Heightened Expression of CTCF in Breast Cancer Cells Is Associated with Resistance to Apoptosis

France Docquier,[1] Dawn Farrar,[1] Vivien D'Arcy,[1] Igor Chernukhin,[1] Abigail F. Robinson,[2] Dmitry Loukinov,[5] Sergei Vatolin,[5] Svetlana Pack,[5] Alan Mackay,[3] Robert A. Harris,[3] Heather Dorricott,[4] Michael J. O'Hare,[3] Victor Lobanenkov,[5] and Elena Klenova[1]

[1]Department of Biological Sciences, Central Campus, University of Essex, Essex; [2]Genetics Unit, Department of Biochemistry, University of Oxford, Oxford; [3]LICR/UCL Breast Cancer Laboratory; [4]Department of Physiology, University College London, London, United Kingdom; and [5]Molecular Pathology Section, Laboratory of Immunopathology, NIAID/NIH, Rockville, Maryland

Abstract

***CTCF* is a candidate tumor suppressor gene encoding a multifunctional transcription factor. Surprisingly for a tumor suppressor, the levels of CTCF in breast cancer cell lines and tumors were found elevated compared with breast cell lines with finite life span and normal breast tissues. In this study, we aimed to investigate the possible cause for this increase in CTCF content and in particular to test the hypothesis that up-regulation of CTCF may be linked to resistance of breast cancer cells to apoptosis. For this purpose, apoptotic cell death was monitored following alterations of CTCF levels induced by transient transfection and conditional knock-down of CTCF in various cell lines. We observed apoptotic cell death in all breast cancer cell lines examined following CTCF down-regulation. In addition, overexpression of CTCF partially protected cells from apoptosis induced by over-expression of Bax or treatment with sodium butyrate. To elucidate possible mechanisms of this phenomenon, we used a proteomics approach and observed that levels of the proapoptotic protein, Bax, were increased following CTCF down-regulation in MCF7 cells. Taken together, these results suggest that in some cellular contexts CTCF shows anti-apoptotic characteristics, most likely exerting its functions through regulation of apoptotic genes. We hypothesize that CTCF overexpression may have evolved as a compensatory mechanism to protect breast cancer cells from apoptosis, thus providing selective survival advantages to these cells. The observations reported in this study may lead to development of therapies based on selective reduction of CTCF in breast cancer cells.** (Cancer Res 2005; 65(12): 5112-22)

Introduction

CTCF (or CCCTC-binding factor) is a ubiquitous 11-zinc finger protein with highly versatile functions. In addition to transcriptional silencing or activation in a context-dependent fashion, it organizes epigenetically controlled chromatin insulators that regulate imprinted genes in soma (1–3). Our previous reports indicate that *CTCF* could be a new tumor suppressor gene (TSG) because (i) it suppresses cell growth (4); (ii) it is localized at the 16q22 chromosomal region associated with loss of heterozygosity in breast malignancies (5); and (iii) functionally significant, tumor-specific mutations in the *CTCF* gene have been identified and characterized in various cancers including breast tumors (6). It is generally acknowledged that the process of tumor development is associated with step-by-step activation of growth-promoting cellular oncogenes and inactivation of TSG, usually resulting in overexpression of oncogenes and under-expression of TSG (7). It was therefore surprising to find that levels of CTCF were elevated rather than reduced in breast cancer cell lines and breast tumors (this study) leading us to further examine this finding. As suppression of apoptosis is one of the critical factors supporting tumor progression (8, 9), one possible function of increased levels of CTCF would be to promote cell survival by protecting cancer cells from apoptosis. The main objective of this study was to test the hypothesis that elevated levels of CTCF in breast cancer cell lines and tumors may indeed be a mechanism mediating resistance to apoptosis. By using different cellular models and independent apoptotic markers, we show that reduction of CTCF levels in breast cancer cells leads to apoptotic cell death and that overexpression of CTCF can partially protect breast cancer cells from induction of apoptosis.

Materials and Methods

Breast tissues. Primary human tumor breast tissues together with paired normal peripheral tissues were collected during surgery from patients treated at Colchester General Hospital (Essex, United Kingdom), with written consent taken before surgery. Normal breast tissue samples were collected after reduction surgery. Tissue specimens were visually examined by an experienced pathologist as fresh material, tumor tissues were selected by conventional pathologic criteria, and the histopathology was further confirmed by microscopic examination. The samples were immediately frozen and stored at −80°C. Histology of the tissues is described in Supplementary Table 1.

