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INTRODUCTION

Germ line mutations in *BRCA1* confer elevated risks in the development of familial breast and ovarian cancers (1) (2). *BRCA1* encodes a 1863-amino acid protein with a highly conserved RING finger domain at the amino terminus and two BRCT repeats at the extreme carboxyl terminus. While most disease-associated mutations of *BRCA1* are predicted to result in gross truncation of the protein, 5-10% of the cancer-predisposing mutations cause single amino acid substitutions (3), many of which are located in the RING domain or BRCT repeats. While it is generally assumed that both types of mutations lead to loss of the biological functions of the protein, several genotype-phenotype correlation studies suggest that *BRCA1* mutations at different locations of the gene may confer different *BRCA1*-dependent cancer risks (4) (5).

Intense research in the past several years has implicated *BRCA1* in regulation of multiple nuclear processes including DNA repair and transcription (6) (7) (8) (2) (9) (10). For example, *BRCA1*-deficient mouse and human cells are hypersensitive to ionizing radiation, due to defects in transcription-coupled repair of oxidative DNA damage, as well as double strand break-induced homologous recombination (11) (12) (13) (14) (15). In addition, *BRCA1* associates with several repair and recombination proteins such as *RAD51* (16), *RAD50/MRE11/NBS1* (17) (18), and *MSH2/MSH6* (18). *BRCA1* also interacts with and is phosphorylated by protein kinases that are key players in the damage checkpoint control, including *ATM*, *ATR*, and *CHK2* (19) (20) (21). Lastly, it has been shown recently that *BRCA1* preferentially binds to branched DNA structures (22). Despite these findings, the exact mechanism that *BRCA1* uses to facilitate the repair process remains to be understood.

In addition to its potential role in DNA repair, *BRCA1* has also been implicated in regulation of transcription (7) (23). When tethered to a transcriptional promoter via a heterologous DNA binding domain, the C-terminal 304-amino-acid region including the BRCT repeats (aa 1560-1863; AD2 in Fig. 1B) can act as a *trans*-activation domain (24) (25). More recent work has revealed a second *trans*-activation domain of *BRCA1* that resides upstream of the BRCT repeats (26) (aa1293-1559; AD1 in Fig. 1B). The two activation domains (AD1 and AD2) can cooperatively activate transcription in many cell lines tested (26). Consistent with its potential role in transcriptional regulation, the *BRCA1* polypeptide is associated with the RNA polymerase II holoenzyme via RNA helicase A (*RHA*) (27) (28). Furthermore, *BRCA1* interacts with a number of site-specific transcription factors and modulates their actions in gene activation (29) (30) (31) (32) (33) (34) (35) (36) (37) (38).

The multifunctional nature of *BRCA1* has raised the possibility that the protein may employ a common mechanism, such as chromatin remodeling, to regulate various chromosomal events. Indeed, the C-terminal region of *BRCA1* (AD2), which is required for *BRCA1* functions in both DNA repair and transcription, can induce changes in nucleosome structure when tethered to chromosomal DNA in *Saccharomyces cerevisiae* (39). Furthermore, *BRCA1* is associated with histone modifying enzymes (*p300* and *HDAC*) (28) (40) (41) and an ATP-dependent chromatin remodeling machine (*hSNF/SWI*) (42). The fact that many cancer-predisposing mutations reduce *BRCA1*'s affinity for these chromatin-modifying proteins suggests that chromatin remodeling may be an important aspect of *BRCA1*-mediated tumor suppression. However, there lacks direct evidence in mammalian cells for *BRCA1*-mediated changes in chromatin structure.

The overall objectives of the study are to explore *BRCA1*'s function in chromatin remodeling and to identify the target proteins that mediate the function of chromatin remodeling. During the second year of the study, we used a lac repressor-based system for visualizing large-scale chromatin dynamics in mammalian cells to demonstrate that *BRCA1* recruits a novel cofactor to induce decondensation of higher levels of chromatin structure. We also show that, unlike several other proteins that are capable of reorganizing large-scale chromatin structure, *BRCA1*-mediated chromatin unfolding is not associated with histone hyperacetylation. In addition, cancer-

predisposing mutations of BRCA1 display allele-specific effects on the chromatin unfolding activity. These data suggest that BRCA1-induced decondensation of higher levels of chromatin structure is an important step in regulation of multiple nuclear processes.

BODY OF THE REPORT

BRCA1-mediated large-scale chromatin decondensation in mammalian cells To assess the impact of BRCA1 on large-scale chromatin structure in mammalian cells, we made use of a Chinese hamster ovary (CHO) cell line, AO3_1, in which multiple copies of the lac operator were engineered to produce a 90 Mb heterochromatic region of the genome (43) (44). Binding of lac repressor, or its derivatives, to this chromosomal site has allowed direct visualization of large-scale chromatin dynamics. Consistent with previous findings, lac repressor-expressing cells stained with the corresponding antibody exhibited a compact nuclear dot (panel a; Fig. 1A). In contrast, expression of lac repressor fused with the full-length BRCA1 induced an irregularly shaped sub-nuclear structure in 14% of transfected cells (panel b; Fig. 1A). Such a staining pattern was not present in any of the cells expressing lac repressor alone. These results suggest that BRCA1, or a BRCA1-associated protein, can induce large-scale chromatin restructuring.

Deletion analysis showed that chromatin-unfolding activity was conferred by the last 570 amino acids of BRCA1 (aa 1293-1863; Fig. 1B). This region of BRCA1, previously designated AD for activation domain (39), consists of two subdomains that act synergistically to stimulate transcription (AD1: aa 1293-1559 and AD2: aa 1560-1863). As illustrated in Fig. 1B, AD2 contains the two BRCT repeats (BRCT1 and BRCT2). Further domain mapping indicated that AD1, BRCT1, and BRCT2 could independently induce large-scale chromatin unfolding (panel c, e, and f in Fig. 1A, and Fig. 1B). However, AD2 failed to cause obvious decondensation of high-order chromatin structure (compare panel d with e and f in Fig. 1A). As explained below, we interpret this as an indication of a negatively regulated chromatin unfolding activity associated with the AD2 region.

Our data also show that half of a BRCT repeat is sufficient for inducing chromatin unfolding (BRCT1C in Fig. 1B). While all lac-fused BRCA1 fragments were expressed at comparable levels (data not shown, but see Fig. 5B), none of the fragments upstream of AD displayed any activity in chromatin unfolding (1-324; 260-554; 554-1293 in Fig. 1B), nor did the N-terminal half of the BRCT1 repeat (BRCT1N). It is of note that AD1 often leads to a ball-shaped structure with smooth edges, whereas BRCT1 and BRCT2 tend to give rise to more extended, fiber-like structures with irregular shapes (compare panel c with e and f in Fig. 1A).

