazolidinyl urea (Sigma), 10 mM Na Citrate, 50 mM EDTA pH 5.7) for 30 min at room temperature. Following fixation, cells are washed in PBS and permeabilized for 15 min using 100 mM Tris-HCl pH 7.4, 50 mM EDTA and 0.5% Triton X-100. Cells are then blocked using 10% FCS in PBS and 0.1% azide and incubated with the primary and secondary antibodies

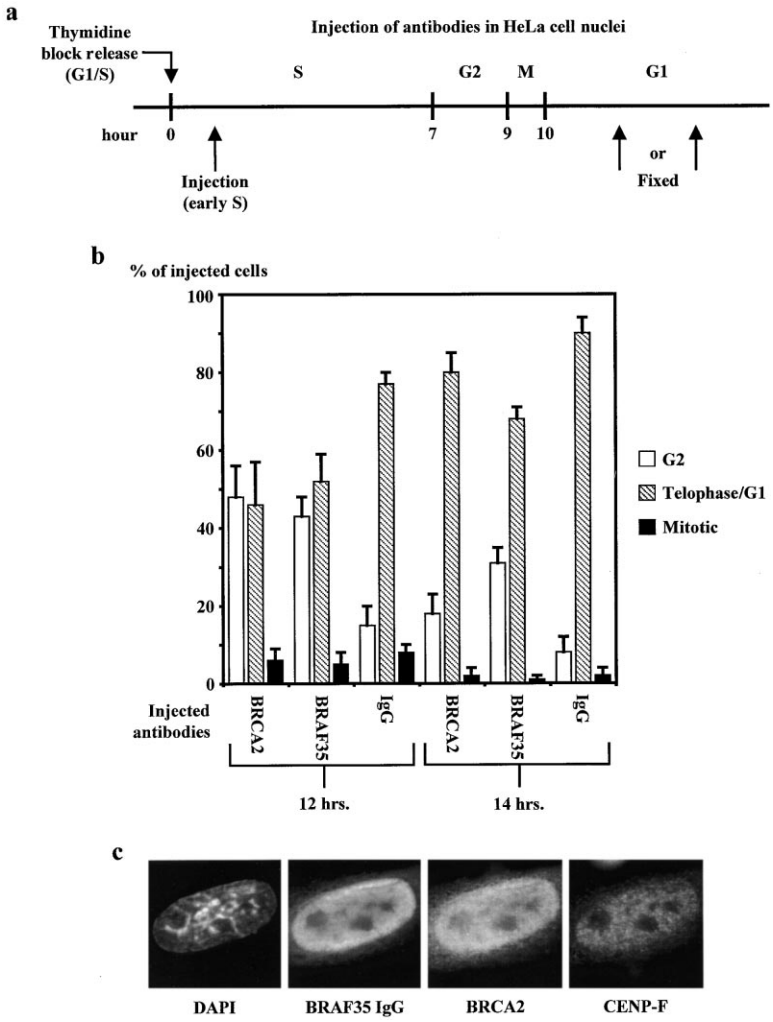

Figure 8. Microinjection of Antibodies against BRAF35 or BRCA2 into Synchronized HeLa Cells Delays Entry into Mitosis

(a) Diagram of microinjection experiments. HeLa cells were synchronized by double thymidine block and released. Anti-BRAF35, anti-BRCA2, or preimmune IgG was injected into the nuclei at S phase, and cells were allowed to proceed through mitosis.

(b) The percent of cells in different stages of the cell cycle following microinjection of anti-BRAF35, anti-BRCA2, or preimmune anti-IgG. The cells were analyzed at 12 and 14 hr following release. Approximately 450 cells were microinjected in four independent experiments.

(c) A typical microinjected cell displaying G2 delay following microinjection of anti-BRAF35 antibody. Each cell was also stained for DAPI, BRCA2, and CENP-F.

Monoclonal anti-BRCA2 antibodies (4.56 and 5.23) were raised against a BRCA2 fusion protein encoding amino acids 2959–3418. We also developed monoclonal antibodies (4.21) to a BRAF35 fusion protein (amino acids 103–317) using The Wistar Institute Hybridoma Facility. Polyclonal antibodies were also developed against the last twenty amino acids of BRCA2 (C20) and BRAF35 and the first twenty amino acid of BRAF35 (Poly (N)). All polyclonal antibodies were affinity purified using the appropriate peptides. Antibodies against phosphorylated S10 and S28 were purchased through UBI and Sigma, respectively.

GST-BRCA2 Fusion Proteins and GST Pull-Down Experiments

GST-BRCA2 fusion protein constructs were a gift from F. J. Couch. GST-BRCA2 fusion proteins #1 to #7 correspond to amino acids 195–784, 705–1217, 1171–1658, 1648–2190, 2114–2608, 2634–2999, and 2959–3418, respectively. GST and GST-BRCA2 (pGEX, Amersham Pharmacia) fusion proteins were expressed in *E. coli* BL21. Cells were harvested and lysed by sonicating in 150 mM NaCl in Buffer G (50 mM Tris, pH 8.0, 10% glycerol, 0.5% Triton X-100, 0.5 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin). The cell lysates were cleared by centrifugation (105,000 × g for 60 min at 4°C). Concentration of GST or GST-BRCA2 fusion proteins in cell lysates was determined by estimation of protein concentration by Coomassie staining of SDS-PAGE samples purified by affinity chromatography on glutathione-Sepharose (Amersham Pharmacia). Cell lysates containing approximately 5 μg of GST-BRCA2 fusion protein were mixed with 10 μl glutathione-Sepharose, incubated for 3 hr at 4°C, and washed three times each with 150 mM NaCl, 500 mM NaCl, and 150 mM NaCl in Buffer G. Approximately 10 μg of overexpressed *E.coli* BRAF35 (pET-28b, Novagen) lysate was added and beads were incubated for 3 hr at 4°C. Beads were washed two times each with 300 mM NaCl, and 150 mM NaCl in Buffer G, and eluted with 30 mM glutathione plus 150 mM NaCl in Buffer G. Samples were subjected to SDS-PAGE followed by Western blotting to determine the presence of BRAF35.

Antibody Microinjection Experiments

Hela cells plated on No. 1 coverslips were synchronized by a double thymidine block. Rabbit anti-BRCA2, BRAF35, and nonimmune antibodies were concentrated to ~5 mg/ml in PBS and injected into nuclei of cells 30 to 60 min following release form the block using an Eppendorf semi-automated microinjector and manipulator mounted on a Nikon TE300 inverted microscope. Samples were taken at approximately 12 to 14 hr after release from the G1/S boundary when neighboring uninjected cells or cells injected with nonimmune IgG had mostly exited mitosis. Cells were fixed in 3.7% paraformaldehyde/PBS for 8 min, permeabilized in 0.2% Triton X-100/PBS/0.1% BSA for 5 min, washed in PBS/0.1% BSA, and stained. Injected antibodies were detected with FITC anti-rabbit secondary antibodies (Jackson Immunoresearch). Cells were also costained with VD human autoimmune serum to detect CENP-F (Rattner et al., 1993), rat anti-phospho Ser-10 H3, and visualized with Cy5 conjugated anti-human (Jackson Immunoresearch) and Alexa594 conjugated anti-rat secondary antibodies (Molecular Probes), respectively. Coverslips were visualized with a Nikon Microphot upright microscope with a 100× objective and images were captured with an 8 bit CCD camera driven by Signal Analytics image processing program.

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