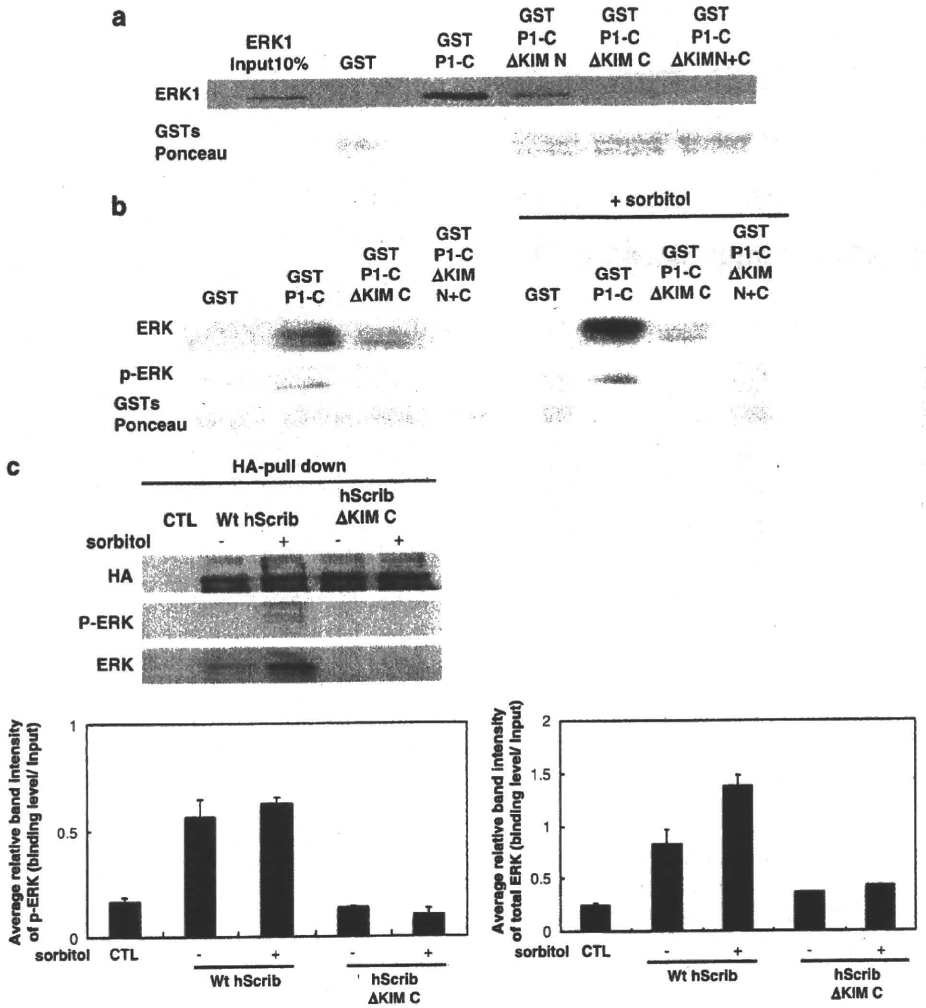

Figure 5 hScrib interacts directly with ERK through the two KIM sites. (**a**) Commercially available purified ERK1 was incubated with the GST-PDZ1-C wild-type and the ΔKIM N, ΔKIM C and the ΔKIM N + C mutants and bound ERK1 ascertained by western blotting. The lower panel shows the Ponceau stain of the nitrocellulose membrane. (**b**) HEK293 cell extracts from untreated and sorbitol-exposed cells were incubated with the GST-PDZ1-C wild-type and the two KIM site mutant GST fusion proteins (single and double) immobilized on glutathione-agarose beads. The bound proteins were analysed by western blotting with the anti-phospho ERK1/2 antibody and the anti-ERK1/2 antibody. The input GSTs are shown in the Ponceau stain of the nitrocellulose membrane. (c) HEK293 cells transfected with pcDNA3.1 (CTL), HA-hScrib or the HA-hScribΔKIM C mutant and the cells were then either incubated with or without 0.3 M sorbitol for 10 min, after which the cells were extracted and immunoprecipitated with anti-HA agarose beads. Co-immunoprecipitated proteins were then analysed by western blotting for anti HA-Scrib and anti-pERK/total ERK. The lower histogram shows the quantitative analyses of the intensities of the pERK and total ERK bindings from three independent experiments with s.d. indicated.

have numerous substrates in common, many of which are nuclear and which participate in the transcriptional regulation of a number of different cellular processes (Treisman, 1996). However, ERK1 and ERK2 are not entirely functionally redundant, and our studies confirm this as we found that hScrib S1448 is preferentially phosphorylated by ERK1. At present, we have no information as to what are the functional consequences of ERK or PKA phosphorylation of hScrib. However, we can speculate that this will most likely affect the ability of hScrib to interact with some of its cellular partners, and studies are currently in progress to investigate these aspects further.

Finally, it is worth noting that only in *Homo sapiens* is the organization of the two KIM sites and the corresponding phospho-acceptor sites perfectly well conserved in Scribble. Figure 8 shows the sequence alignment of Scribble from a number of different organisms. It can be seen from this that although the C-terminal KIM and phospho-acceptor site are well conserved among vertebrate species, the N-terminal site is somewhat divergent, whereas in lower organisms neither of the two regulatory elements seem to be conserved. This is particularly true for *Drosophila*, which has been the model organism of choice for many of the studies on hScrib, and suggests a very different

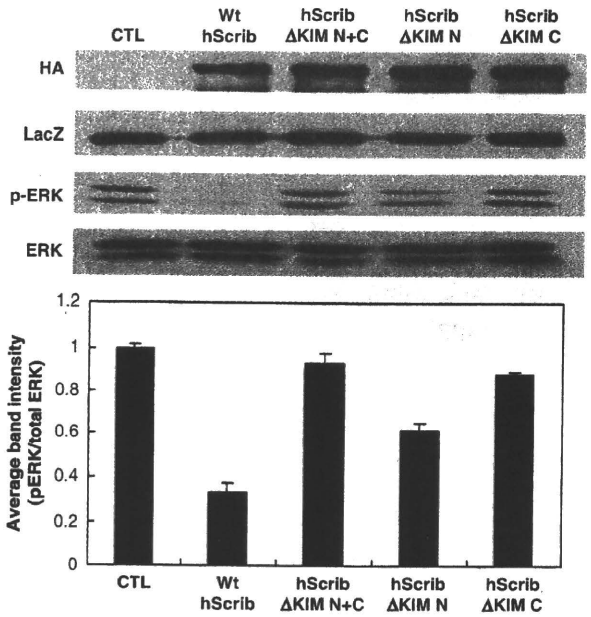

Figure 6 hScrib downregulates ERK activation through a direct interaction. HEK293 cells were transfected with pcDNA3.1 (CTL), HA-tagged wild-type hScrib, ΔKIM N + C, ΔKIM N and ΔKIM C mutants. After 24 h, the cells were harvested and the levels of ERK and phospho-ERK were analysed by western blotting. LacZ was monitored as a control for transfection efficiency. The lower panel shows the quantifications of the pERK/total ERK ratios from at least three independent experiments.

form of regulation and function of hScrib between flies and higher organisms. It is also worth noting that only in *Homo sapiens* is the potential regulatory PKA site so closely juxtaposed to the carboxy terminal ERK phospho-acceptor site, and further studies are warranted to determine whether there are any co-regulatory effects in humans of these two kinases on hScrib.

In summary, we have identified a novel regulatory mechanism by which the cell polarity regulator hScrib can directly control the MAPK signalling cascade through a direct protein interaction with ERK. These studies suggest that loss of hScrib expression, which is observed in many tumours, can directly affect continued cell proliferation and cell survival by increasing MAPK activation and nuclear translocation.

Materials and methods

Cells and treatments

HEK293 (human embryonic kidney cells), HaCaT (human keratinocyte) and BRK cells were cultured in Dulbecco's modified Eagles's medium supplemented with 10% fetal bovine serum, penicillin–streptomycin (100 U/ml) and glutamine (300 μg/ml) in a humidified 5% CO_2 incubator. Transfection was carried out using calcium phosphate precipitation as described previously (Graham and van der Eb, 1973) or using Lipofectamine 2000 (Invitrogen, Milan, Italy) according to the manufacturer's protocol. To generate the depleted Scribble cell lines, HaCaT cells were transfected using a pool of short hairpin RNA constructs against hScrib (S2, ScribC) using Lipofectamine 2000 (Invitrogen). The cells were then selected with puromycin (500 ng/ml) and after 4 weeks single colonies were analysed for hScrib expression by immunofluorescence and western blotting, and two such separate colonies (S2, ScribC) were used in this analysis. Parallel transfections and selections were performed using empty vector to generate control clones (TR) that had been subjected to the drug selection. For induction of osmotic shock, the cells were exposed to 0.3 M sorbitol for the times indicated in the text.

Cell transformation assays were performed using BRK cells obtained from 9-day-old Wistar rats with a combination of human papillomavirus-16 E7 and EJ-ras plus the appropriate hScrib expression plasmids. Cells were placed under G418 selection for 3 weeks, and then fixed and stained as described previously (Thomas *et al*., 2005).

Plasmids

The wild-type HA-tagged pcDNA hScrib expression plasmid and the truncated mutant pGEX hScrib PDZ1-C, PDZ1-4

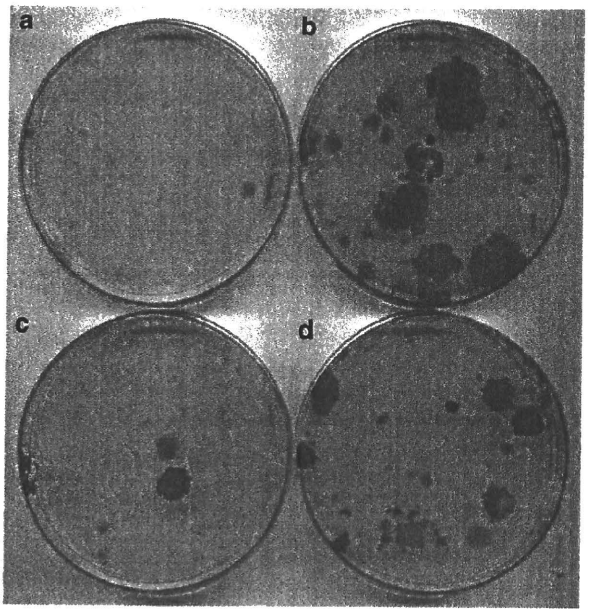

Figure 7 hScrib suppresses human papillomavirus (HPV)-16 E7 and EJ-ras oncogene cooperation in a KIM site-dependent manner. BRK cells were transfected with EJ-ras alone (**a**), HPV-16 E7 plus EJ-ras (**b**), HPV-16 E7 plus EJ-ras and wild-type hScrib (**c**) and HPV-16 E7 plus EJ-ras and the ΔKIM C hScrib mutant (**d**). After 3 weeks, the dishes were fixed and stained and the colonies counted.

Table 1 Suppression of HPV-16 E7 and EJ-ras cooperation by hScrib is KIM site-dependent

	Number of cell colonies		
	Exp 1	*Exp 2*	*Exp 3*
EJ-ras	10	0	0
EJ-ras + 16 E7	52	34	63
EJ-ras + 16 E7 + hScrib	22	11	44
EJ-ras + 16 E7 + hScrib ΔKIMC	30	33	59

Abbreviations: HPV, human papillomavirus; hScrib, human Scribble; KIM, kinase interaction motif; KIM C, KIM C-terminal.
Number of colonies obtained after 3 weeks of cultivation in three independent assays.