Cell lines. A panel of breast cell lines of different origins ranging from immortalized to transformed human breast cells was obtained from M. O'Hare (LICR/UCL Breast Cancer Laboratory, London, United Kingdom) and B. Gusterson (Department of Pathology, University of Glasgow, Glasgow, United Kingdom). It included two cell lines, HBL100 and HB4a, which originated from normal breast epithelium and were immortalized with SV40 T antigen. HB4a was established from normal breast luminal cells (10) and HBL100 was derived from cells obtained from the milk of a nursing mother (11). Six estrogen receptor–positive (ER+) cell lines originating from human breast carcinomas were T47D, MCF7, BT474, CAMA1, ZR75-1, and ZR75-30. Nine ER-negative breast

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/). A. Robinson is currently at Elsevier Ltd., The Boulevard, Langford Lane, Kidlington, Oxford, OX5 1GB, United Kingdom. A. Mackay is currently at The Breakthrough Toby Robins Breast Cancer Research Centre, Chester Beatty Laboratories, Institute of Cancer Research, Fulham Road, London, SW3 6JB, United Kingdom. R. Harris is currently at Arrow Therapeutics, Britannia House 7, Trinity Street, London, SE1 1DA, United Kingdom. H. Dorricott is currently at Centre for Auditory Research, 332 Grays Inn Road, London, WC1X 8EE, United Kingdom.

Requests for reprints: Elena Klenova, Department of Biological Sciences, University of Essex, Wivenhoe Park, Colchester, Essex CO4 3SQ, United Kingdom. Phone: 44-0-1206-874868; Fax: 44-0-1206-872592; E-mail: klenovae@essex.ac.uk.

cancer cell lines (ER−) included HMT3522 (a cell line derived from fibrocystic breast tissue); adherent carcinomas MDA175, MDA231, MDA435, MDA453, MDA468, SKBR5, and SKBR7; and nonadherent carcinoma DU4475. Finite life span human mammary epithelial cells (HMEC; refs. 12, 13) were obtained from Cambrex (Walkersville, MD). Cell lines from two individuals were termed HMEC1 and HMEC2. More detailed information on these cell lines is presented in Supplementary Table 2. All cell lines were maintained in RPMI 1640 supplemented with HEPES, GlutaMAX, sodium bicarbonate, 20 μg/mL gentamicin, 10% FCS (all from Life Technologies, Gaithersburg, MD). For HB4a cells, 5 μg/mL of insulin (Sigma, St. Louis, MO) and 5 μg/mL of hydrocortisone (Sigma) were included in the medium. HMEC were grown according to the manufacturer's instructions.

A panel of human cell lines not derived from breast tissues included embryonic kidney 293T, prostate carcinoma DU145, prostate adenocarcinoma LNCaP, testicular carcinoma NTERA2, cervix carcinoma HeLa, bladder carcinomas J82 and T24, osteosarcoma UTA6, hepatocellular carcinoma HepG2, melanoma G361, rhabdomyosarcoma RD, and lung carcinoma A549 (details on these cell lines and their growth conditions are summarized in Supplementary Table 3).

Expression vectors and transient transfections. The vectors for transient transfection were made by insertion of the full-length CTCF cDNA (14) in both orientations into the pcDNA3 vector (Invitrogen, Carlsbad, CA). The pSFFV-Bax construct was a kind gift from S. Korsmeyer (Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA). Transient transfections were done using a calcium phosphate transfection protocol (15).

Isopropyl-L-thio-B-D-galactopyranoside-inducible antisense CTCF system. MCF/LAP5 cells expressing an isopropyl-L-thio-B-D-galactopyranoside (IPTG)–dependent transactivator LAP267 were provided by L. Lau and D. Pestov (Department of Biochemistry and Molecular Genetics, University of Illinois, Chicago, IL; ref. 16). Construction of the IPTG-inducible episomal vector pEpiLac3 and pEpiLac*CTCF* (in both orientations) was described previously (17, 18). A schematic outline of the antisense construct, pEpiLacCTCFanti, is shown in Fig. 4*A*. The antisense CTCF construct and the empty pEpiLac3 were transfected into the MCF/LAP5 cells. The Hygro-resistant cells were selected, cloned, and screened for IPTG-controlled expression of CTCF. One of the clones, termed MCF/LAP5/antiCTCF, was used in this study.