Distinction between BRCT and other well-characterized trans-activation domains in large-scale chromatin unfolding Previous study has shown that the VP16-induced chromatin unfolding is accompanied by local histone hyperacetylation, a property frequently observed for transcriptionally active or competent chromatin (44)(also see panels m-o in Fig. 2A). Here we extended the previous work by examining the *trans*-activation domains of two cellular transcription factors, E2F1 and p53. Like lac-VP16, lac-E2F1 and lac-p53 also induced significant chromatin unfolding in 60% and 45% of transfected cells, respectively (panel g and j in Fig. 2A). Furthermore, the lac-E2F1 and lac-p53-unfolded chromatin regions were enriched with hyperacetylated histone H3 and H4 (panels g-i and j-l in Fig. 2A; and data not shown). Thus, all three well-characterized *trans*-activation domains (VP16, E2F1, and p53) can simultaneously induce large-scale chromatin unfolding and histone hyperacetylation. This result provides important validation for the known function of these proteins in transcriptional activation.

The extent of chromatin decondensation induced by a single BRCT repeat is comparable to that exhibited by the potent transcriptional activation domains (compare panel a and d with g, j, and m in Fig. 2A). However, no obvious histone H3 or H4 hyperacetylation was detected in the

BRCT1- and BRCT2-unfolded chromatin regions (panels a-c and d-f in Fig. 2A). It is known that a single BRCT repeat is not sufficient to strongly activate transcription when tethered to a transcriptional promoter (24) (25)(data not shown). Therefore, unlike the first category of chromatin unfolding domains (VP16, E2F1, and p53), the BRCT-mediated large-scale chromatin decondensation appears to be independent of transcriptional activation per se and histone hyperacetylation. It has been reported that BRCA1 interacts with the chromatin remodeling complex hSWI/SNF (42). However, the BRCT-mediated chromatin unfolding observed here is unlikely to involve hSWI/SNF either, as the chromatin unfolding domains defined in the current study are distinct from the hSWI/SNF-interacting region in BRCA1 (42).

In addition to acetylation, histones are subject to other posttranslational modifications under various physiological conditions. Of particular interest, phosphorylation of H2AX, a histone H2A variant, at serine 139 (γ -H2AX) is rapidly stimulated following ionizing radiation (45). Before irradiation, a subset of γ -H2AX nuclear foci colocalize with BRCA1 foci (46). Following DNA damage, the number of both γ -H2AX and BRCA1 nuclear foci increases significantly, and furthermore, the majority of BRCA1 foci overlapped γ -H2AX foci (46). Using an antibody that specifically recognizes the phosphorylated form of H2AX (γ H2AX), we detected colocalization of the endogenous γ -H2AX with lac-BRCA1 in a sub-population (15%) of lac-BRCA1-transfected cells (panel a-c in Fig. 2B). In contrast, lac-VP16 did not display any colocalization with γ -H2AX (panel d-f in Fig. 2B). Consistent with previous reports (45) (46), ionizing radiation significantly increased the number and overall intensity of γ H2AX foci (data not shown). However, the strong γ -H2AX signal over the entire nucleus made it difficult to examine the effect of DNA damage on the colocalization between γ -H2AX and lac-BRCA1 at the lac binding sites. While the functional significance of the γ -H2AX-BRCA1 colocalization remains to be explored, the finding suggests a possible role of γ -H2AX in BRCA1-mediated chromatin unfolding.

Allele-specific effects of cancer-predisposing mutations of BRCA1 on chromatin unfolding To determine the effect of cancer-associated mutations on the BRCA1-dependent chromatin unfolding, we introduced a series of common cancer-predisposing mutations (3) into either full-length BRCA1 or AD2. Based on their behaviors in the chromatin unfolding assay, mutations were classified into three phenotypic categories. The first includes nonsense mutations resulting in truncation of the entire C-terminus (group a in Fig. 3B). Consistent with our domain mapping data, this group of mutations completely abolished the chromatin unfolding activity of the full-length BRCA1. Mutations in group b, most of which are missense ones located upstream of AD2 (e.g. C61G, S1040N, and R1347G), do not significantly affect the BRCA1-mediated chromatin unfolding. Contrary to the first two groups, mutations in the last group markedly enhanced the ability of lac-BRCA1 to induce chromatin unfolding (group c in Fig. 3B). Strikingly, all mutations in this group are located within the AD2 region. For example, A1708E, M1775R, and Y1853X led to a pronounced enlargement of the unfolded chromatin structure (compare panel b with c and d; and panel e with f-h in Fig. 3A). The same mutations also significantly increased the percentage of transfected cells that showed chromatin unfolding (Fig. 3B). For instance, 79% of the cells that expressed the M1775R mutant displayed significant chromatin unfolding, compared to 14% for the wild-type full-length protein. As discussed below, the allele-specific effects of cancer-predisposing mutations on the chromatin unfolding activity could account for the variations in clinical phenotypes associated with different BRCA1 mutations.

Identification of a novel BRCA1-interacting protein To identify co-factors that were recruited by the BRCT repeats to mediate the chromatin unfolding, we used BRCT1 as the bait in a yeast two-hybrid screen. One candidate gene, designated *COBRA1* (for cofactor of BRCA1), was

isolated from a human ovary cDNA library. It encodes a novel 580-amino acid protein rich in leucine residues (17%; Fig. 4). COBRA1 also contains three repeats of the LXXLL motif, which is often present in many transcription co-activators and responsible for mediating their ligand-dependent interactions with steroid hormone receptors (47). Database searches revealed COBRA1-related hypothetical proteins in mouse and fly, which share 96% and 51% amino acid identity with the human protein, respectively (Fig. 4). *COBRA1* mRNA is ubiquitously expressed, with enrichment in testis (data not shown). This expression profile is similar to the expression patterns of *BRCA1* (48) and *BACH1*, a recently identified gene that encodes a BRCA1-interacting protein (49).

To confirm the interaction between BRCA1 and COBRA1, a lysate of human HEK293T cells that ectopically expressed FLAG-tagged COBRA1 was immunoprecipitated with an anti-FLAG antibody, followed by immunoblotting with an anti-BRCA1 antibody. As shown in Fig. 5A, the endogenous human BRCA1 was co-precipitated in a FLAG-COBRA1-dependent manner (lane 1 and 2). As a control, addition of an excess of FLAG peptide to the immunoprecipitation reaction abolished the BRCA1 signal in the immunoprecipitate (lane 3 of Fig. 5A).

To further assess the binding specificity of COBRA1 to the BRCT1 region of BRCA1, we co-transfected HEK293T cells with FLAG-COBRA1 and lac repressor fused with various fragments of BRCA1. The cell lysates were then immunoprecipitated with the anti-FLAG antibody and subsequently immunoblotted with the anti-lac antibody. As shown in Fig. 5B, lac-BRCT1 was capable of binding to the FLAG-COBRA1 (lane 2). Consistent with their activity in chromatin unfolding, the C-terminal, but not the N-terminal half of BRCT1, interacted with COBRA1 (lanes 3 and 4). None of the BRCA1 fragments upstream of the BRCT repeat, including AD1, displayed any significant affinity for COBRA1 (lanes 5-8). Taken together, our data show that the COBRA1 binding correlates with the BRCT1-mediated large-scale chromatin unfolding.