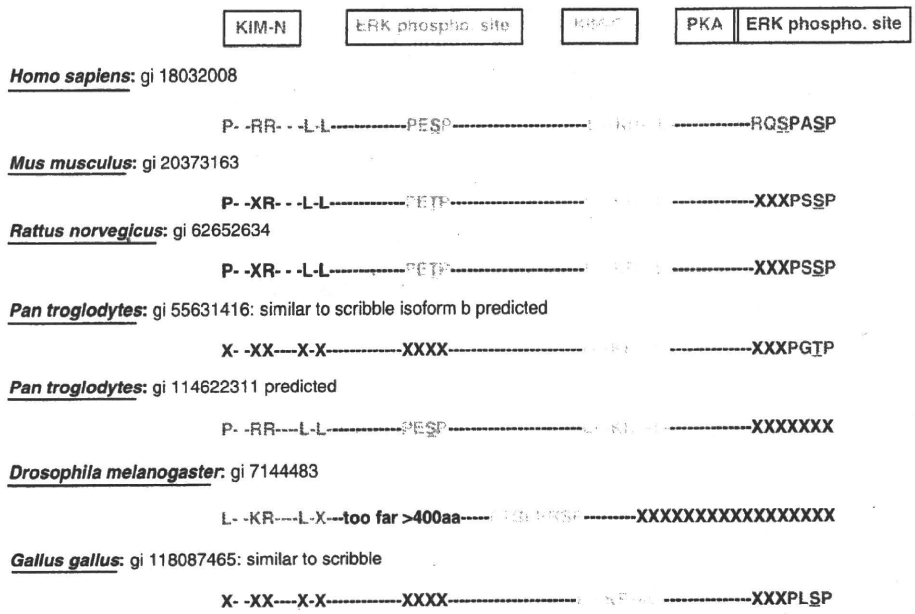

Figure 8 Comparison and sequence alignment of the region of hScrib containing the consensus ERK phosphorylation/binding sites in humans, chimpanzees, mice, rats, chickens and *Drosophila*. There is no evidence for the conservation of the C-terminal hScrib-dependent ERK signalling cascade in non-vertebrate species; however, interestingly, only human Scribble has the two ERK sites.

expression plasmids have been described previously (Thomas *et al*., 2005; Nagasaka *et al*., 2006). The mutations of Ser 853, 1445 and 1448 to either singly, doubly alanine(A) or aspartate(D) or KR, RR to alanine AA mutants in hScrib were performed using the QuikChange XL site-directed mutagenesis kit from Stratagene Cloning Systems (La Jolla, CA, USA) (Celbio, Milan, Italy) according to the manufacturer's instruction. The mutants were confirmed by DNA sequencing.

Antibodies

The following commercial antibodies were used at the dilution indicated: anti-hScrib goat polyclonal antibody (Santa Cruz, Santa Cruz, CA, USA; western blot (WB) 1:1000), anti-p44/42 MAPK (Erk1/2) antibody (Cell Signalling Technology, Danvers, MA, USA; WB 1:1000), anti-phospho p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody (Cell Signaling Technology, WB 1:1000), anti-HA monoclonal antibody 12CA5 (Roche, Milan, Italy; WB 1:500), anti-β-galactosidase antibody (Promega, Milan, Italy; WB 1:5000), anti-γ-tubulin monoclonal antibody (Sigma, Milan, Italy; WB 1:5000), anti-p84 mouse monoclonal antibody (Abcam, Cambridge, UK; WB 1:1000), anti-α-tubulin mouse monoclonal antibody (Abcam, WB 1:1000) and anti-E-Cadherin rabbit polyclonal antibody (Santa Cruz, WB 1:500).

Immunofluorescence and microscopy

For immunofluorescence, cells were grown on glass coverslips and fixed in 3.7% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min at room temperature. After washing in PBS, the cells were permeabilized in PBS/0.1% Triton for 5 min, washed extensively in PBS and then incubated with primary antibody diluted in PBS for 1 h followed by the appropriately conjugated secondary antibodies. Secondary antibodies conjugated to Alexa Fluor 488 or 548 were obtained from Invitrogen. The cells were then washed several times in water and mounted on glass slides. Cells were visualized using a Zeiss Axiovert 100 M microscope (Zeiss, Milan, Italy) attached to a LSM 510 confocal unit.

Small interfering RNA transfection

HEK293 cells were seeded on 6 cm dishes and transfected using Lipofectamine 2000 (Invitrogen) with control small interfering RNA against Luciferase (siLuc), or small interfering RNA against hScrib sequences (Dharmacon, Lafayette, CO, USA). At 48 h after transfection, cells were harvested and total cells extracts or cell fractionated extracts were then analysed by western blotting.

In vitro *kinase assays*

Purified GST fusion proteins were incubated with commercially purified ERK1, ERK2, JNK1 (Cell Signaling Technology) or PKA (Promega) for 20 min at 30 °C in phosphorylation buffer (0.25 M Tris pH 7.5, 1 M $MgCl_2$, 3 M NaCl, 0.3 mM aprotinin and 1 mM Pepstatin) supplemented with 56 nM [^{32}P] γ-ATP (Perkin Elmer, Waltham, MA, USA) and 10 mM ATP following the manufacturer's instruction. After extensive washing, the phosphorylated proteins were monitored by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and autoradiography.

Phospho-mapping analyses

HEK293 cells were transfected with HA-tagged Scrib and after 24 h left untreated or exposed to sorbitol for 30 min. After this time, the cells were extracted and proteins immunoprecipitated with anti-HA agarose beads, separated on SDS–PAGE and the silver-stained gel slice corresponding to hScrib was excised. Phospho-mapping mass spectroscopy was then performed using NextGen Sciences (Ann Arbor, MI, USA).

Subcellular fractionation assays

Differential extraction of HEK293 cells to obtain cytoplasmic, nuclear and membrane fractions was performed using the

Calbiochem ProteoExtract Fractionation Kit (Calbiochem, Milan, Italy) according to the manufacturer's instructions. To inhibit phosphatase activity during the preparation of cell lysates, phosphatase inhibitors (1 mM Na_3VO_4, 1 mM β-glycerophosphate, 2.5 mM sodium pyrophosphate and 1 mM sodium fluoride) were also included.

Immunoprecipitation and western blotting

Total cellular extracts were prepared by directly lysing cells from dishes in SDS lysis buffer. Alternatively, cells were lysed in either E1A buffer (25 mM HEPES pH 7.0, 0.1% NP-40, 150 mM NaCl, plus protease inhibitor cocktail; Calbiochem) or RIPA buffer (50 mM Tris–HCl pH 7.4, 1% NP-40, 150 mM NaCl, 1 mM EDTA, plus protease inhibitor cocktail; Calbiochem). The supernatant (soluble fraction), pellet (insoluble fraction) and the whole cells extracts were analysed by SDS–PAGE and western blotting. For immunoprecipitations, total cell lysates were transferred into a tube of equilibrated EZview Red Anti-HA Affinity Gel beads (Sigma), and incubated for 2 h at 4 °C. Immunoprecipitates were extensively washed four times in lysis buffer and solubilized in SDS–PAGE sample buffer. For western blotting, 0.45 μm nitrocellulose membrane (Schleicher and Schuell, Milan, Italy) was used and membranes were blocked for 1 h at 37 °C in 10% milk/PBS followed by incubation with the appropriate primary antibody diluted in 10% milk/0.5% Tween 20 for 1 h. After several washings with PBS 0.5% Tween 20, secondary antibodies conjugated with horseradish peroxidase (DAKO, Milan, Italy) in 10% milk/0.5% Tween 20 were incubated for 1 h. Blots were developed using Amersham enhanced chemiluminescence reagents (Amersham, Milan, Italy) according to the manufacturer's instructions.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

This work was supported by the Yoshida (YKK) Scholarship Foundation (to KN) and by a research grant from the Associazione Italiana per la Ricerca sul Cancro (to LB). We are also very grateful to David Allen (Nextgen Sciences) for his kind support and advice on the phospho-mapping analyses.

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British Journal of Cancer (2010) 102, 1061 – 1067

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Identification of DBC1 as a transcriptional repressor for BRCA1

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BACKGROUND: DBC1/KIAA1967 (deleted in breast cancer 1) is a putative tumour-suppressor gene cloned from a heterozygously deleted region in breast cancer specimens. Caspase-dependent processing of DBC1 promotes apoptosis, and depletion of endogenous DBC1 negatively regulates p53-dependent apoptosis through its specific inhibition of SIRT1. Hereditary breast and ovarian cancer susceptibility gene product BRCA1, by binding to the promoter region of SIRT1, is a positive regulator of SIRT1 expression.

METHODS: A physical interaction between DBC1 and BRCA1 was investigated both *in vivo* and *in vitro*. To determine the pathophysiological significance of DBC1, its role as a transcriptional factor was studied.

RESULTS: We found a physical interaction between the amino terminus of DBC1 and the carboxyl terminus of BRCA1, also known as the BRCT domain. Endogenous DBC1 and BRCA1 form a complex in the nucleus of intact cells, which is exported to the cytoplasm during ultraviolet-induced apoptosis. We also showed that the expression of DBC1 represses the transcriptional activation function of BRCT by a transient expression assay. The expression of DBC1 also inhibits the transactivation of the SIRT1 promoter mediated by full-length BRCA1.

CONCLUSION: These results revealed that DBC1 may modulate the cellular functions of BRCA1 and have important implications in the understanding of carcinogenesis in breast tissue.

British Journal of Cancer (2010) **102,** 1061 – 1067. doi:10.1038/sj.bjc.6605577 www.bjcancer.com
Published online 16 February 2010

Keywords: DBC1; BRCA1; interaction; repression

The gene encoding DBC1 (deleted in breast cancer 1) was identified during a representative differential analysis to search for candidate breast tumour-suppressor genes on a human chromosome 8p21 region that is frequently deleted in breast cancers (Hamaguchi *et al*, 2002). In this study, the expression of DBC2 (deleted in breast cancer 2) was substantially decreased in breast and lung cancer specimens. On the other hand, the expression of DBC1 was not substantially abrogated in cancers from any source. Molecular and cellular functions of DBC1 are currently extensively investigated to reveal the physiological role of DBC1 (Sundararajan *et al*, 2005; Kim *et al*, 2008; Zhao *et al*, 2008; Cha *et al*, 2009). Endogenous DBC1 is a nuclear protein and is thought to localise in the nucleus depending on its nuclear localisation signal (NLS) at the amino terminus. During tumor necrosis factor-α-induced apoptosis, DBC1 is translocated to the cytoplasm with loss of the NLS by caspase-dependent cleavage and this cleavage promotes apoptosis because of the death-promoting activity of its carboxyl-terminal coiled-coil domain (Sundararajan *et al*, 2005). Therefore, caspase-dependent cleavage of DBC1 may function as a positive feedback mechanism to promote apoptosis and this would explain how DBC1 functions as a tumour suppressor. A recent study demonstrated that DBC1 promotes p53-mediated apoptosis through specific inhibition of SIRT1, the mammalian homologue of yeast silent information regulator 2 (Sir2) (Kim *et al*, 2008; Zhao *et al*, 2008). However, functions of DBC1 in living cells still remain largely unknown and it should be determined whether DBC1 has a pivotal role in tumour suppression.

It is well known that the germ-line mutation of BRCA1 predisposes women to early-onset breast and ovarian cancer. BRCA1 is predominantly located in the nucleus and is involved in the basal transcriptional machinery (Scully *et al*, 1997; Anderson *et al*, 1998). BRCA1 regulates stress-inducible gene expressions such as p21 (Ouchi *et al*, 1998), p53 (Somasundaram *et al*, 1999), and GADD45 (Jin *et al*, 2000). The carboxyl-terminal BRCA1, referred to as the BRCT domain, has been shown to be involved in double-stranded DNA repair and homologous recombination (Callebaut and Mornon, 1997; Moynahan *et al*, 1999; Zhong *et al*, 1999). BRCT is indispensable for normal cellular growth

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Received 27 October 2009; revised 21 January 2010; accepted 25 January 2010; published online 16 February 2010

because the targeted deletion of the BRCT domain results in embryonic lethality (Hohenstein *et al*, 2001). The major function of BRCT is thought to be a gene regulator, mediating BRCA1 function as a tumour suppressor. This hypothesis is based on several lines of evidence, including that the autonomous transactivation function of BRCT was preserved in a recombinant protein consisting of the BRCT domain fused to a GAL4 DNA binding domain (Miyake *et al*, 2000). In addition, point mutations in the BRCT domain derived from patients with inherited breast cancer result in loss of transcriptional activity, and BRCA1 can also function as a negative regulator on some gene promoters (Chapman and Verma, 1996; Monteiro *et al*, 1996). This domain has already been shown to be an interaction surface with a number of transcription factors and co-regulators (Saka *et al*, 1997; Yarden and Brody, 1999; Wada *et al*, 2004; Oishi *et al*, 2006). A recent study revealed the interplay between SIRT1 and BRCA1 (Wang *et al*, 2008). BRCA1 was shown to stimulate the expression level of SIRT1 through binding to the specific promoter region of SIRT1, and this interplay prompted us to search for the cross talk between DBC1 and BRCA1.