Western blotting analysis, two-dimensional gel electrophoresis, and mass spectrometry. Lysates from different cell lines were prepared according to Klenova et al. (19). Western blotting was done as described previously (20). In brief, cell lysates were resolved in SDS-PAGE, immunoblotted, and probed with anti-CTCF (Abcam, Cambridge, Cambs, United Kingdom), anti-Bax (Cell Signaling, Beverly, MA), anti-α-tubulin and anti-β-actin (both from Sigma) antibodies. Immunocomplexes were detected by enhanced chemiluminescence reagent (Amersham Biosciences, Little Chalfont, Bucks, United Kingdom) according to the manufacturer's instructions. For normalization of the data, membranes were reprobed with the anti-α-tubulin or anti-β-actin antibodies to enable cross-comparison between individual samples. Quantification of band intensities was done by using the Bio-Rad Quantity One Quantitation software.

Preparation of protein extracts for two-dimensional gel electrophoresis was carried out as described earlier (21). Samples for mass spectrometry were prepared according to Chernukhin et al. (22). The MALDI-TOF (Bruker Daltonics Reflex 4) was used to map the mass of the tryptic digests. The spectra were analyzed using Xtof 5.1.5 Bruker Daltonics software and the sequence retrieval was done using MASSCOT sequence query (http://www.matrixscience.com) on the SWISS-PROT database.

Immunofluorescence, apoptosis monitoring, apoptosis induction, and immunohistochemistry. For indirect immunofluorescent staining, standard protocols were used (23). For CTCF staining, an additional modification that involved a step of microwave heating after fixation of cells in formaldehyde was included (24). The primary anti-CTCF antibody and the secondary FITC (green fluorescence)– or TRITC (red fluorescence)–conjugated swine anti-rabbit antibodies (DAKO, Carpinteria, CA) were used at dilution of 1:25 (primary antibody) and 1:40 (secondary antibodies). Bromodeoxyuridine (BrdUrd) incorporation by cells containing IPTG-inducible antisense *CTCF* mRNA was monitored at selected times during culture by pulse labeling for 3 hours. Staining with anti-BrdUrd antibody was done according to the manufacturer's instructions (Amersham Pharmacia, Piscataway, NJ). Several techniques were used to monitor apoptotic cell death. The terminal deoxynucleotidyl transferase–mediated nick end labeling (TUNEL) method was employed for detection of apoptosis using the *in situ* cell detection kit, tetramethylrhodamine red (TMR, red fluorescence) from Roche Applied Science (Lewes, East Sussex,