As shown in Fig. 3, cancer-predisposing mutations in the 3' region of *BRCA1* caused significant enhancement of the chromatin unfolding activity. Intriguingly, the same mutations (A1708E, M1775R, and Y1853X) also increased the affinity for COBRA1 in the co-immunoprecipitation assay (compare lane 9 with 10-12 in Fig. 5B). A similar result was also observed in an in vitro glutathione S-transferase (GST) pull-down assay (Fig. 5C). In this case, ³⁵S-labeled in vitro translated COBRA1 was pulled down by both GST-AD and GST-AD2, but not by GST-AD1 (lane 3-5; bottom panel of Fig. 5C). Furthermore, COBRA1 displayed a higher affinity for the mutant (M1775R) than the wild-type GST-AD2 fusion (lane 5 and 6; bottom panel of Fig. 5C). As a control, we also used ³⁵S-labeled CtIP, a transcriptional corepressor that binds to the C-terminus of BRCA1 (50) (51) (52). Consistent with previous findings, CtIP binds specifically to AD2 but, unlike COBRA1, its association with AD2 is abolished by the M1775R mutation (lane 5 and 6; top panel of Fig. 5C). Thus, the same cancer-predisposing mutations exert opposite effects on BRCA1 binding to two different partners.

Involvement of COBRA1 in BRCT1-mediated chromatin unfolding To explore the role of COBRA1 in the BRCT1-mediated chromatin unfolding, we co-transfected FLAG-COBRA1 with various lac-BRCA1 fusion constructs into AO3_1 cells. As detected by confocal immunofluorescent microscopy, FLAG-COBRA1 and lac-BRCT1 colocalized in 96% of the cells that expressed both proteins (panel a-c in Fig. 6A). In contrast, we did not detect any enrichment of the FLAG-COBRA1 signal at either the BRCT2- or AD1-unfolded chromatin regions (panel d-f for BRCT2; g-i for AD1). Thus, while all three domains of BRCA1 are capable of inducing large-scale chromatin unfolding, they appear to recruit distinct co-factors to mediate this process.

Wild-type AD2, which failed to induce chromatin unfolding (Fig. 1), did not display any obvious colocalization with COBRA1 (panels j-l in Fig. 6A). However, two 3' cancer-predisposing mutations in the same context led to pronounced recruitment of COBRA1 to the

unfolded chromatin regions (panels m-o for A1708E; p-r for M1775R). Colocalization of COBRA1 and the mutant lac-AD2 fusion proteins was observed in over 90% cells that expressed both proteins. Thus, the effect of the 3' mutations on COBRA1 recruitment correlates with their stimulatory effects on chromatin unfolding.

To directly assess the impact of COBRA1 on large-scale chromatin structure, we used lac repressor to target COBRA1 to the lac binding sites in AO3_1 cells. As shown in Fig. 6B, 61% of the cells that expressed lac-COBRA1 led to a similar extent of chromatin unfolding as did lac-BRCT1 (compare panel c, d with e, f). This finding strongly implicates COBRA1 in BRCT1-mediated chromatin restructuring.

KEY RESEARCH ACCOMPLISHMENTS

- 1) Demonstration of a chromatin unfolding activity of BRCA1.
- 2) Localization of three independent chromatin unfolding domains in BRCA1.
- 3) Characterization of the effect of cancer-predisposing mutations of BRCA1 on chromatin unfolding.
- 4) Isolation of a novel human gene the product of which mediates the BRCA1 chromatin unfolding.

REPORTABLE OUTCOMES

Ye, Y., Hu, Y-H., Belmont, A., and Li, R. BRCA1-mediated high-order chromatin unfolding and its deregulation by cancer predisposing mutations. under review by *J. Cell Biol.*

CONCLUSIONS

BRCA1 has been implicated in regulation of multiple nuclear events, including transcription and DNA repair. However, its exact biochemical function remains to be elucidated. It has been suggested that BRCA1 may regulate multiple nuclear processes by modulating chromatin structure, but there lacks direct evidence for BRCA1-induced changes in chromatin structure in mammalian cells. In the current work, we utilized a unique targeting system to assess the impact of BRCA1 on the higher levels of chromatin structure in mammalian cells. Our work leads to the following three important findings of BRCA1 function in chromatin remodeling.

First, we demonstrate that BRCA1 contains multiple functional domains, including the BRCT repeats, for decondensing large-scale chromatin in mammalian cells. This unfolding activity appears to be separable from transcriptional activation and the well-characterized chromatin modification events such as histone acetylation. Therefore, the large-scale chromatin decondensation observed in our study may represent a distinct step in BRCA1-mediated reorganization of chromatin structure.

Second, we show that cancer-predisposing mutations display an allele-specific effect on the chromatin unfolding activity: mutations that result in truncation of the protein abolish chromatin unfolding, whereas mutations in the BRCT repeats significantly enhance this activity. This result provides a possible molecular explanation for the phenotype-genotype correlation among various cancer-predisposing mutations of BRCA1.

Last but not least, we identify a novel cofactor of BRCA1 that is recruited to the BRCA1-induced unfolded chromatin region and by itself sufficient to induce chromatin unfolding. An

interesting feature of this novel factor is its high affinity for those BRCA1 mutants that exhibit enhanced chromatin unfolding activity. Thus, the discovery of this BRCA1-interacting protein provides mechanistic insights into the reorganization of higher levels of chromatin structure.