To better understand the functional significance and the transcriptional regulation of BRCA1, we investigated the physical interaction between BRCA1 and DBC1. We found that DBC1 directly interacted with the BRCT domain. Our findings revealed that the amino terminus of DBC1 binds directly to the BRCT domain both *in vitro* and *in vivo*. We studied the effect of the transcriptional regulation of BRCA1 driven by DBC1. These findings establish a principal biological function of DBC1 in the modulation of BRCA1 function, and further identify DBC1 as a possible determinant and potential therapeutic target in breast cancer.

MATERIALS AND METHODS

Cell culture

Human cervical adenocarcinoma HeLa (CCl-2), human breast cancer MCF-7 (HTB-22), and human kidney 293T (CRL-11268) cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). These cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum.

Plasmid construction

BRCA1 expression vectors, BRCT vectors, and reporter constructs (17M8-AdMLP-luc) were described previously by Wada *et al*, 2004. DBC1 (Clone ID 5496068) and SIRT1 (Clone ID 4518906) expression vectors were purchased from Thermo Fisher Scientific Open Biosystems (Huntsville, AL, USA). Fragments of DBC1 were inserted into pcDNA-Myc vector derived from pcDNA3 (Invitrogen, Carlsbad, CA, USA).

Chemicals and antibodies

Rabbit polyclonal antibodies were anti-DBC1 (produced in our laboratory) and anti-acetyl-p53 (Upstate, Temecula, CA, USA, catalogue no. 06-758). Mouse monoclonal antibodies were anti-BRCA1 (Calbiochem, EMD Biosciences, Inc., LaJolla, CA, USA, catalogue no. OP93T), anti-Myc (Invitrogen, catalogue no. R95025), and anti-SIRT1 (Abnova, Taipei, Taiwan, catalogue no. H00023411-M01). Anti-BRCA1 (catalogue no. sc-642), anti-p21 (catalogue no. sc-397), anti-p53 (catalogue no. sc-126), and anti-actin (catalogue no. sc-47778) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Alexa Fluor 488-conjugated donkey anti-mouse IgG (A-21202) and Alexa Fluor 555-conjugated goat anti-rabbit IgG (A-21428) were purchased from Invitrogen.

Immunoprecipitation and western blot

The formation of a DBC1–BRCA1 complex in HeLa and 293T cells was analysed by immunoprecipitation. The whole-cell extracts of HeLa cells were immunoprecipitated with anti-BRCA1 antibodies, and subsequently immunoblotted by anti-DBC1 antibodies. Reciprocal immunoprecipitation was also performed. Cells (293T) transfected with indicated plasmids were lysed and subjected to anti-FLAG M2 agarose (Sigma Aldrich, St Louis, MO, USA). Immunoprecipitated materials were blotted with anti-Myc antibodies to identify DBC1-containing complexes.

RNAi

The ablation of DBC1 and BRCA1 was performed by transfection of HeLa cells with small interfering RNA (siRNA) duplex oligos synthesised by Qiagen (Hilden, Germany). Control siRNA (AllStars Negative Control siRNA, Qiagen, 1027281), DBC1-specific siRNA (DBC1-RNAi: 5′-AAACGGAGCCUACUGAACA-3′, which covered mRNA regions of nucleotides 1379–1397 (amino acids 460–466) of DBC1, and KIAA1967-RNAi, SI00461853), and BRCA1-specific siRNA (#14 (SI02664361) and #15 (SI02664368)) were transfected using HyperFect reagent (Qiagen).

GST pull-down assay

Glutathione *S*-transferase (GST) fusion proteins or GST alone were expressed in *Escherichia coli* and immobilised on glutathione–sepharose 4B beads (GE Healthcare UK Ltd., Buckinghamshire, UK). GST proteins were incubated with [^{35}S] methionine-labelled proteins using a TNT-coupled transcription–translation system (Promega Co., Madison, WI, USA). Unbound proteins were removed and specifically bound proteins were eluted and analysed by SDS polyacrylamide gel electrophoresis.

Luciferase assay and mammalian two-hybrid assay

Transfection was performed with Effectene reagent (Qiagen) according to the manufacturer's recommendation. For luciferase assay, indicated expression vectors and GAL4 vectors were co-transfected with 17M8-AdMLP-luc or SIRT1-luc. For mammalian two-hybrid assay, GAL4 vectors and VP16 vectors were co-transfected. As an internal control to equalise transfection efficiency, phRL CMV-*Renilla* vector (Promega Co.) was also transfected in all experiments. Individual transfections, each consisting of triplicate wells, were repeated at least three times (Wada *et al*, 2004).

Fluorescence microscopy

Cells (MCF-7) were grown on 12 mm BD BioCoat glass coverslips (BD Biosciences, NJ, USA, 354085) in six-well plates before induction of apoptosis. The cells were treated or not treated with irradiation of ultraviolet (UV) light (0.24 J), fixed with phosphate-buffered saline (PBS) containing 4% paraformaldehyde, and permeabilised in PBS with 0.2% (v/v) Triton X-100. After blocking, the cells were incubated sequentially with anti-BRCA1 and anti-DBC1 antibodies. Secondary antibodies were Alexa Fluor 488-conjugated donkey anti-mouse IgG and Alexa Fluor 555-conjugated goat anti-rabbit IgG. The slides were briefly counter-stained and analysed under a confocal fluorescence microscope (Carl-Zeiss MicroImaging Inc., Oberkochen, Germany). Colocalisation was quantified using LSM7 series-ZEN200x software (Carl-Zeiss MicroImaging Inc.), and the ratio of colocalisation pixels *vs* total pixels in the target area was determined. The degree of colocalisation signal is expressed as mean ± standard deviation.

Immunohistochemistry

The procedure for immunohistochemical study has been described by Wada-Hiraike *et al*, 2006. The primary antibody used was anti-DBC1, and the ChemMate EnVision Detection system (DAKO, Carpinteria, CA, USA) was used to visualise the signal.

Chromatin immunoprecipitation assay

Soluble HeLa chromatin for PCR amplification was essentially prepared as described by Oishi *et al*, 2006. Subconfluent HeLa cells were crosslinked with 1.5% formaldehyde at room temperature for 15 min, and washed twice with ice-cold PBS. The cell pellet was then resuspended in 0.2 ml lysis buffer and sonicated by Bioruptor UCD-250 (Cosmo Bio, Co., Ltd., Tokyo, Japan). The sheared soluble chromatin was then subjected to immunoprecipitation with specific antibodies and protein G-sepharose with salmon sperm DNA (Upstate). After an extensive wash, the beads were eluted. The eluate was incubated for 6 h at 65°C to reverse the formaldehyde crosslink. The extracted DNA was purified using the QIAquick PCR purification kit (Qiagen). PCR was performed using specific primers (Wang *et al*, 2008).

RESULTS

DBC1 and BRCA1 interact *in vivo* and *in vitro*

To determine the interaction between endogenous DBC1 and BRCA1 in cultured human cells, cell extracts from HeLa cells were immunoprecipitated with anti-BRCA1 antibodies or with preimmune IgG. The immunoblotting analysis using anti-DBC1 antibodies revealed the existence of DBC1 in cell lysate immunoprecipitates (Figure 1A), which indicates that DBC1 physically associates with BRCA1 in living cells. Reciprocal immunoprecipitation analysis confirmed this association (Figure 1A). In addition, Flag-tagged BRCA1 and Myc-tagged DBC1 were each transfected in 293T cells and extracts of transfected cells were immunoprecipitated with anti-FLAG M2 agarose beads. Western blotting analysis with anti-Myc antibodies revealed the existence of Myc-tagged DBC1 in the protein extract of immunoprecipitates (Figure 1B),

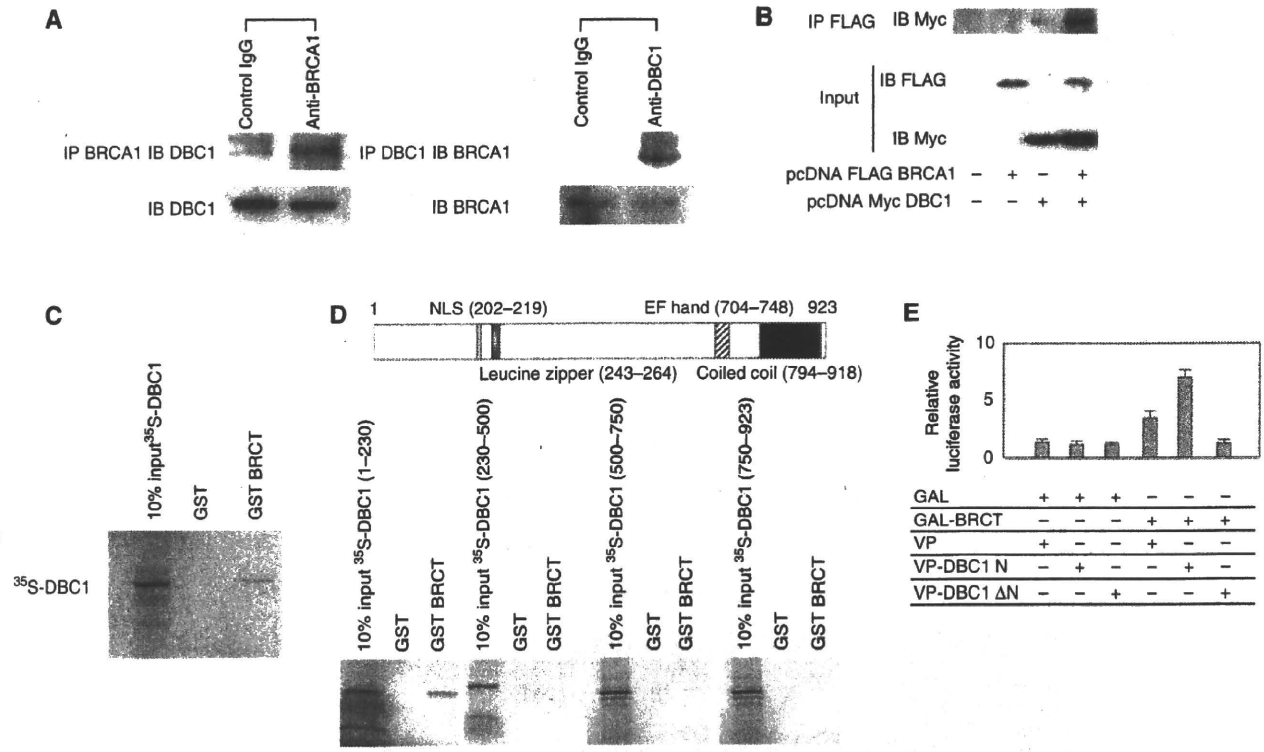

Figure 1 *In vivo* and *in vitro* association between DBC1 and BRCA1, and mapping of the BRCT-interacting region of DBC1. (**A**) The formation of a DBC1–BRCA1 complex in HeLa cells was analysed by co-immunoprecipitation (IP) with antibodies to BRCA1 or preimmune IgG, followed by immunoblotting (IB) using anti-DBC1 antibodies. The immunoprecipitates were subjected to 30 μl of protein G sepharose 4 Fast Flow and bound proteins were detected by western blotting. Reciprocal co-immunoprecipitation with antibodies to DBC1 and subsequent IB confirmed the complex formation of DBC1 and BRCA1. (**B**) The formation of a DBC1–BRCA1 complex in 293T cells was analysed by IP with anti-Flag M2 agarose beads, followed by IB using anti-Myc antibodies. Bound proteins were detected by western blotting. (**C**) Mapping of the BRCT-interacting region of DBC1 using glutathione *S*-transferase (GST)–BRCT and DBC1. Bacterially expressed GST fusion proteins immobilised on beads were used in *in vitro* pull-down assays. Full-length DBC1 was *in vitro* translated in the presence of [^{35}S] methionine using a TNT-coupled *in vitro* translation system. Labelled DBC1 was then incubated with GST–BRCT. The mixtures were washed and subjected to SDS polyacrylamide gel electrophoresis (PAGE) and analysed. Polyacrylamide gels were stained briefly with Coomassie Brilliant Blue to verify the loading amounts of fusion proteins. (**D**) A schematic diagram of the structure of DBC1 is shown. Fragments of DBC1 ((amino acids 1–230), (230–500), (500–750), and (750–923)) were *in vitro* translated using a TNT-coupled *in vitro* translation system. Labelled DBC1 was incubated with GST–BRCT. The mixtures were extensively washed and subjected to SDS–PAGE and then analysed by autoradiography. (**E**) Mammalian two-hybrid interaction analysis. Cells (293T) were transfected with the indicated combinations of mammalian expression vectors encoding GAL4, GAL4–BRCT, the herpes simplex virus VP16 transactivation domain (VP16), and VP16-DBC1 chimera. At 24 h after transfection, cells were harvested, and transfected whole-cell lysates were assayed for luciferase activity produced from a co-transfected GAL4 DNA binding site-driven reporter template (17M8-AdMLP-luc). GAL–BRCT shows additive transactivation when co-transfected with VP-DBC1 N, suggesting the interaction between BRCT and DBC1 (1–230) *in vivo*.

confirming that BRCA1 was able to form a complex with DBC1. To address the functional importance of the DBC1–BRCA1 interaction, *in vitro*-translated DBC1 in the presence of [^{35}S] methionine was incubated with GST and GST fusion BRCT. As clearly shown in Figure 1C, [^{35}S]-labelled DBC1 bound the GST-fused BRCT protein, consistent with the results from the immunoprecipitation assay. These data indicated that DBC1 directly interacted with the BRCT domain. To map the region of DBC1 that interacts with the BRCT domain, GST pull-down assays were performed to test for the interaction with GST–BRCT and fragments of *in vitro*-translated DBC1 (Figure 1D). The amino-terminal region of DBC1 including the NLS interacted with the BRCT domain. These findings indicate that the DBC1 amino terminus including the NLS and the BRCT domain are both necessary and sufficient for the interaction between DBC1 and BRCA1. We further confirmed the binding of the BRCT domain to the DBC1 amino terminus using mammalian two-hybrid assays. In this assay, the VP16-fused amino terminus of DBC1 containing the NLS (VP-DBC1 N) exhibited a prominent interaction with the BRCT domain, whereas DBC1 lacking the amino terminus (VP-DBC1 ΔN) showed no interaction, underscoring the results of immunoprecipitation and GST pull-down experiments (Figure 1E).