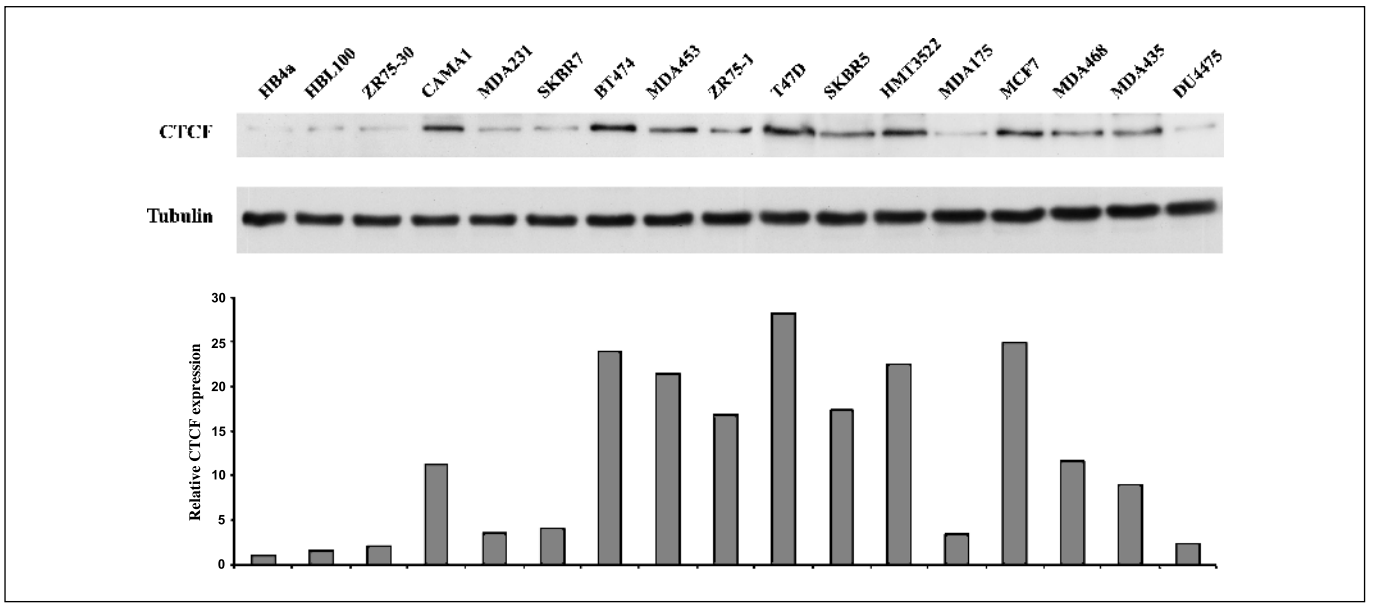

Figure 1. Western blot analysis of CTCF protein levels in human breast cell lines. Cellular extracts were prepared from 5×10^6 cells and equal amounts (40 μg) of total protein were loaded onto SDS-PAGE. Samples were electrophoretically separated, blotted, and probed with the anti-CTCF antibody. The membrane was reprobed with the anti-α-tubulin antibody, which served as an internal control for protein loading. The developed films were scanned and quantified. The ratios of the intensity of the CTCF bands over the intensity of the corresponding α-tubulin bands were determined and expressed as fold change relative to the lowest CTCF/α-tubulin ratio found in HB4a (designated as 1.0). Columns in the histogram are the results.