REFERENCES

1. Rahman, N., and Stratton, M. R. *Annu Rev Genet* **32**, Page (1998).
2. Welcsh, P. L., and King, M.-C. *Hum. Mol. Genet.* **10**, Page (2001).
3. Core, B. C. I.
4. Gayther, S. A., Warren, W., Mazoyer, S., Russell, P. A., Harrington, P. A., Chiano, M., Seal, S., Hamoudi, R., van Rensburg, E. J., Dunning, A. M., Love, R., Evans, G., Easton, D., Clayton, D., Stratton, M. R., and Ponder, B. A. J. *Nature Genet.* **11**, Page (1995).
5. Risch, H. A., McLaughlin, J. R., Cole, D. E., Rosen, B., Bradley, L., Kwan, E., Jack, E., Vesprini, D. J., Kuperstein, G., Abrahamson, J. L., Fan, I., Wong, B., and Narod, S. A. *Am J Hum Genet* **68**, Page (2001).
6. Zhang, H., Tomblin, G., and Weber, B. L. *Cell* **92**, Page (1998).
7. Scully, R., and Livingston, D. M. *Nature* **408**, Page (2000).
8. Zheng, L., Li, S., Boyer, T. G., and Lee, W. H. *Oncogene* **19**, Page (2000).
9. Parvin, J. P. *Proc. Natl. Acad. Sci. USA* **98**, Page (2001).
10. Baer, R., and Lee, W.-H. *J. Mammary Gland Biol. Neoplasia* **3**, Page (1998).
11. Gowen, L. C., Avrutskaya, A. V., Latour, A. M., Koller, B. H., and Leadon, S. A. *Science* **281**, Page (1998).
12. Moynahan, M. E., Chiu, J. W., Koller, B. H., and Jasin, M. *Mol Cell* **4**, Page (1999).
13. Xu, X., Weaver, Z., Linke, S. P., Li, C., Gotay, J., Wang, X. W., Harris, C. C., Ried, T., and Deng, C. X. *Mol Cell* **3**, Page (1999).
14. Scully, R., Ganesan, S., Vlasakova, K., Chen, J., Socolovsky, M., and Livingston, D. M. *Mol Cell* **4**, Page (1999).
15. Abbott, D. W., Thompson, M. E., Robinson-Benion, C., Tomlinson, G., Jensen, R. A., and Holt, J. T. *J Biol Chem* **274**, Page (1999).
16. Scully, R., Chen, J., Plug, A., Xiao, Y., Weaver, D., Feunteun, J., Ashley, T., and Livingston, D. M. *Cell* **88**, Page (1997).
17. Zhong, Q., Chen, C.-F., Li, S., Chen, Y., Wang, C.-C., Xiao, J., Chen, P.-L., Sharp, Z. D., and Lee, W.-H. *Science* **285**, Page (1999).
18. Wang, Y., Cortez, D., Yazdi, P., Neff, N., Elledge, S. J., and Qin, J. *Genes & Dev.* **14**, Page (2000).
19. Cortez, D., Wang, Y., Qin, J., and Elledge, S. J. *Science* **286**, Page (1999).
20. Tibbetts, R. S., Cortez, D., Brumbaugh, K. M., Scully, R., Livingston, D., Elledge, S. J., and Abraham, R. T. *Genes & Dev.* **14**, Page (2000).
21. Lee, J.-S., K.M., C., Brown, A. L., Lee, C.-H., and Chung, J. H. *Nature* **404**, Page (2000).
22. Paull, T. T., Cortez, D., Bowers, B., Elledge, S. J., and Gellert, M. *Proc Natl Acad Sci U S A* **98**, Page (2001).
23. Monteiro, A. N. A. *TIBS* **25**, Page (2000).
24. Chapman, M. S., and Verma, I. M. *Nature* **382**, Page (1996).
25. Monteiro, A. N., August, A., and Hanafusa, H. *Proc Natl Acad Sci U S A* **93**, Page (1996).
26. Hu, Y.-F., Miyake, T., Ye, Q., and Li, R. *J. Biol. Chem.* **275**, Page (2000).
27. Scully, R., Anderson, S. F., Chao, D. M., Wei, W., Ye, L., Young, R. A., Livingston, D. M., and Parvin, J. D. *Proc Natl Acad Sci U S A* **94**, Page (1997).
28. Neish, A. S., Anderson, S. F., Schlegel, B. P., Wei, W., and Parvin, J. D. *Nucleic Acids Res* **26**, Page (1998).
29. Somasundaram, K., Zhang, H., Zeng, Y. X., Houvras, Y., Peng, Y., Wu, G. S., Licht, J. D., Weber, B. L., and El-Deiry, W. S. *Nature* **389**, Page (1997).

30. Zhang, H., Somasundaram, K., Peng, Y., Tian, H., Zhang, H., Bi, D., Weber, B. L., and El-Reiry, W. S. *Oncogene* **16**, Page (1998).
31. Ouchi, T., Monteiro, A. N., August, A., Aaronson, S. A., and Hanafusa, H. *Proc Natl Acad Sci U S A* **95**, Page (1998).
32. Harkin, D. P., Bean, J. M., Miklos, D., Song, Y.-H., Truong, V. B., Englert, C., Christians, F. C., Ellisen, L. W., Maheswaran, S., Oliner, J. D., and Haber, D. A. *Cell* **97**, Page (1999).
33. MacLachlan, T. K., Somasundaram, K., Sgagias, M., Shifman, Y., Muschel, R. J., Cowan, K. H., and El-Deiry, W. S. *J. Biol. Chem* **275**, Page (2000).
34. Wang, Q., Zhang, H., Kajino, K., and Greene, M. I. *Oncogene* **17**, Page (1998).
35. Zheng, L., Pan, H., Li, S., Flesken-Nikitin, A., Chen, P.-L., Boyer, T. G., and Lee, W.-H. *Mol. Cell* **6**, Page (2000).
36. Houvras, Y., Benezra, M., Zhang, H., Manfredi, J. J., Weber, B. L., and Licht, J. D. *J Biol Chem* **275**, Page (2000).
37. Fan, S., Wang, J., Yuan, R., Ma, Y., Meng, Q., Erdos, M. R., Pestell, R. G., Yuan, F., Auborn, K. J., Goldberg, I. D., and Rosen, E. M. *Science* **284**, Page (1999).
38. Ouchi, T., Lee, S. W., Ouchi, M., Aaronson, S. A., and Horvath, C. M. *Proc Natl Acad Sci U S A* **97**, Page (2000).
39. Hu, Y. F., Hao, Z. L., and Li, R. *Genes Dev* **13**, Page (1999).
40. Yarden, R. I., and Brody, L. C. *Proc Natl Acad Sci U S A* **96**, Page (1999).
41. Pao, G. M., Janknecht, R., Ruffner, H., Hunter, T., and Verma, I. M. *Proc Natl Acad Sci U S A* **97**, Page (2000).
42. Bochar, D. A., Wang, L., Beniya, H., Kinev, A., Xue, Y., Lane, W. S., Wang, W., Kashanchi, F., and Shiekhata, R. *Cell* **102**, Page (2000).
43. Li, G., Sudlow, G., and Belmont, A. S. *J. Cell Bio.* **140**, Page (1998).
44. Tumber, T., Sudlow, G., and Belmont, A. S. *J. Cell. Biol.* **145**, Page (1999).
45. Rogakou, E. P., Boon, C., Redon, C., and Bonner, W. M. *J. Cell Biol.* **146**, Page (1999).
46. Paull, T. T., Rogakou, E. P., Yamazaki, V., Kirchgessner, C. U., Gellet, M., and Bonner, W. M. *Curr. Biol.* **10**, Page (2000).
47. Heery, D. M., Kalkhoven, E., Hoare, S., and Parker, M. G. *Nature* **387**, Page (1997).
48. Miki, Y., Swensen, J., Shattuck-Eidens, D., Futreal, P. A., Harshman, K., Tavtigian, S., Liu, Q., Cochran, C., Bennett, L. M., Ding, W., and et al. *Science* **266**, Page (1994).
49. Cantor, S. B., Bell, D. W., Ganesan, S., Kass, E. M., Drapkin, R., Grossman, S., Wahrer, D. C. R., Sgroi, D. C., Lane, W. S., Haber, D. A., and Livingston, D. M. *Cell* **105**, Page (2001).
50. Wong, A. K., Ormonde, P. A., Pero, R., Chen, Y., Lian, L., Salada, G., Berry, S., Lawrence, Q., Dayananth, P., Ha, P., Tavtigian, S. V., Teng, D. H., and Bartel, P. L. *Oncogene* **17**, Page (1998).
51. Li, S., Chen, P. L., Subramanian, T., Chinnadurai, G., Tomlinson, G., Osborne, C. K., Sharp, Z. D., and Lee, W. H. *J Biol Chem* **274**, Page (1999).
52. Yu, X., Wu, L. C., Bowcock, A. M., Aronheim, A., and Baer, R. *J Biol Chem* **273**, Page (1998).