DBC1 and BRCA1 colocalise in intact and apoptotic cells

Immunohistochemical studies using human breast specimens showed nuclear staining of DBC1 in the duct and adipose tissue (Figures 2A and B, respectively). Most breast cancer cells exhibited nuclear staining (Figure 2C), but this nuclear staining of DBC1 was not observed in cancer cells with an enlarged nucleus (Figure 2D, arrows). As previously shown, during tumor necrosis factor-α-induced apoptosis, DBC1 is translocated to the cytoplasm with a loss of the amino terminus containing the NLS (Sundararajan *et al*, 2005), and the expression of BRCA1 is downregulated by caspase-3-mediated cleavage during UV-induced apoptosis (Zhan *et al*, 2002). The changes in cellular distribution of DBC1 and BRCA1 during apoptotic processes were examined comprehensively under a confocal microscope in MCF-7 cells treated by UV-mediated death signalling. Both DBC1 (Alexa Fluor 555-conjugated anti-rabbit IgG, red) and BRCA1 (Alexa Fluor 488-conjugated anti-mouse IgG, green) were abundantly expressed and colocalised in the nuclei of control cells (Figure 2E, 1–4). In contrast to healthy cells, both DBC1 and BRCA1 are translocated to the cytoplasm in cells showing apoptotic morphological changes (Figure 2E, 5–8). The degree of colocalisation signal was quantified in both control and apoptotic cells, and these data indicated that UV-mediated apoptosis signalling prompted the translocation of these proteins (Figure 2F).

DBC1 represses the transcriptional activation function of BRCT

The result that the BRCT domain interacts with DBC1 led us to examine the role of DBC1 in the transactivation function of GAL4-fused BRCT. Transient transfection assays were performed using a 17M8-AdMLP-luc luciferase reporter plasmid, carrying

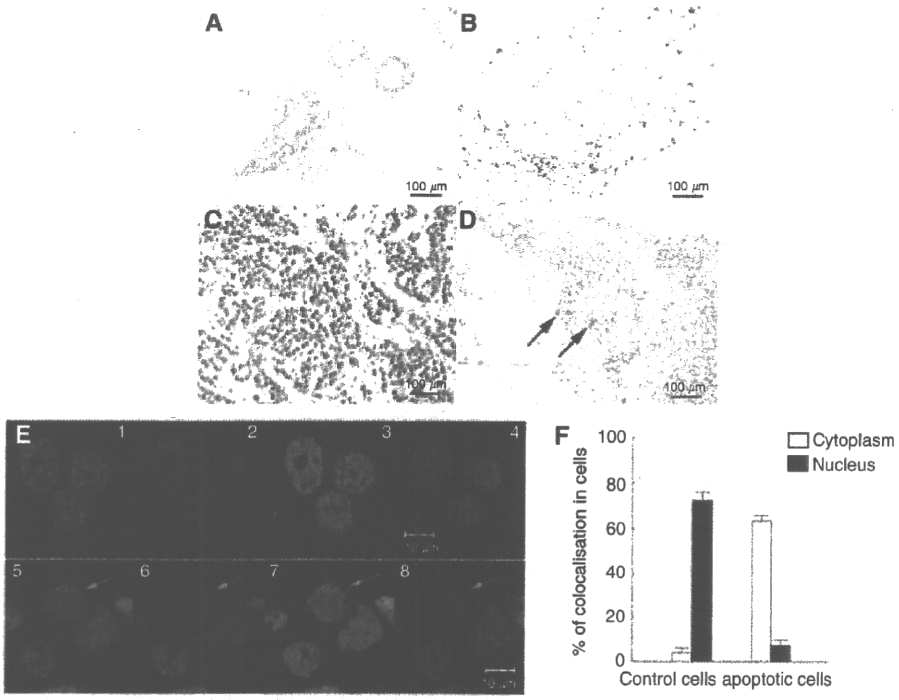

Figure 2 Immunohistochemical detection of DBC1 in human breast tissues and colocalisation of BRCA1 and DBC1 in MCF-7 human breast cancer cells. (**A**–**D**) Breast specimens were obtained at the time of diagnosis of breast cancer in accordance with the guidelines of the Ethical Board of Komagome Hospital. DBC1 showed nuclear staining of ductal epithelium (**A**) and adipose tissue (**B**) in breast specimens. DBC1 expression was observed in the nuclei of cancer tissues (**C**). Cancer cells exhibiting an enlarged nucleus showed a complete loss of DBC1 expression (**D**, arrow). (**E**) MCF-7 cells were either treated or not treated by ultraviolet (UV) light (0.24 J), fixed, and permeabilised. Cells were incubated with primary antibodies and subsequently with secondary antibodies. The expression of DBC1 (red) and BRCA1 (green) was investigated under confocal fluorescence microscopy (Carl-Zeiss). Representative immunofluorescence studies are shown (E, 1–4; control, 5–8; UV exposure for 10 min, E3 and E7; merge, E4 and E8; 4′,6-diamino-2-phenylindole staining). Arrows in E5–8 indicate a cell showing apoptotic morphological changes with the cytoplasmic expression of DBC1 and BRCA1. Bars indicate 10 μm. (**F**) The degree of colocalisation (BRCA1 and DBC1) was measured using a confocal microscope. The colocalisation signal was quantified in the nucleus and cytoplasm of cells separately.

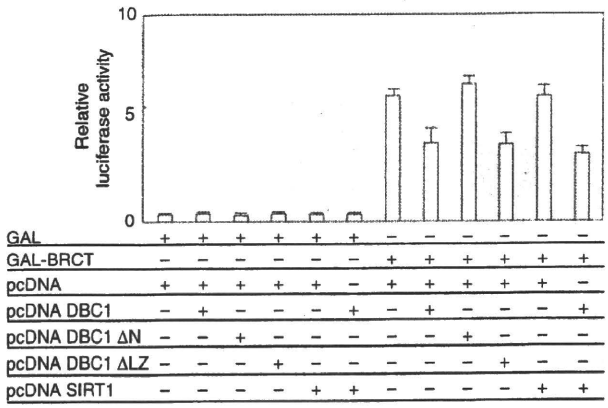

Figure 3 DBC1 represses transcription of GAL4–BRCT through its amino-terminal domain. Transient transfection assays were performed to examine the co-factor activity of DBC1 in the transactivation function of GAL4-fused BRCT. Cells (293T) were transfected with the indicated combinations of mammalian expression plasmids. At 24 h after transfection, the cells were harvested, and transfected whole-cell lysates were assayed for luciferase activity produced from the reporter plasmid (17M8-AdMLP-luc). DBC1 showed a specific repression of the transactivation function of BRCT. The amino terminus of DBC1 was indispensable for this inhibition of BRCT. SIRT1, a binding partner that has roles in cell senescence and tumourigenicity, had no effect on the transactivation function of BRCT. phRL *Renilla* CMV-luc vector was transfected as a control of transfection efficiency. Each experiment was repeated at least three times in triplicate. Error bars represent s.d.

eight tandem repeat GAL4 DNA binding sites (17M × 8) upstream of the major late promoter of adenovirus (AdMLP) driving the expression of the firefly luciferase gene. Although the GAL4-BRCT fusion protein (GAL-BRCT) activated the promoter activity of the reporter plasmid in 293T cells, the transcriptional activity of BRCT was significantly decreased by the expression of DBC1 in luciferase assays (Figure 3). DBC1 lacking an interaction domain with BRCA1 (DBC1 ΔN) lost its ability to inhibit the BRCT-mediated transactivation function (Figure 3). DBC1 lacking a binding region with SIRT1 deacetylase (DBC1 ΔLZ) suppressed the GAL-BRCT transactivation function. SIRT1 showed no influence on the GAL-BRCT transactivation function and on the repression of GAL-BRCT by DBC1. The BRCT-repression function of DBC1 was unaffected in the presence of resveratrol, a major activator of SIRT1, and trichostatin A, a histone deacetylase inhibitor (data not shown). These results suggest that the amino terminus of DBC1 has a significant role in the repression of GAL-BRCT and SIRT1 has no role in regulating the GAL-BRCT function.

DBC1 disrupts BRCA1-mediated SIRT1 expression

The previous chromatin immunoprecipitation assay showed that BRCA1 interacted with the SIRT1 promoter region between 1354 and 1902 and this binding resulted in an elevated expression of SIRT1 (Wang *et al*, 2008). We investigated whether DBC1 has an effect on the BRCA1-mediated stimulation of the SIRT1 promoter. An analysis of the effect of DBC1 on SIRT1-luciferase constructs containing various lengths of SIRT1 promoter regions upstream of the luciferase gene was performed and DBC1 demonstrated a specific downregulation of BRCA1-mediated stimulation on the SIRT1 promoter (SIRT1 1-2852 Luc), evidenced by the expression of DBC1 (Figure 4A). As expected, DBC1 ΔN showed no influence to inhibit the BRCA1-mediated transactivation function of SIRT1-luciferase reporter constructs, whereas DBC1 ΔLZ showed repression on the SIRT1-luciferase transactivation function mediated by BRCA1. We next examined the effect of siRNA-mediated depletion of DBC1 or BRCA1 on their downstream genes. As expected, knockdown of BRCA1 expression by BRCA1-specific siRNA completely abrogated the expression of SIRT1 (Figure 4B, lanes 4 and 5), validating the previous report that the expression of SIRT1 is indeed dependent on BRCA1 (Wang *et al*, 2008). As shown in Figure 4B, lanes 2 and 3, depletion of endogenous DBC1 increased the expression of SIRT1. To demonstrate that SIRT1 functions on the p53 acetylation level (Zhao *et al*, 2008), we tested whether depletion of BRCA1 or DBC1 indeed influences the expression of acetylated p53. Consistent with previous studies (Kim *et al*, 2008; Zhao *et al*, 2008), RNAi-mediated knockdown of DBC1 expression resulted in hypoacetylation of p53 (Figure 4B, lanes 2 and 3), whereas depletion of endogenous BRCA1 had no influence on the expression level of acetylated p53 (Figure 4B, lanes 4 and 5). Depletion of DBC1 resulted in a downregulation of p21, a transcriptional target of BRCA1 (Figure 4B, lanes 2 and 3). Thus, our data demonstrate that DBC1 has a critical role in regulating downstream gene expressions dependent on BRCA1 *in vivo* such as SIRT1 and p21. To test whether DBC1 and BRCA1 were indeed recruited to the SIRT1 promoter, we performed a chromatin immunoprecipitation assay using the SIRT1 gene promoter 1354-1902, a region known to recruit BRCA1 (Wang *et al*, 2008). As expected, a clear recruitment of endogenous BRCA1 to the target sequence (1354-1902) in the SIRT1 promoter was observed in HeLa cells (Figure 4C). Besides this BRCA1 recruitment, DBC1 and SIRT1 were also detected in the promoter region, presumably reflecting the complex formation of BRCA1-DBC1 on the SIRT1 promoter (Figure 4C).