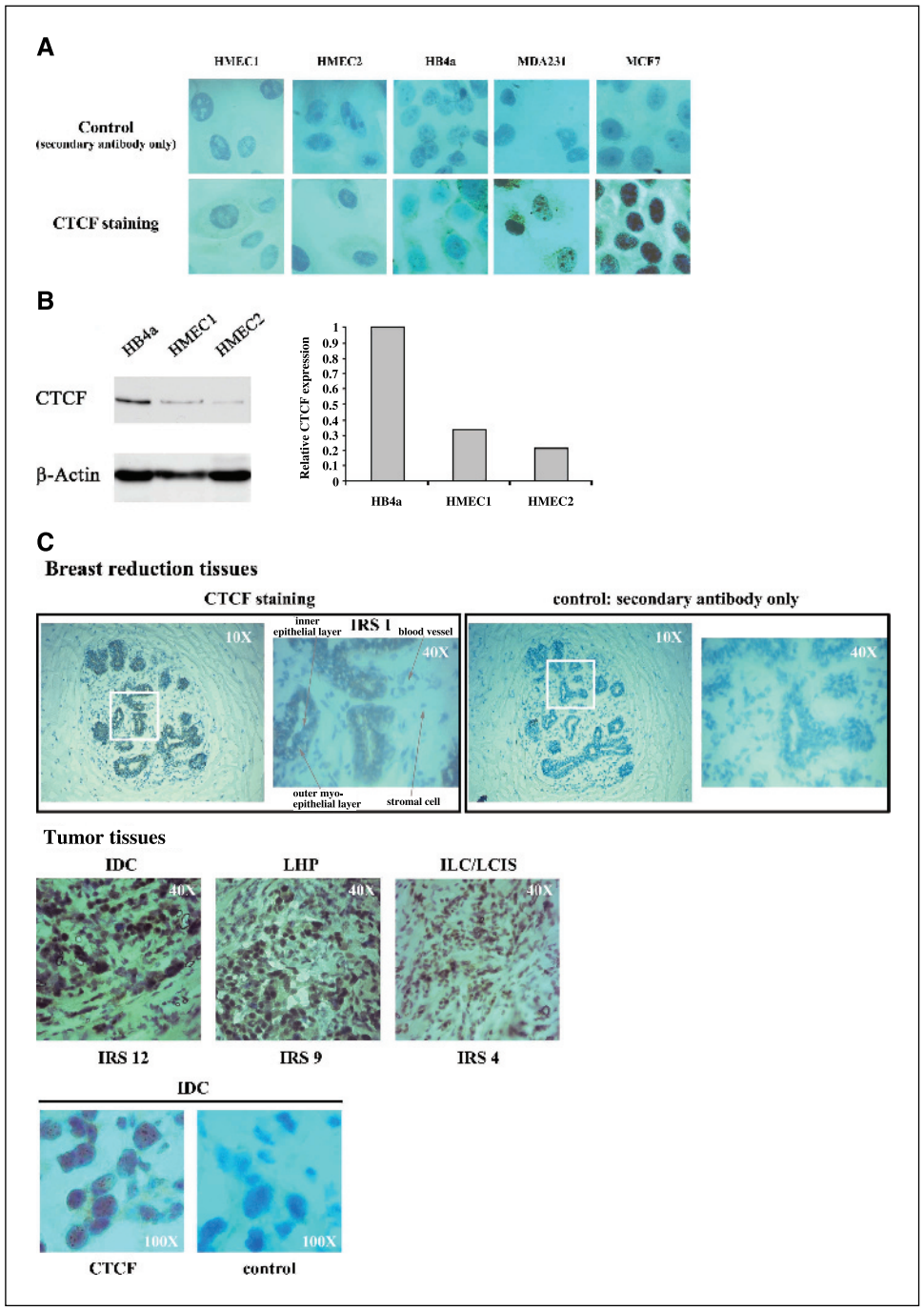

Figure 2. CTCF is expressed at higher levels in breast cancer cell lines and primary tumors than in breast cells with finite life span and normal tissues. *A,* immunohistochemical staining of CTCF in breast cell lines showing very low (HMEC from two individuals and HB4a), medium (MDA231), and high (MCF7) expression levels of CTCF (*bottom*). Background staining with hematoxylin plus secondary antibodies only (*top*). *B,* Western blot analysis of HMEC1 and HMEC2 cells compared to HB4a. Cellular extracts were prepared from 5×10^6 cells and equal amounts (60 μg) of total protein were loaded onto SDS-PAGE. Samples were electrophoretically separated, blotted, and probed with the anti-CTCF antibody. The same membrane was reprobed with the anti-β-actin antibody, which served as an internal control for protein loading. The developed films were scanned and quantified. The ratios of CTCF/β-actin were calculated and expressed as fold change relative to HB4a (designated as 1). Columns in the histogram are the results. *C,* immunohistochemical staining of CTCF in normal and tumour breast tissues. *Top,* breast reduction tissues stained with the anti-CTCF antibody (*left*). Background staining with hematoxylin plus secondary antibodies (*right*). Two cell layers (*inner epithelial* and *myoepithelial*) of breast duct, blood vessel, and stromal cell (*arrows*). *Middle,* examples of tumors with different IRS immunostained with the anti-CTCF antibody are presented. *Bottom,* section of an IDC with IRS12 at higher (×100) magnification. *Right,* background staining with hematoxylin plus secondary antibodies only. Abbreviations: *IDC*, invasive ductal carcinoma; *LHP*, lobular hyperplasia; *ILC*, infiltrating lobular carcinoma; *LCIS*, lobular carcinoma *in situ*.

United Kingdom) according to the manufacturer's instructions. For detection of the characteristic chromatin condensation and nuclear fragmentation associated with apoptosis, staining with 4′,6-diamidino-2-phenylindole dilactate (DAPI; Sigma) at 5 μg/mL in 1× PBS for 15 minutes was conducted (25). Apoptosis was also monitored by staining with the M30 monoclonal antibody that is specific for an epitope of cytokeratin 18 uncovered by caspase cleavage (Roche Molecular Biochemicals, Lewes, East Sussex, United Kingdom). Apoptotic cell death in MCF7 cells was induced with 5 mmol/L sodium butyrate as described previously (26). Immunofluorescence was visualized using confocal laser scanning microscopy (Bio-Rad, Hercules, CA).

Immunohistochemical analysis was done by staining with the Vectastain Elite avidin-biotin complex method standard kit (Vector Laboratories, Burlingame, CA) as suggested by the manufacturer. The dilution of the anti-CTCF antibody applied to the sections was 1:50. Several breast cell lines, HMEC and frozen tissues sections were tested by immunohistochemistry. The histologic types of breast tissues analyzed in this assay included 18 invasive ductal carcinomas, three lobular carcinomas, three ductal carcinomas *in situ*, four adenocarcinomas, one medullary carcinoma, one lobular hyperplasia, and paired peripheral normal breast tissue samples, in addition to normal breast reduction tissues. The immunologic staining was evaluated by using the immunoreactivity score (IRS) as previously described (27). In brief, the percentage of CTCF-positive cells was divided into four categories (<10%, 11-50%, 51-80%, and >80%), whereas the staining intensity was given a scale from 0 (no detectable immunostaining) to 3 (strong immunostaining). The IRS (0-12) was then calculated by multiplying the score values. A minimum of 400 cells was counted in several fields. Statistical analysis was done using Student's *t* test.