APPENDICES

Figure 1. BRCA1 induces large-scale chromatin decondensation. **(A)** The AO3_1 CHO cell line was transiently transfected with expression vectors for the following proteins: lac repressor (a), lac-BRCA1(b), lac-AD1(c), lac-AD2(d), lac-BRCT1(e), and lac-BRCT2(f). A polyclonal anti-lac repressor antibody (Stratagene) and a Cy3-conjugated secondary anti-rabbit IgG (Vector Laboratories) were used for immunostaining. Nuclei were visualized by DNA staining with 4',6'-diamidino-2-phenylindole (DAPI). **(B)** The ability of various BRCA1 fragments to unfold chromatin was measured by the percentage of transfected cells that displayed enlarged lac staining

and the degree of unfolding. Over 100 transfected cells were surveyed for each construct. Single, double, and triple plus signs indicate various degrees of chromatin unfolding, as exemplified by images for lac-BRCA1 (+), lac-AD1 (++), and lac-BRCT1 (+++) (Fig.1A). Also shown are schematic diagrams and amino acid coordinates for various BRCA1 fragments.

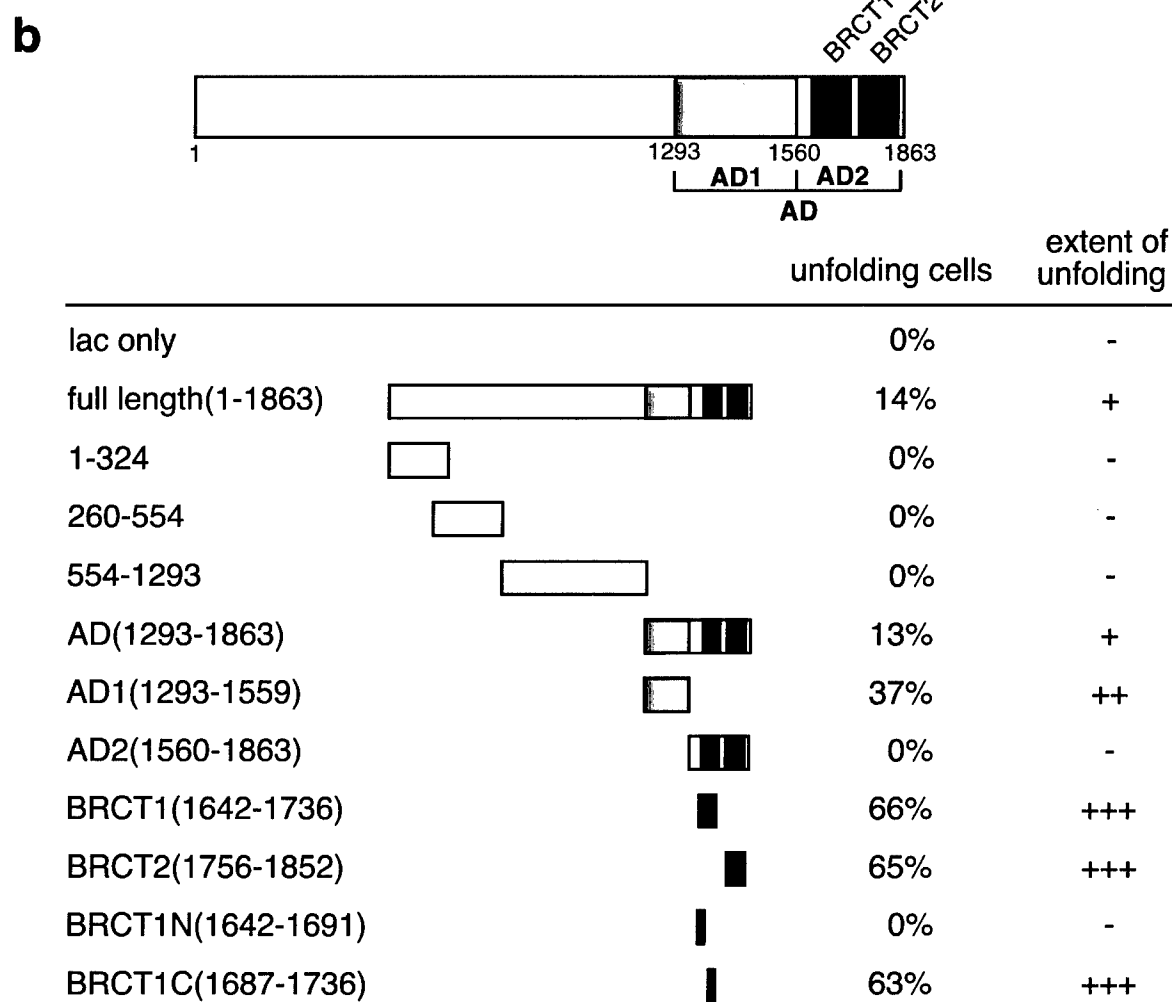
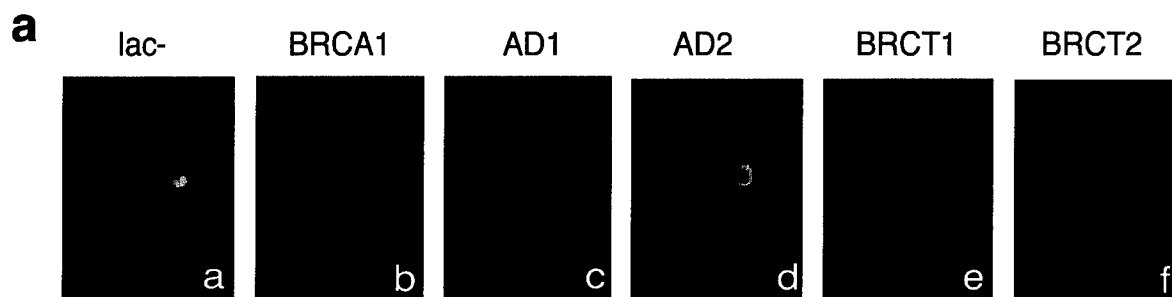
Figure 2. Comparison of chromatin unfolding by various lac fusion proteins. (A) Absence of histone hyperacetylation associated with BRCT-mediated chromatin unfolding. AO3_1 cells were transfected with the expression vectors for lac fused with BRCT1 (a-c), BRCT2 (d-f), E2F1 (g-i), p53 (j-l), or VP16 (m-o). Cells were double stained with a mouse polyclonal anti-lac antibody (1:1,000) and a rabbit anti-acetylated histone H3 antibody (1:500), followed by anti-mouse Cy3- and anti-rabbit FITC-conjugated secondary antibodies. The lac (green), acetylated histone H3 (red), and the merged images were captured by confocal immunofluorescence microscopy. (B) Association of lac-BRCA1 with phosphorylated H2AX. AO3_1 cells were transfected with the lac-BRCA1 expression vector. Cells were double stained with the mouse anti-lac antibody and a rabbit anti- γ -H2AX antibody (Upstate Biotechnology; 1:100 dilution). The images were captured by confocal immunofluorescence microscopy.