DISCUSSION

The transcriptional activation function of BRCT is believed to be a key to its tumour-suppressor activity (Chapman and Verma, 1996; Monteiro *et al*, 1996). The importance of BRCT for transcriptional control and growth suppression is also highlighted by the fact that cancer-associated mutations attenuated both, but a neutral polymorphism did not (Humphrey *et al*, 1997; Yarden and Brody, 1999). BRCT possesses an autonomous folding unit defined by conserved clusters of hydrophobic amino acids, and BRCT is likely to represent a protein interaction surface (Saka *et al*, 1997). Although a number of proteins have been identified to interact with the BRCT domain, most of them activate the transcriptional function of BRCT (Wada *et al*, 2004; Oishi *et al*, 2006), and the repressors of BRCT have been poorly studied until now (Chen *et al*, 2001). Here, we clearly showed that endogenous DBC1 associated with BRCA1 *in vivo* and *in vitro*, which suggests the possibility that DBC1 might have a functional relationship with BRCA1-related phenotypical changes. This interaction between BRCA1 and DBC1 was physiologically functional because our results indicated that the DBC1-containing complex might modulate a role of BRCA1 in living cells, repressing BRCT function. In this respect, DBC1 seems to have a tumourigenic role in living cells. The BRCT domain is found in a diverse group of proteins implicated in DNA repair and cell-cycle checkpoint control (Bork *et al*, 1997; Callebaut and Mornon, 1997). A point mutation within the BRCT domain (A1708E) was shown to be critical for DNA damage response by treatment with DNA-damaging agent methylmethane sulphonate (Zhong *et al*, 1999). Thus, repression of BRCT has implications both in tumourigenic and in defective DNA repair processes. Our results also indicated that DBC1 suppressed BRCA1-dependent transcriptional regulation, because SIRT1-luciferase activity was attenuated by the expression of DBC1. Together with the result of chromatin immunoprecipitation assay, these data suggest the possibility that DBC1 might be involved in the basal transcriptional machinery because BRCA1 associates with RNA polymerase II holoenzyme (Scully *et al*, 1997; Anderson *et al*, 1998). DBC1 would serve as a transcriptional repressive factor to manipulate transcriptions,

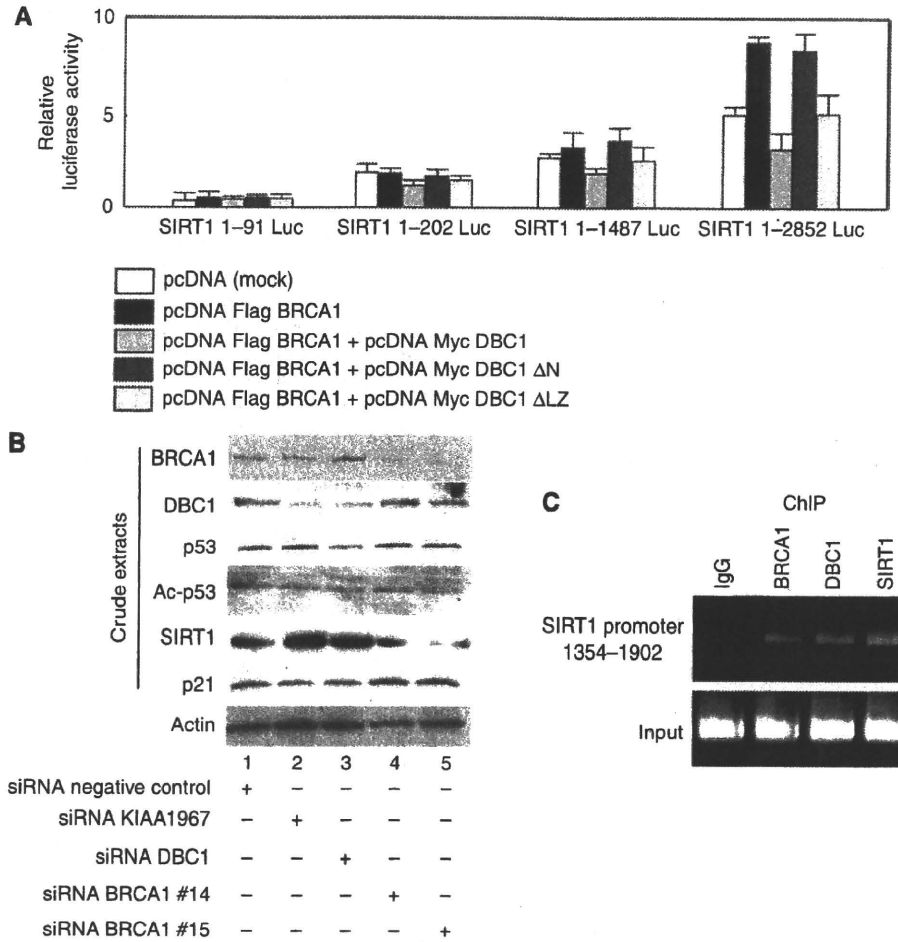

Figure 4 DBC1 represses transcription by BRCA1 through its amino-terminal domain. (**A**) Transient transfection assays were performed to examine the influence of DBC1 using an artificial luciferase reporter construct. Cells (293T) were transfected with the indicated combinations of mammalian expression plasmids. At 24 h after transfection, cells were harvested, and transfected whole-cell lysates were assayed for luciferase activity produced from reporter plasmids. Various lengths of SIRT1 promoter (1–91, 1–202, 1–1487, and 1–2852) were fused upstream of the firefly luciferase reporter plasmid. Full-length DBC1 and DBC1ΔLZ showed specific downregulation of 1–2852 SIRT1-luciferase activity mediated by BRCA1. (**B**) Small interfering RNA (siRNA)-mediated knockdown of BRCA1 decreased the expression of SIRT1. Knockdown of DBC1 resulted in downregulation of acetylated p53 and p21. The expression of SIRT1 was increased by depletion of DBC1. HeLa cells were transfected with indicated siRNA. At 48 h after transfection, cells were harvested and analysed by western blotting. (**C**) Chromatin immunoprecipitation assay was performed to confirm the recruitment of BRCA1 and DBC1 at the SIRT1 gene promoter (1354–1902), a region known to recruit BRCA1 (Wang *et al*, 2008).

thereby influencing transcriptional products such as SIRT1 and p21. Consistent with these results, our recent data have also shown that DBC1 suppresses the ligand-dependent transcriptional activation function of ERβ (Koyama *et al*, 2010).

Apoptosis is a normal physiological process that has an important role in embryonic development and in tissue homoeostasis maintenance. As apoptosis is genetically programmed, its dysfunction contributes to tumour promotion. The previous study showed that BRCA1 is cleaved at amino acid 1151–1154 (DLLD) by caspase-3 during UV-C-induced apoptosis, and the cleaved fragment of BRCA1, containing the BRCT domain, induced cell death through activation of BRCA1 downstream effectors, GADD45 and JNK (Jin *et al*, 2000). Another study reported that DBC1 is translocated from the nucleus to the mitochondria during apoptosis (Sundararajan *et al*, 2005). Thus, our results of immunofluoresence showing that the cleavage of DBC1 and BRCA1 after death signalling promotes their cytoplasmic shuttling indicated that BRCA1 and DBC1 may function synergistically in the apoptotic pathway. It seems reasonable to hypothesise that the cells devoid of nuclear DBC1 staining may be apoptotic. Loss of nuclear DBC1 staining in tissues would be speculated as a marker of therapy efficiency.

The accumulation of DNA damage activates p53 and induces cell-cycle arrest and apoptosis. Acetylation of p53 has been shown to augment p53 DNA binding and to regulate the stability of p53 by inhibiting its ubiquitination by MDM2. In response to DNA damage, acetylation of p53 is stimulated and acetylated p53 enhances its ability to induce cell-cycle arrest, apoptosis, and DNA damage repair (Smith and La Thangue, 2005). Consistent with the previous report by Zhao *et al*, 2008, inactivation of endogenous DBC1 leads to hypoacetylation of p53. This would suggest that abrogation of DBC1 causes malfunctions of p53, including defective DNA repair activities. Furthermore, as we discussed above, repression of the BRCT transactivation function may have significance in impaired DNA damage response. The mechanism by which DBC1 regulates DNA damage machinery seems to be complicated, as DBC1 may possess dual roles in promoting and inhibiting DNA repair, because depletion of DBC1 also results in an increased expression of SIRT1, which possesses DNA repair activity (Jeong *et al*, 2007). We have to further confirm the effect of

DNA damage response when DBC1 is abrogated. Altogether, our results provide new insight into the fact that DBC1 may serve, at least in part, as a DNA damage response machinery.

In conclusion, our data indicate that DBC1 has an important role in regulating BRCA1-mediated functions through binding to the BRCT domain. In addition to its inhibition of the deacetylase activity of SIRT1, DBC1 represses the expression of SIRT1 by associating with BRCA1. DBC1 may be involved both in tumourigenic and anti-tumourigenic processes. This conflicting mechanism can be the reason why expression of DBC1 was not substantially abrogated in various cancers from any type of tissue (Hamaguchi *et al*, 2002). Therefore, both inhibitors and activators of DBC1 would be therapeutically beneficial, by affecting different DBC1-mediated regulatory pathways together with BRCA1. These results suggest that the failure of binding between BRCA1 and DBC1 may be a key event in cancer predisposition.

ACKNOWLEDGEMENTS

This study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, JMS Bayer Schering Pharma Grant, and Kowa Life Science Foundation, Japan. We thank Dr Rui-Hong Wang and Chu-Xia Deng for SIRT1-luciferase vectors, and Dr Ja-Eun Kim for DBC1 ΔLZ construct.

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Biochemical and Biophysical Research Communications 392 (2010) 357–362

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Repression of estrogen receptor β function by putative tumor suppressor DBC1

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ARTICLE INFO

Article history:
Received 5 January 2010
Available online 13 January 2010

Keywords:
DBC1
ERβ
Breast cancer
Transcription
Repression

ABSTRACT

It has been well established that estrogen is involved in the pathophysiology of breast cancer. Estrogen receptor (ER) α appears to promote the proliferation of cancer tissues, while ERβ can protect against the mitogenic effect of estrogen in breast tissue. The expression status of ERα and ERβ may greatly influence on the development, treatment, and prognosis of breast cancer.

Previous studies have indicated that the deleted in breast cancer 1 (DBC1/KIAA1967) gene product has roles in regulating functions of nuclear receptors. The gene encoding DBC1 is a candidate for tumor suppressor identified by genetic search for breast cancer. Caspase-dependent processing of DBC1 promotes apoptosis, and depletion of the endogenous DBC1 negatively regulates p53-dependent apoptosis through its specific inhibition of SIRT1. In addition, DBC1 modulates ERα expression and promotes breast cancer cell survival by binding to ERα.

Here we report an ERβ-specific repressive function of DBC1. Immunoprecipitation and immunofluorescence studies show that ERβ and DBC1 interact in a ligand-independent manner similar to ERα. *In vitro* pull-down assays revealed a direct interaction between DBC1 amino-terminus and activation function-1/2 domain of ERβ. Although DBC1 shows no influence on the ligand-dependent transcriptional activation function of ERα, the expression of DBC1 negatively regulates the ligand-dependent transcriptional activation function of ERβ *in vivo*, and RNA interference-mediated depletion of DBC1 stimulates the transactivation function of ERβ. These results implicate the principal role of DBC1 in regulating ERβ-dependent gene expressions.

Introduction

Estrogen elicits its biological responses via estrogen receptor (ER) α/β-mediated genomic and/or non-genomic pathways. ER is a member of the nuclear receptor (NR) gene superfamily and acts as a ligand-induced transcription factor [1]. ERα and ERβ are stimulated by two distinct activation regions, activation function-1 (AF-1) and AF-2. AF-1, which is located in the amino-terminal A/B domain, is constitutively activated in cell-type and promoter specific manner [2]. AF-2 is located in the carboxyl-terminal ligand binding domain and exerts a ligand-dependent transcriptional activation. AF-1 and AF-2 activate transcription independently and act synergistically [3]. The ligand-dependent activation of ERs requires ligand-dependent association of AF-2 coactivators [4]. An increasing number of molecules which can interact with ERs have been identified and these molecules can modulate biological behavior, e.g. proliferation, growth, sensitivity to apoptotic stimuli, and invasiveness.

Previous studies using knockout mice of ERβ have shown that deficiency of ERβ leads to hyperproliferation and loss of differentiation in epithelia of the uterus [5], ventral prostate [6], and colon [7]. In consistent with *in vivo* studies, *in vitro* studies also suggest the pro-differentiative and anti-proliferative functions of ERβ.

The gene encoding DBC1 was identified during a representative differential analysis to search for candidate breast tumor suppressor genes on a human chromosome 8p21 region frequently deleted in breast cancers [8]. Molecular and cellular function of DBC1 is currently extensively investigated to reveal its physiological role.

Abbreviations: E_2, 17β-estradiol; ER, estrogen receptor; FBS, fetal bovine serum; NLS, nuclear localization signal; NR, nuclear receptor; AF, activation function; GST, glutathione-S-transferase

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0006-291X/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2010.01.025

Endogenous DBC1 is a nuclear protein and is thought to localize in the nucleus depending on its nuclear localization signal (NLS) at the amino-terminus. During TNF-α induced apoptosis, DBC1 is exported to the cytoplasm with loss of the NLS by caspase-dependent cleavage and this processing promotes apoptosis due to the death-promoting activity of its carboxyl-terminal coiled-coil domain [9]. Therefore, caspase-dependent cleavage of DBC1 may act as a positive feedback mechanism to promote apoptosis and possibly also tumor suppression. Recent studies have demonstrated that DBC1 promotes p53-mediated apoptosis through specific inhibition of deacetylase activity of SIRT1, the mammalian homologue of yeast Sir2 (silent information regulator 2) [10,11]. However, the functions of DBC1 in breast cancer still remain largely unknown and it should be determined whether DBC1 plays a pivotal role in tumor suppression.

Current studies have shown that DBC1 interacts with several NRs. DBC1 associates with androgen receptor and facilitates transcriptional activation of androgen receptor [12]. DBC1 stabilizes the interaction between chicken ovalbumin upstream promoter transcription factor I (COUP-TFI) and NCoR by interacting directly with both proteins [13]. DBC1 also interacts with NR complex interacting factor (NRC)-1 to drive the transcription regulation of NRs [14]. On the basis of the report showing that DBC1 is a principal determinant of unliganded ERα expression and promotes proliferation of human breast cancer cells [15], we investigated the functional interaction between DBC1 and ERβ. Our findings reveal that the amino terminus of DBC1 binds directly to ERβ both *in vitro* and *in vivo*. The expression of DBC1 results in a repression of ligand-dependent transcriptional activation function of ERβ. These findings thus may establish a principal biological function for DBC1 in the repression of ERβ function and further underscore DBC1 as a possible endocrine response determinant and potential therapeutic target in breast cancer.

Materials and methods

Cell culture and chemical reagent. ERα positive MCF-7 (HTB-22), ERαβ-positive T47D (HTB-133), and ERβ-positive MDA-MB-231 (HTB-26) human breast cancer cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). These cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). DMEM, FBS, and 17β-estradiol (E_2) were purchased from Sigma–Aldrich (St. Louis, MO, USA). ERβ selective ligand, 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN), was from Tocris Bioscience (Ellisville, MO, USA).

Immunoprecipitation. The formation of a DBC1–ERβ complex in MDA-MB-231 cells was analyzed by co-immunoprecipitation using the specific antibodies raised against human ERβ (H-150, Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by immunoblotting using the anti-human DBC1 (produced in our laboratory). Immunoprecipitation, Western blot analysis, and immunostaining were performed as described previously [16].

Plasmid construction. DBC1 (Clone ID 5496068) and SIRT1 (Clone ID 4518906) expression vectors were purchased from Thermo Fisher Scientific Open Biosystems (Huntsville, AL, USA). Full-length and fragments of DBC1 were inserted into pcDNA-Myc vector derived from pcDNA3 (Invitrogen, Carlsbad, CA, USA). Human ERα AF-1, ERα AF-2, ERβ AF-1, ERβ AF-2 vectors, and a reporter construct (ERE-tk-luc) were described previously [17].

Fluorescence microscopy. MCF-7 and T47D cells were grown on 12 mm BD BioCoat glass coverslips in 6-well plates. These cells

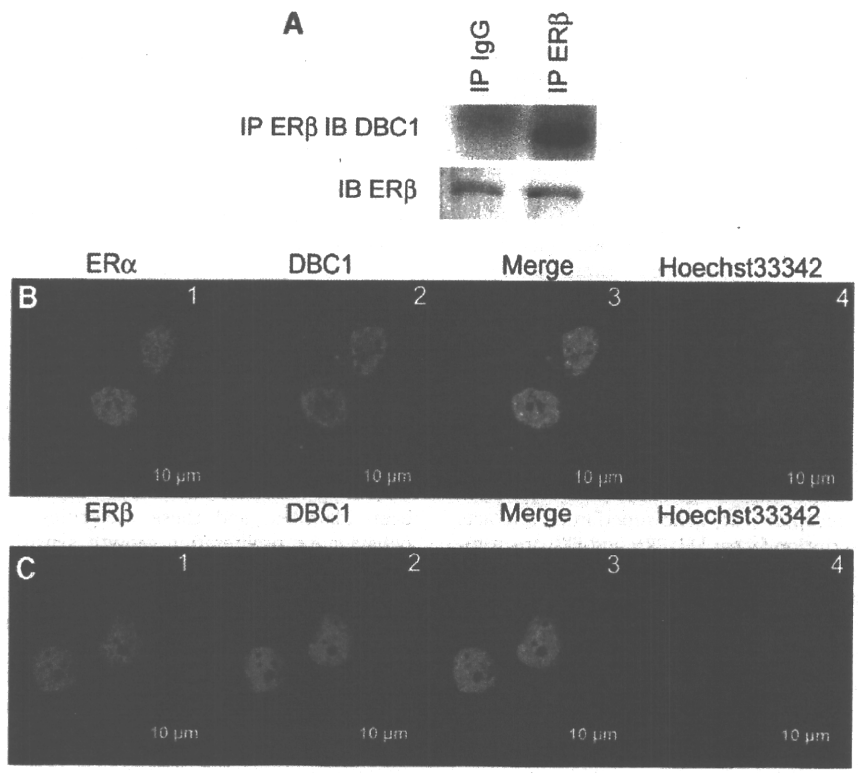

Fig. 1. *In vivo* association between DBC1 and ERα/β. (A) The formation of a DBC1 and ERβ complex in MDA-MB-231 cells was analyzed by co-immunoprecipitation (IP) with the antbodies to ERβ or preimmune IgG, followed by immunoblotting (IB) using the anti-DBC1 antibody. The immunoprecipitates were subjected to 30 μl of protein G sepharose™ 4 Fast Flow and detected by Western blotting. (B) MCF-7 cells were fixed and permeabilized. The cells were incubated with primary antibodies and subsequently with secondary antibodies. B1, ERα; B2, DBC1; B3, merge; B4, Hoechst 33342 staining. (C) T47D cells were incubated with primary antibodies and secondary antibodies. C1, ERβ; C2, DBC1; C3, merge; C4, Hoechst 33342 staining. Expression of DBC1 (red) and ERs (green) were investigated under the confocal fluorescence microscopy. Bars indicate 10 μm.

were maintained in DMEM supplemented with 10% FBS. Cells were fixed with PBS containing 4% paraformaldehyde and permeabilized in PBS with 0.2% (v/v) Triton X-100. After blocking, MCF-7 cells were incubated with anti-ERα (D-12, Santa Cruz Biotechnology) and anti-DBC1 antibodies. T47D cells were incubated with anti-ERβ (14C8, Novus Biologicals Inc., Littleton, CO, USA) and anti-DBC1 antibodies. Secondary antibodies were Alexa fluor 488 conjugated donkey anti-mouse IgG, and Alexa fluor 555 conjugated goat anti-rabbit IgG. The slides were briefly counter-stained and analyzed under the confocal fluorescence microscope (Carl-Zeiss MicroImaging Inc., Oberkochen, Germany).

GST-pull down assay. GST fusion proteins or GST alone were expressed in *Escherichia coli* and bound to glutathione-sepharose 4B beads (GE healthcare UK Ltd., Buckinghamshire, UK). Immobilized GST-ERα/β AF-2 fusion proteins were preincubated for 30 min in GST binding buffer (20 mM Tris–HCl pH 7.5, 200 mM NaCl, 1 mM EDTA) with E_2 (10^{-6} M). The GST proteins were incubated at 4 °C with indicated [^{35}S] methionine-labeled proteins. After 1 h incubation, unbound proteins were removed by washing the beads in GST binding buffer containing 0.5% Nonidet P-40 and proteases inhibitor cocktail. Specifically bound proteins were eluted by boiling in SDS sample buffer and analyzed by 7~12% SDS–polyacrylamide gel electrophoresis and autoradiography.

Luciferase assay. Two days before transfection, the medium was changed to phenol red-free DMEM containing 5% charcoal stripped FBS. Transfection was performed with Effectene reagent (Qiagen, Hilden, Germany) according to the manufacturer's recommendation. For luciferase assay, 250 ng ERE-tk-luc plasmid was cotransfected with indicated expression vectors. As an internal control to equalize transfection efficiency, 1 ng of phRL CMV luc vector (Promega Co., Madison, WI, USA) was cotransfected in all the experiments. Individual transfections, each consisting of triplicate wells, were repeated at least three times [16].

RNAi. The ablation of DBC1 was performed by the transfection of MDA-MB-231 cells with either of two siRNA duplex oligos synthesized by Qiagen. Either of two DBC1-specific siRNA (DBC1-RNAi: 5′ AAACGGAGCCUACUGAACA 3′, which covered mRNA regions of nucleotides 1379–1397 (amino acids 460–466) of DBC1 or KIAA1967-RNAi: 5′-CGCUUAUAGUUCGAAGGUA-3′, SI00461853), or control siRNA (All Stars Negative Control siRNA, 1027281) was transfected by using HyperFect reagent (Qiagen).

RNA extraction and real-time quantitative PCR. MDA-MB-231 cells were transfected with pcDNA3 (control) or pcDNA Myc DBC1. The cells were treated with vehicle (Ethanol), E_2 (10^{-9} M), or DPN (10^{-8} M) and incubated for subsequent 24 h. Total RNA was extracted from the cells using RNeasy Mini Kit (Qiagen) and real-time quantitative PCR was performed. The amplification of Bcl-2 mRNA was performed using primers for cDNA of Bcl-2, upstream 5′-AGGATTGTGGCCTTCTTTGAG-3′ and downstream 5′-CCTGCAGCTTTGTTTCATGGT-3′ [18]. Bcl-2 mRNA levels were normalized to RNA loading for each sample using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA as an internal standard. Data analyses were performed using a Light Cycler (Roche Applied Science, Mannheim, Germany).

Results

ERβ and DBC1 interact in vivo

The fact that endogenous ERα and DBC1 interacts in the absence of E_2 led us to examine whether DBC1 protein interacts with ERβ in cultured human cells. ERβ was immunoprecipitated in MDA-MB-231 cell lysates and a complex formation of the precipitated proteins was confirmed by Western blotting. Immunoblotting revealed the existence of DBC1 in the cell lysate immunoprecipitates (Fig. 1A), which supports our hypothesis that DBC1 physically associates with ERβ in living cells. This result was further confirmed by immunofluorescence studies using the specific antibodies raised against ERβ and DBC1. Immunofluorescence staining revealed the ligand-independent colocalization of ERβ and DBC1 in the nucleus of T47D cells (Fig. 1C, 1–4). As expected from the previous study, ERα and DBC1 showed ligand-independent colocalization in the nucleus of MCF-7 cells (Fig. 1B, 1–4).