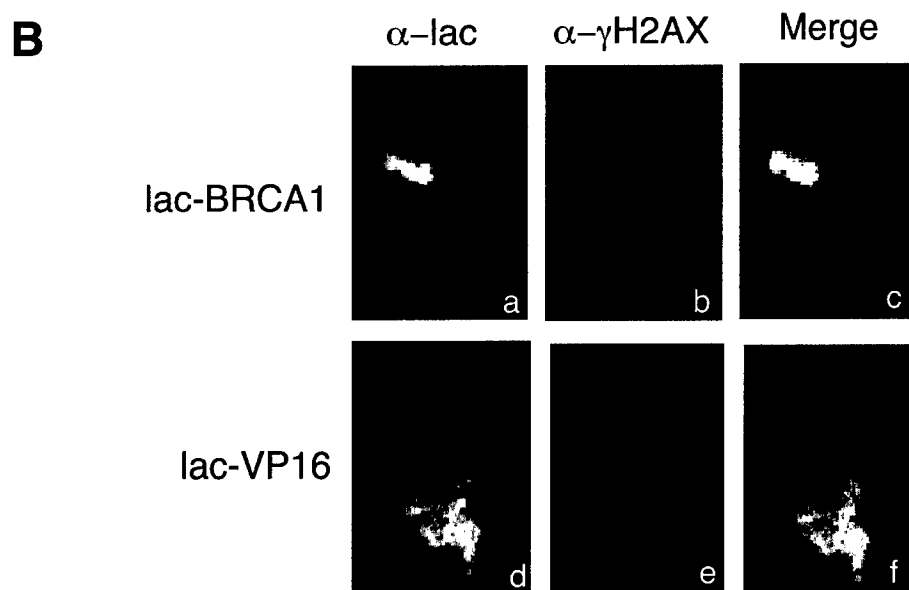
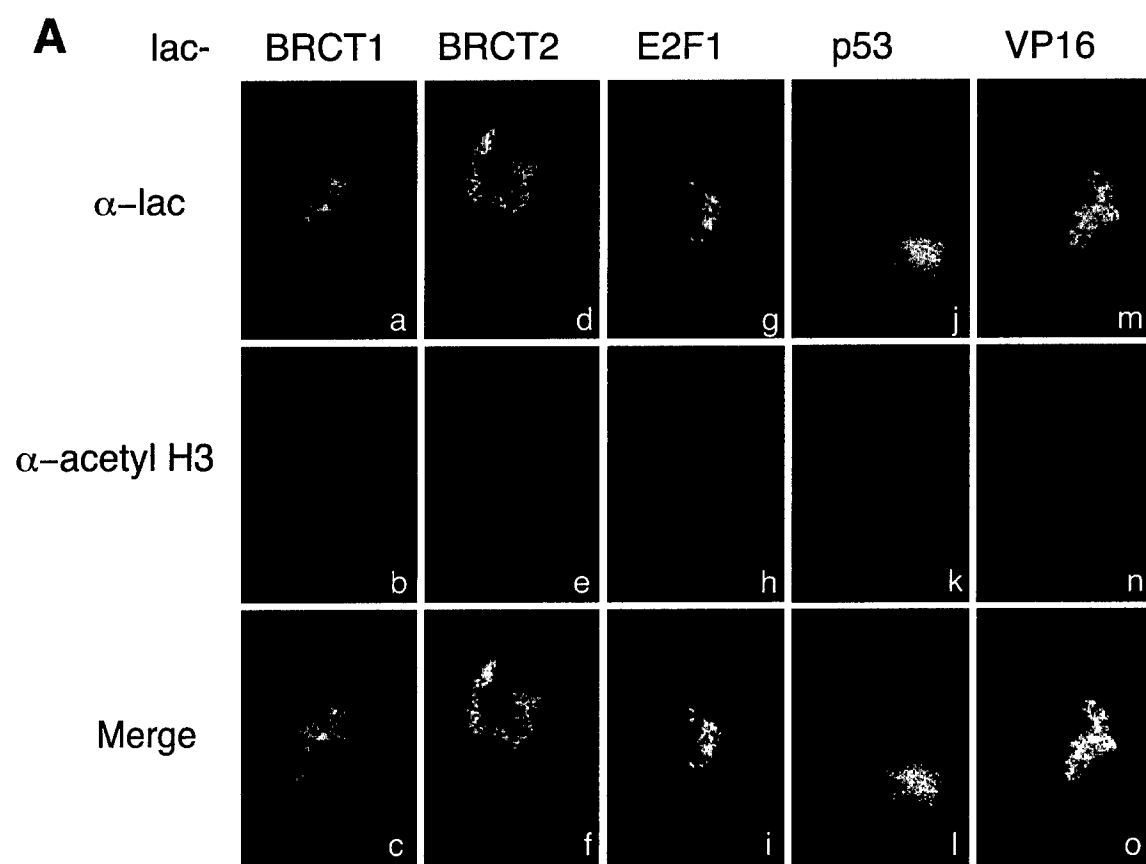
Figure 3. A subset of cancer predisposing mutations in the C terminal domain of BRCA1 cause increased chromatin unfolding (A) Cancer-predisposing mutations were introduced into either the full-length BRCA1 (panels a-d) or AD2 (panels e-h). The corresponding expression vectors were transfected into AO3_1 cells and immunostaining was performed as described in Fig.1. (B) Summary of the effects of different cancer-associated mutations on chromatin unfolding. All mutants shown in this table were tested in the context of full-length BRCA1. Locations of missense mutations are indicated by asterisks, whereas those of nonsense and frameshift mutations are indicated by wavy lines. All mutations are grouped into three (a-c) as discussed in the text.

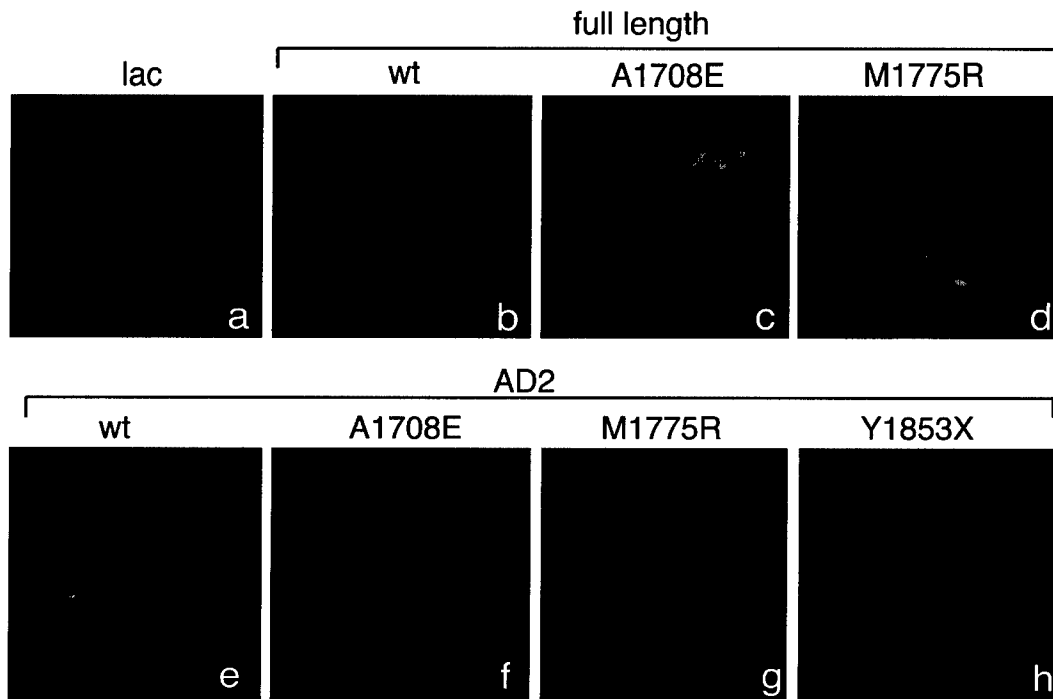
Figure 4. Sequence alignment of human COBRA1 and its homologues from mouse and fly. The conserved amino acid residues are highlighted in black, and the similar residues in gray. The locations of the LXXLL motif are indicated by asterisks.

Figure 5. Identification of COBRA1 as a novel BRCA1-interacting protein. (A). COBRA1 interacts with endogenous full-length BRCA1. Human HEK293T cells were transfected with either an empty vector (lane 1) or expression vector for the FLAG-tagged COBRA1 (F-COBRA1; lanes 2 and 3). Cell lysates were immunoprecipitated (IP) with an anti-FLAG antibody conjugated to Protein A agarose beads (Sigma), in the absence (lane 2) or presence (lane 3) of the FLAG peptide at a final concentration of 0.8 μ g/ml. The immunoprecipitates were immunoblotted (IB) with a monoclonal anti-BRCA1 antibody (AB1 from Oncogene), the results of which are shown in the top panel. As controls, the crude lysates were immunoblotted for the endogenous BRCA1 (middle panel) and the ectopically expressed FLAG-COBRA1 (bottom panel). (B) Further characterization of the interaction between BRCA1 and COBRA1. Various lac-BRCA1 fusion constructs and the FLAG-COBRA1 expression vector were co-transfected into HEK293T cells. Cell lysates were immunoprecipitated with an anti-FLAG antibody and subsequently immunoblotted with an anti-lac antibody, the results of which are shown in the top panel. Expression of the lac fusion proteins was determined by immunoblotting of the crude lysates with the anti-lac antibody (bottom panel). (C) In vitro GST pull-down assay to characterize the BRCA1-COBRA1 interaction. Various GST fusion proteins were expressed in bacteria and coupled to glutathione agarose beads (data not shown). An equal amount of the GST fusion proteins was used to pull down the 35 S labeled, in vitro translated CtIP (top panel) or COBRA1 (bottom panel).

Figure 6. COBRA1 colocalizes with lac-BRCT1 and can induce large-scale chromatin unfolding. **(A)** Co-localization of lac-BRCT1 (red) and FLAG-COBRA1 (green) at the unfolded chromatin region. AO3_1 cells were co-transfected with the expression vectors for FLAG-COBRA1 and lac fused with various fragments of BRCA1. Double immunostaining was performed using a rabbit polyclonal anti-lac and mouse monoclonal anti-FLAG antibody, following by Cy3-conjugated anti-rabbit and FITC-conjugated anti-mouse secondary antibodies. The lac (red), COBRA1 (green), and merged images were captured by confocal immunofluorescence microscopy. **(B)** COBRA1 induces chromatin unfolding when directly targeted to the chromosome. AO3_1 cells were transfected with the expression vectors for lac repressor alone (a and b), lac-BRCT1 (c and d), or lac-COBRA1 (e and f). Chromatin unfolding was detected as described in the previous figures.





a**b**

			unfolding cells	extent of unfolding
lac only			0%	-
BRCA1wt			14%	+
(a)	Q563X		0%	-
	E908X		0%	-
(b)	C61G		19%	+
	S1040N		9%	+
	R1347G		12%	+
	R1443X		14%	+
(c)	A1708E		59%	++
	1756insC		38%	+
	M1775R		79%	++
	R1835X		51%	++
	Y1853X		47%	++

hs 1 -----
 Mm 1 -----
 Dm 1 MIMSTPAKNNNGT E VNI PGQAY R A A L S D L K A I E S F L L A N S I L L P S I R P M L L L

hs 51 -----
 Mm 51 -----
 Dm 61 VR D H T I M E L I R E P L I A H I N E M G Q K - E P R E R D H I K E L I V A E P V E V K L R

hs 111 -----
 Mm 111 -----
 Dm 120 A I R N T Q I I D D Y I I I V R R E L A D T D T V F Q I R S I L F G D E V S P L L S A I R