ERαβ and DBC1 interact in vitro in a ligand-independent manner

To address the functional importance of the DBC1–ERβ interaction, *in vitro* translated [^{35}S] methionine labeled DBC1 was incu-

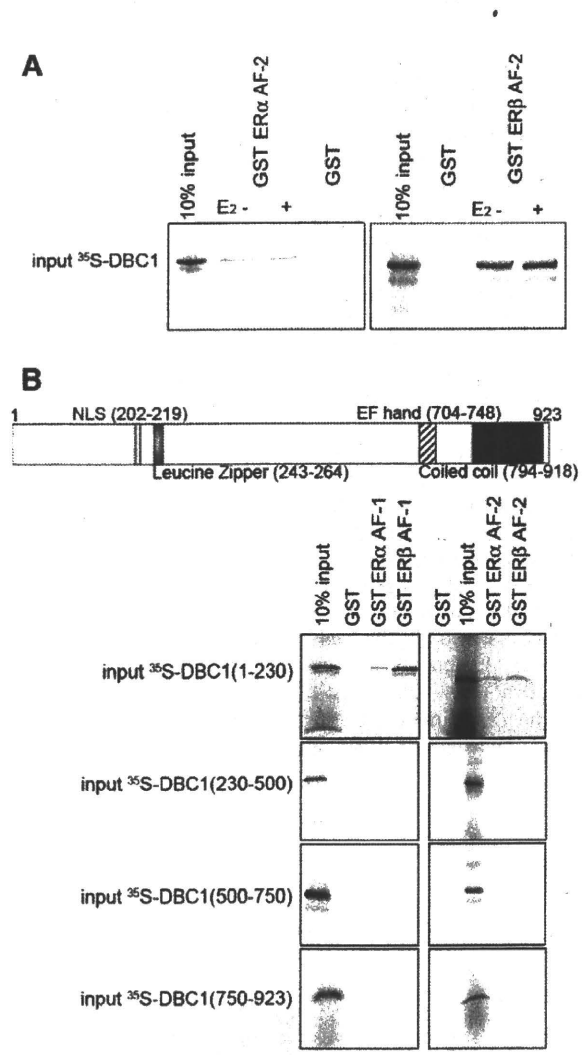

Fig. 2. *In vitro* association between DBC1 and ERαβ, and mapping of the ERβ-interacting region of DBC1. (A) Ligand independent association between full-length DBC1 and AF-2 region of ERαβ using GST-ERαβ AF-2 and DBC1. Bacterially expressed GST fusion proteins immobilized on beads were used in *in vitro* pull-down assays. Full-length DBC1 was *in vitro* translated in the presence of [^{35}S] methionine using a TNT coupled *in vitro* translation system. Labeled DBC1 was then incubated with GST-ERαβ AF-2. GST-ERαβ AF-2 was treated with or without 10^{-6} M of E_2. The mixture was washed and subjected to SDS–PAGE and analyzed. (B) Mapping of the DBC1-interaction region of ERαβ using GST-ERαβ AF-1, GST-ERαβ AF-2, and fragments of DBC1. DBC1 amino acids 1–230, 230–500, 500–750, and 750–923 were *in vitro* translated. The fragments of DBC1 and GST-ERαβ AF-1 and AF-2 were tested for interaction. The mixture was washed and subjected to SDS–PAGE and then visualized by autoradiography. Polyacrylamide gels were stained briefly with Coomassie Brilliant Blue to verify the loading of equal amounts of fusion proteins prior to drying and autoradiography.

bated with GST-fused ERαβ AF-2. As clearly shown in Fig. 2A, GST-fused ERαβ AF-2 protein possessed the ability to retain DBC1 on the column in the presence and absence of E_2.

To map the region of DBC1 that interacts with ERαβ, GST-fused ERαβ AF-1 or AF-2 and *in vitro* translated DBC1 fragments, (1–230 amino acids), (230–500), (500–750), and (750–923), were incubated and tested for the interaction. Only amino-terminus of DBC1 including the NLS interacted with ERαβ AF-2 in a ligand-independent manner. Interestingly, ERαβ AF-1 showed different interaction pattern because the GST ERβ AF-1 column exhibited significantly stronger interaction with amino-terminus of DBC1 compared with GST ERα AF-1 column (Fig. 2B).

DBC1 exhibits no influence on the transcriptional activation function of ERα

To examine the cofactor activity of DBC1 in the transactivation function of ERα, transient transfection assays were performed using a luciferase reporter plasmid driven by the thymidine kinase promoter containing estrogen responsive element (ERE-tk-luc). Although ERα showed a ligand-dependent transactivation function in 293T cells, a transient coexpression of DBC1 showed no influence on the luciferase activity of ERα (Fig. 3A). Role of SIRT1 in regulating transactivation of ERα was confirmed and the expression of SIRT1 decreased luciferase activity of ERα (Fig. 3A). The transcriptional regulation of ERα played by DBC1 was further confirmed in MDA-MB-231 cells (Fig. 3B). The ligand-induced transactivation function of ERα was unaffected by short-interference RNA (siRNA) mediated depletion of DBC1 (Fig. 3B).

DBC1 represses the transcriptional activation function of ERβ

The cofactor activity of DBC1 in the transactivation function of ERβ was further confirmed. Although ERβ showed a ligand-dependent transactivation function in 293T cells, a transient coexpression of DBC1 led to a significant decrease in luciferase activity of ERβ (Fig. 4A and B). Role of SIRT1 in regulating transactivation of ERβ was analyzed but the luciferase activity of ERβ was unaffected by the expression of SIRT1 unlike ERα. This downregulation of transactivation by DBC1 was further confirmed in MDA-MB-231 cells. The ligand-induced transactivation function of ERβ was stimulated by transfecting siRNA of DBC1 (Fig. 4B). To evaluate the effect of DBC1 on the endogenous gene expression, mRNA expression of Bcl-2 was examined because this anti-apoptotic gene has been shown to be an ERβ-repressed gene in the ventral prostate [6]. The transient expression of DBC1 resulted in an increase of Bcl-2 mRNA in the presence of ERβ ligands, namely, E_2 or DPN (Fig. 4C). These data indicate a significant role of DBC1 in the ligand-dependent repression function of ERβ.

Discussion

The recent study has shown that the overexpression of DBC1 and SIRT1 are related to the poorer prognosis in gastric cancer patients [19]. In addition, the expression of DBC1 was not substantially abrogated in various cancers from any tissue [15]. Even though DBC1 was originally identified as a candidate for breast tumor suppressor gene, it remains unknown whether the aberrant expression of DBC1 is correlated with carcinogenesis. The precise molecular and cellular mechanisms of DBC1 in tumorigenesis should be solved to understand the physiological function of DBC1. Not only the interaction between ERα and DBC1 [15], but the interaction between ERβ and DBC1 may have a significant role in the pathogenesis of breast cancer. ERβ plays a multifaceted role in the functional differentiation of various epithelial and non-epithelial cell types and ERβ seems to be essential for driving cellular differentiation and apoptosis. Many studies to find correlations between breast tumorigenesis and ERs have been conducted, and an increase in ERα/ERβ ratio in breast cancer as compared with benign tumors and normal tissues has been reported [20–22]. These studies indicate that healthy mammary glands express more ERβ mRNA than do breast cancer samples. A number of *in vitro* functional studies have been performed to examine the effect of ERβ expression on the proliferation of breast cancer cells [23,24]. Although the results are not unanimous, the majority of studies conclude that an increase in ERβ expression decreases cell proliferation. All these observations suggest that loss of the expression of ERβ may be involved in tumor progression. The American Association for Cancer Research Task Force Report has mentioned ERβ as a possible target for chemoprevention in a number of cancers, including breast cancer [25]. Several ERβ selective ligands such as DPN [26], ERB-41 [27], and TAS-108 [28] have been developed and evaluated *in vivo*. Among them, TAS-108 is a selective ERα antagonist with partial ERβ agonist activity, and a Phase I clinical trial using

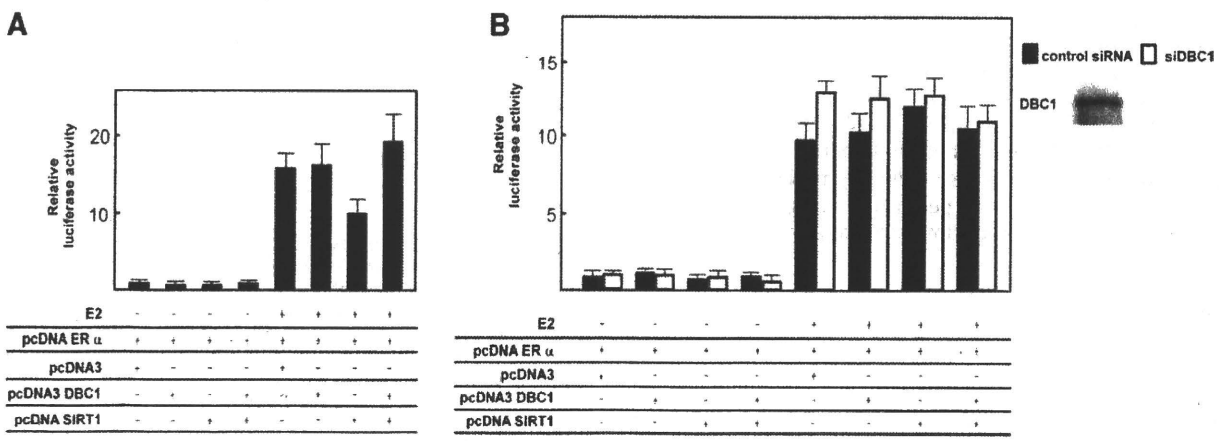

Fig. 3. DBC1 shows no influence on ligand-dependent transcriptional activation function of ERα. (A) Transient transfection assays were performed to examine the activity of DBC1 in the transactivation function of ERα. The expression of DBC1 showed no effect on the ligand-dependent transactivation function of ERα in 293T cells. (B) siRNA-mediated depletion of the endogenous DBC1 was performed in MDA-MB-231 cells. The cells were transfected with siRNA specific for DBC1. Forty-eight hours after transfection, the cells were transfected with indicated expression vectors and reporter constructs. Twenty-four hours after transfection of expression vectors and reporter constructs (ERE-tk-luc), cells were harvested, and transfected whole cell lysates were assayed for luciferase activity produced from the reporter plasmid.

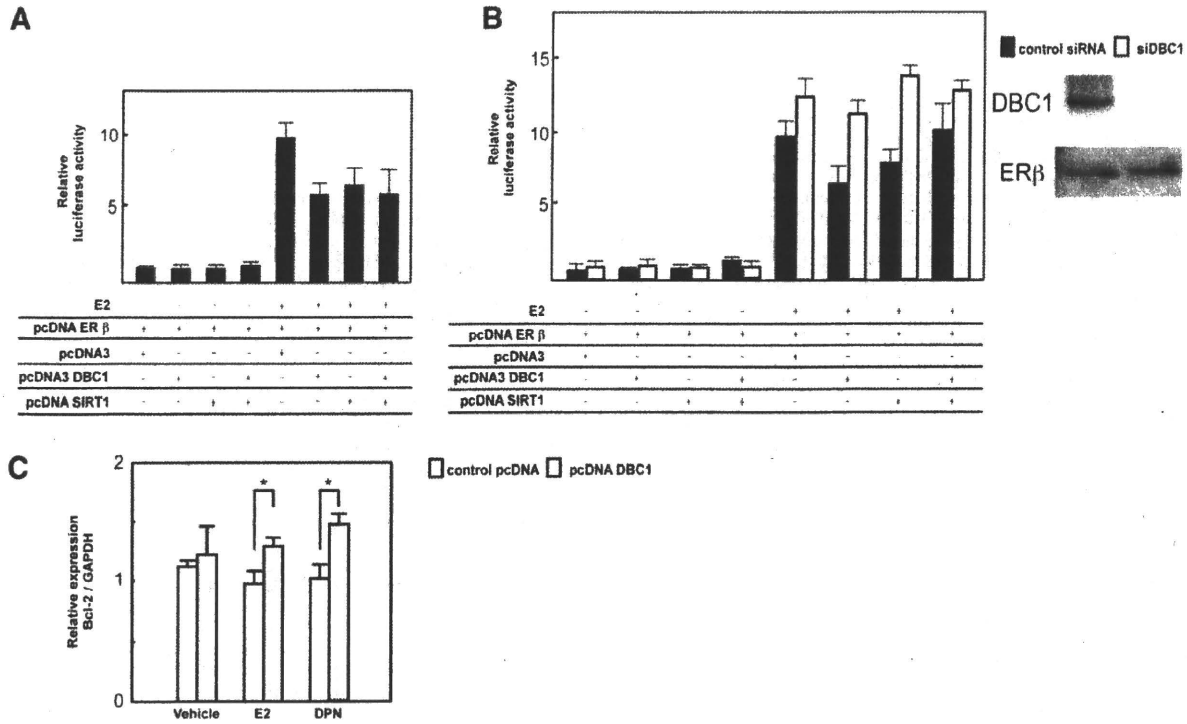

Fig. 4. DBC1 attenuates ligand-dependent transcription activation function of ERβ. (A) Transient transfection assays were performed to examine the activity of DBC1 in the transactivation function of ERβ. DBC1 showed a specific repression of ligand-dependent transactivation function of ERβ in 293T cells. (B) The repression function of DBC1 was reversed by siRNA-mediated depletion of the endogenous DBC1. MDA-MB-231 cells were transfected with siRNA specific for DBC1. Forty-eight hours after transfection, the cells were transfected with indicated expression vectors and reporter constructs. The expression of ERβ was unaffected by the siRNA-mediated knockdown of DBC1. (C) In the presence of E_2 and DPN, the expression of Bcl-2 mRNA was stimulated by transfecting the expression vector of DBC1.