hs 171 -----
 Mm 171 -----
 Dm 180 H I D H T N L N N L H T I V P G E V K L A N L T S F L D E N L Q F L R T L F L K T R

hs 231 -----
 Mm 231 -----
 Dm 239 A L H O L E Q E I S I F Q H E T M A L A C I R E K N I I S R E L Q G F L D N T

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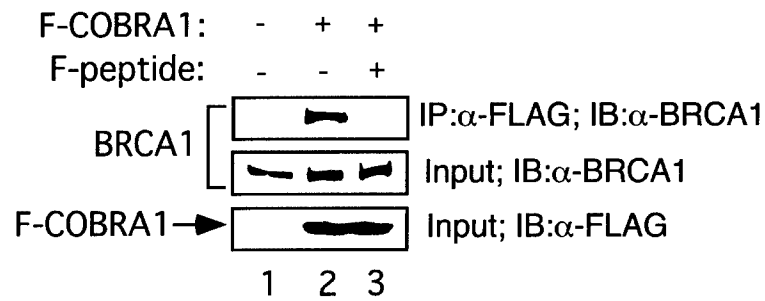
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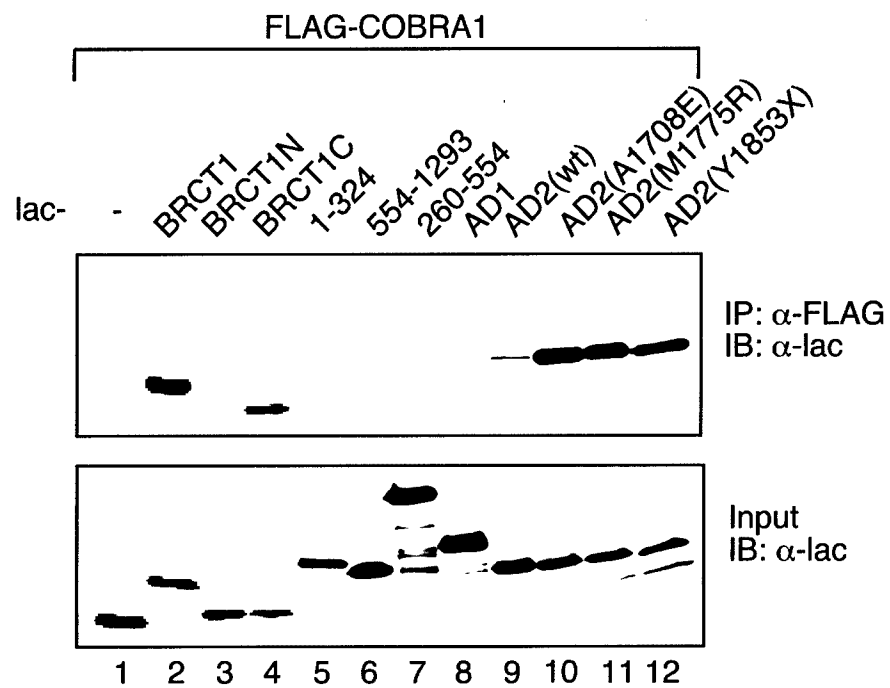
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hs 527 -----
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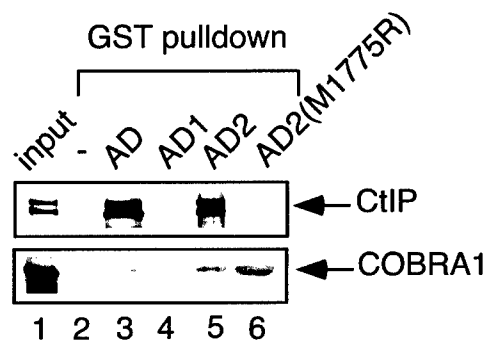
a

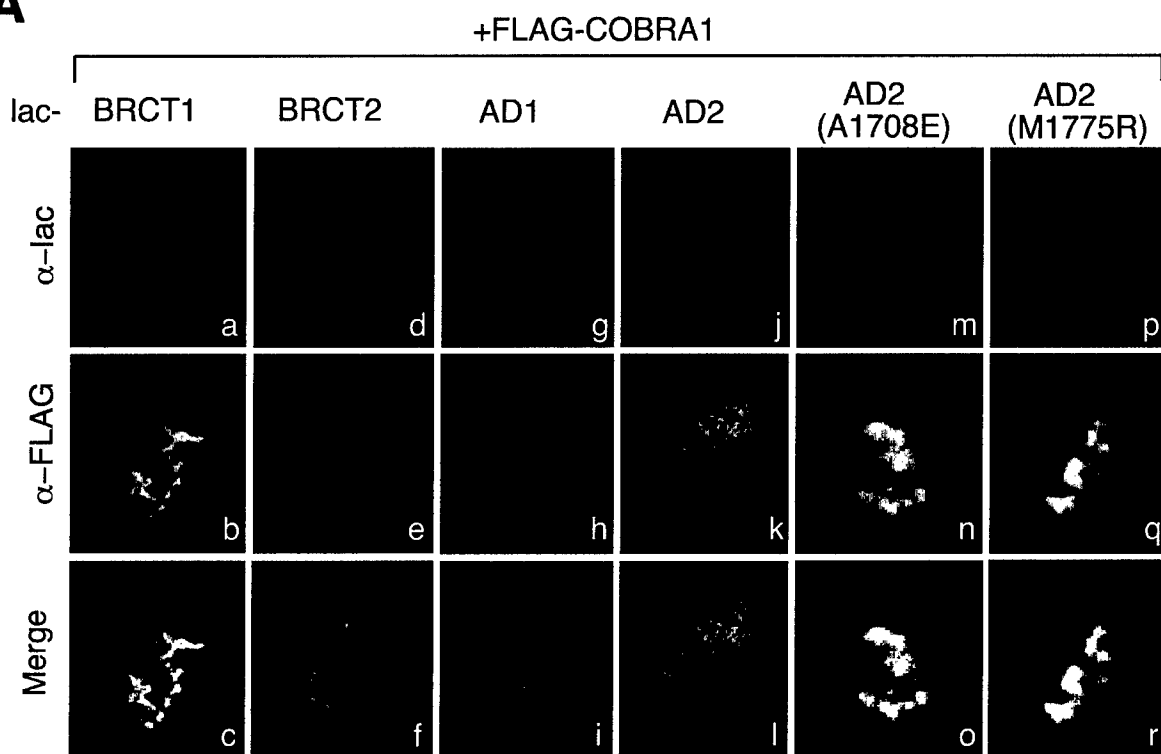
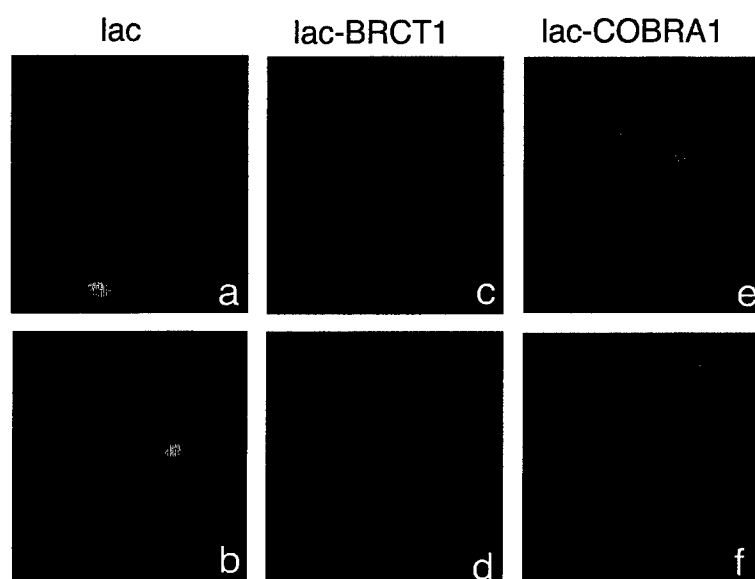


b



c



A**B**



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REPLY TO
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