TAS-108 has been conducted with promising results [29]. Therefore, the expression of ERβ and the stimulation of ERβ transcriptional activity have significant roles in the pathophysiology of tumor development. Our findings have demonstrated a novel role of DBC1 in repressing the transcriptional function of ERβ. This ligand-dependent repression function of DBC1 was confirmed by the expression of Bcl-2, an ERβ-repressed gene, and Bcl-2 was increased in a ligand-dependent manner. In contrast to the previous report [15], ERα AF-2 exhibited physical association with DBC1 irrespective of the presence of E_2. Although ERβ AF-2 also associated with DBC1 in a ligand-independent manner, DBC1 exhibited completely different transcriptional regulation of ERβ compared to that of ERα. This difference may be attributed to the fact that ERβ AF-1 showed robust interaction with DBC1. Taking into account of these data, DBC1 might be an ERβ-specific transcriptional repressor. We speculate that the DBC1–ERα complex formation would be related to hormone-independent tumor growth and the DBC1–ERβ complex formation would be related to hormone-dependent tumor growth. These complex formations between ERαβ and DBC1 would be tumorigenic and this fact would have implications for breast cancer prognosis and/or treatment. It is believed that a balance between proliferation (ERα) and apoptosis (ERβ) influences the response of breast tumors to hormonal therapy, and dysregulation of apoptotic signaling pathways has been suggested as a possible basis for treatment failure. Alterations in the expression and activity of ERs by DBC1 could tip the balance between breast tumor formation and death signaling. Further investigations are needed to elucidate the molecular mechanisms that underlie the formation of ERαβ–DBC1 in normal cellular growth, thereby evaluating DBC1 as a possible target for modality of prevention and medical treatment of breast cancer.

Acknowledgments

This study was supported by Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, JMS Bayer Schering Pharma Grant, and Kowa Life Science Foundation, Japan.

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Oncogene (2010) 29, 1897–1908

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ORIGINAL ARTICLE

Genome-wide single-nucleotide polymorphism arrays in endometrial carcinomas associate extensive chromosomal instability with poor prognosis and unveil frequent chromosomal imbalances involved in the PI3-kinase pathway

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Endometrial cancer is one of the tumor types in which either chromosomal instability (CIN) or microsatellite instability (MSI) may occur. It is known to possess mutations frequently in the Ras-PI3K (phosphatidylinositol 3′-kinase) pathway. We performed a comprehensive genomic survey in 31 endometrial carcinomas with paired DNA for chromosomal imbalances (25 by the 50K and 6 by the 250K single-nucleotide polymorphism (SNP) array), and screened 25 of the 31 samples for MSI status and mutational status in the Ras-PI3K pathway genes. We detected five or more copy number changes (classified as CIN-extensive) in 9 (29%), 1 to 4 changes (CIN-intermediate) in 17 (55%) and no changes (CIN-negative) in 5 (16%) tumors. Positive MSI was less common in CIN-extensive tumors (14%), compared with CIN-intermediate/negative tumors (50%), and multivariate analysis showed that CIN-extensive is an independent poor prognostic factor. SNP array analysis unveiled copy number neutral LOH at 54 loci in 13 tumors (42%), including four at the locus of *PTEN*. In addition to eight (26%) tumors with *PTEN* deletions, we detected chromosomal imbalances of *NF1*, *K-Ras* and *PIK3CA* in four (13%), four (13%) and six (19%) tumors, respectively. In all, 7 of the 9 CIN-extensive tumors harbor deletions in the loci of *PTEN* and/or *NF1*, whereas all the 10 MSI-positive tumors possess *PTEN*, *PIK3CA* and/or *K-Ras* mutations. Our results showed that genomic alterations in the Ras-PI3K pathway are remarkably widespread in endometrial carcinomas, regardless of the type of genomic instability, and suggest that the degree of CIN is a useful biomarker for prognosis in endometrial carcinomas.

Oncogene (2010) **29**, 1897–1908; doi:10.1038/onc.2009.474; published online 11 January 2010

Keywords: chromosomal instability; microsatellite instability; copy number neutral LOH; homozygous deletions; Ras-PI3K pathway; endometrial carcinoma

Introduction

Genomic alterations, such as DNA sequence changes, genomic instability and epigenetic gene silencing, cooperate to develop and progress human malignancies. Understanding the molecular basis of cancer has now become feasible through the application of genome analysis technologies, as in a project started by The Cancer Genome Atlas (Cancer Genome Atlas Research Network, 2008).

Endometrial cancer is the fourth most frequent cancer in women and its incidence is increasing (Parkin, 2001). There are two different pathogenetic types of endometrial carcinomas: estrogen-dependent type I and estrogen-independent type II (Ryan *et al.*, 2005; Doll *et al.*, 2008). Approximately, 80% of endometrial carcinomas are endometrioid adenocarcinomas, generally considered as type I tumors. Type II is composed of high-grade tumors (such as serous adenocarcinomas or clear cell carcinomas) with aggressive behavior. Even among type I tumors, recurrent tumors respond limitedly to systemic therapy and the prognosis is very poor. Microsatellite instability (MSI) is associated with mutator phenotype, and is reported approximately at 15–20% in endometrial and colorectal cancer (Atkin, 2001; Woerner *et al.*, 2003). MSI in endometrial cancer is much more frequent in type I endometrioid adenocarcinomas than in type II tumors (Tashiro *et al.*, 1997; An *et al.*, 2004). Chromosomal instability (CIN) is measured by the number of chromosomal copy alterations within entire tumor genomes, and the CIN phenotypes can be classified according to the level of alterations, such as CIN-high, CIN-low and CIN-very low (Rowan *et al.*, 2005; Weber *et al.*, 2007; Geigl *et al.*, 2008). CIN is inversely correlated with MSI in colorectal cancer (Choi

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Received 27 June 2009; revised 7 November 2009; accepted 19 November 2009; published online 11 January 2010

et al., 2002; Grady, 2004); however, the relationship between CIN and MSI and its prognostic effect in type I endometrioid adenocarcinomas is still controversial (Hirasawa *et al.*, 2003; Arabi *et al.*, 2009). We especially focused on endometrioid adenocarcinomas to clarify whether the status of genomic instability, determined by CIN and MSI analyses, is associated with morphologically indistinguishable tumor aggressiveness.

Copy number neutral (CNN) LOH is a type of genomic alteration caused by the loss of one allele and gain of the opposite allele. Recently, single-nucleotide polymorphism (SNP) arrays, which could provide allele-specific copy number information, have been applied to detect segmental uniparental structures (Fitzgibbon *et al.*, 2005; Teh *et al.*, 2005; Midorikawa *et al.*, 2006). Somatic CNN LOH has been increasingly recognized as a common molecular defect in various types of cancer. In addition, improvement of resolution by SNP arrays is also useful in identifying microdeletions, including homozygous deletions (HDs) (Komura *et al.*, 2006; Gorringe *et al.*, 2007). SNP array analyses have unveiled that regions surrounding various tumor suppressor genes (such as *CDKN2A*, *RB1*, *TP53*, *BRCA1*, *BRCA2*, *WT1*, *CEBPA*, *RUNX1* and *NF1*) frequently show CNN LOH in many types of tumors (Fitzgibbon *et al.*, 2005; Raghavan *et al.*, 2005; Flotho *et al.*, 2007; Walsh *et al.*, 2008). Recent studies also indicate that CNN LOH regions may carry activated oncogenes, such as mutated *JAK2*, *HRAS* and *NRAS* (Kralovics *et al.*, 2005; Kratz *et al.*, 2007; Dunbar *et al.*, 2008). Thus, SNP array is a useful methodology to identify novel genomic alterations in specific genes and pathways.

The phosphatidylinositol 3′-kinases (PI3Ks) are widely expressed lipid kinases that catalyze the production of the second messenger phosphatidylinositol 3,4, 5-triphosphate, which activates a wide range of downstream targets, including Akt (Stokoe *et al.*, 1997). Ras-PI3K signaling is activated through various genetic alterations, such as mutations in *Ras*, *PTEN*, *EGFR*, *PIK3CA* and *AKT1* (Vogelstein *et al.*, 1988; Li *et al.*, 1997; Lynch *et al.*, 2004; Samuels *et al.*, 2004; Carpten *et al.*, 2007), and amplifications in *ERBB2*, *AKT2* and *PIK3CA* (Slamon *et al.*, 1989; Cheng *et al.*, 1992; Shayesteh *et al.*, 1999). In endometrial cancer, high prevalence of mutations of the genes in the Ras-PI3K pathway is reported, including *K-Ras*, *PTEN* and *PIK3CA* (Enomoto *et al.*, 1991; Kong *et al.*, 1997; Oda *et al.*, 2005). We reported that more than 70% of endometrial carcinomas contain one or more mutations in the Ras-PI3K pathway (Oda *et al.*, 2008). However, the role of chromosomal imbalances in this pathway is not fully understood. Various kinds of inhibitors targeting the PI3K pathway have been developed and are now under clinical trials (Kong and Yamori, 2008; Maira *et al.*, 2009). Detailed analysis of genomic alterations in this pathway might clarify the possibility of these molecular targeted therapies in endometrial carcinomas.

In this study, we attempted to comprehensively figure out genomic alterations in endometrial carcinomas. First, we classified endometrial carcinomas into three subgroups according to the CIN status, and found an inverse trend between the high degree of CIN (CIN-extensive) and positive MSI. Second, we show that CIN-extensive is an independent poor prognostic predictor in endometrial carcinomas. Third, we focused on the genes involved in the Ras-PI3K pathway for mutations, CNN LOH, HD and other chromosomal imbalances and clarified that genomic alterations associated with the Ras-PI3K pathway are exceedingly widespread in endometrial carcinomas.

Results

Chromosomal gains, losses, CNN LOH and HDs in endometrial carcinomas

As endometrioid adenocarcinoma is the most common histological type showing a high ratio of MSI, we specifically focused on endometrioid adenocarcinomas in this study. We evaluated chromosomal imbalances in a total of 31 endometrioid adenocarcinomas with paired DNA (tumor and normal) by SNP arrays (25 samples on the Affymetrix 50K array and 6 on the 250K array). Chromosomal imbalances with one or more loci were detected in 26 (84%) out of 31 tumors. Gains on chromosome arms were observed on 1q (29%), 2p (10%), 7q (13%), 8p (10%), 8q (19%), 10p (10%), 10q (13%), 12p (16%), 12q (16%), 17q (10%), 20p (13%) and 20q (10%). LOH was detected on 1p (19%), 1q (10%), 2q (13%), 5q (13%), 6q (19%), 9p (16%), 9q (16%), 10q (29%), 16q (19%), 17p (16%) and 22p (10%) (Figure 1 and Supplementary Table 2). The regions of these gains and losses were compatible with the reports by comparative genomic hybridization (CGH) (Suehiro *et al.*, 2000; Micci *et al.*, 2004; Levan *et al.*, 2006).

CNN LOH involves allelic changes, including hemizygous deletion with a gain of the opposite allele. In addition to chromosomal gains (16/31; 52%) and LOH (19/31; 61%), we detected CNN LOH in 13 samples (42%) at 54 regions (Table 1). All these CNN LOH are somatic events, as these regions in the tumors were identified by retention of heterozygsity in the paired germline DNA. In addition, we assessed known copy number variations (CNVs) from the Database of Genomic Variants v8 (http://projects.tcag.ca/variation/), and confirmed that all of these 54 CNN LOH included non-CNV regions. The regions of CNN LOH include 8q and 10q, at which copy number gains are frequently detected in endometrial carcinomas. Five of the 54 CNN LOH (9%) occur in the whole arm of each chromosome (1p, 1q, 6p, 18p and 18q). The minimal CNN LOH region in 51 out of the 54 was more than 100 kb, and the other three regions with <100 kb were detected in both the 50K and the 250K SNP array (Table 1), suggesting that the 50K SNP probes distinguish CNN LOH at a comparable level with the 250K probes. We found that the CNN LOH regions frequently include the loci of several well-known tumor suppressor genes, such as *CDKN2A* (9p21.3), *PTEN* (10q23.3) and *TP53* (17p13.1).