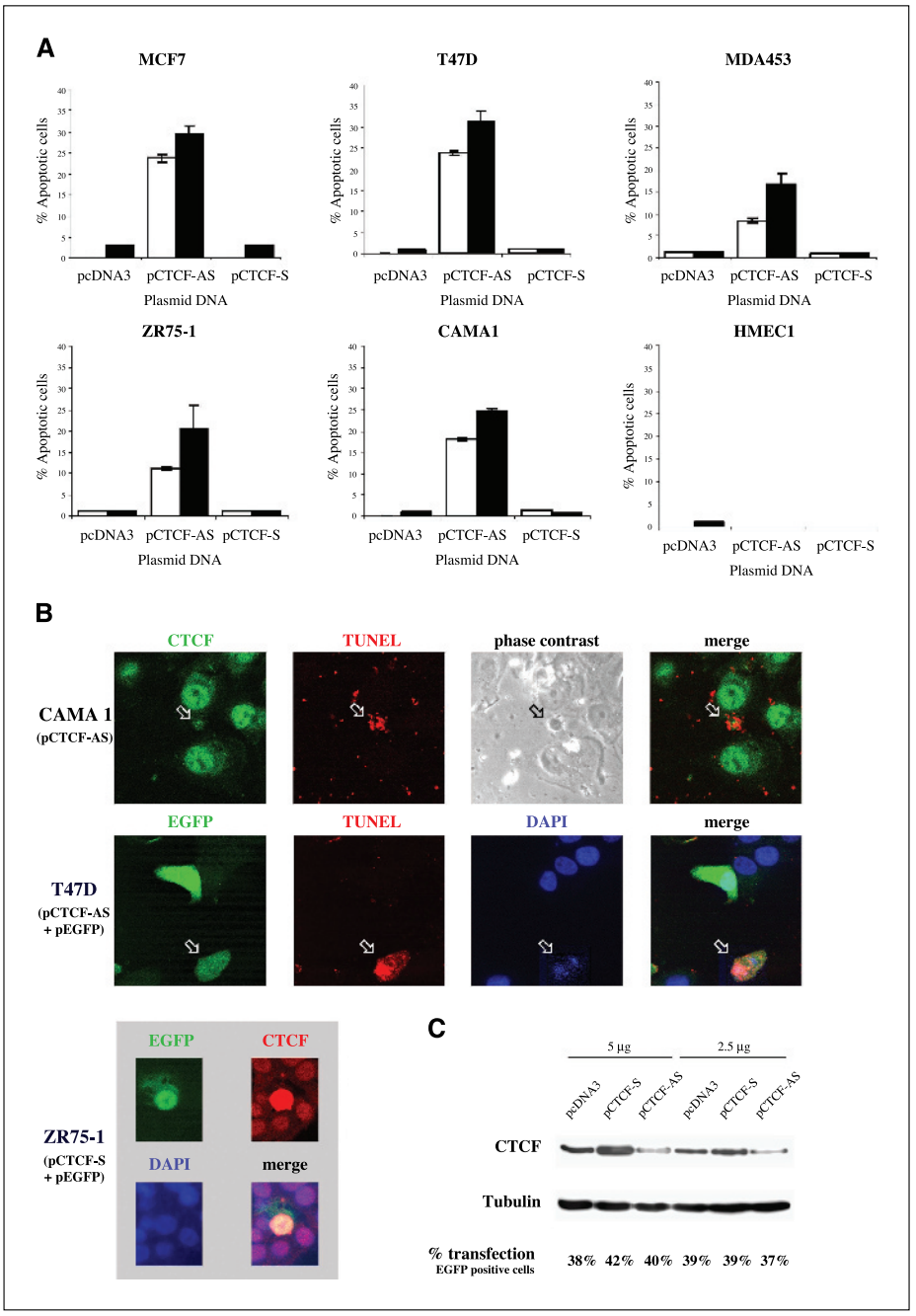

Figure 3. Transient knockdown of CTCF induces apoptotic cell death in breast cancer cells. *A,* apoptotic cell death is induced by CTCF knockdown in breast cancer cells with high (MCF7, T47D, and MDA453) and moderate (ZR75-1 and CAMA1) levels of CTCF but not in HMEC with low levels of CTCF. Cells were transiently transfected with 2.5 μg pCTCF-S, pCTCF-AS, or pcDNA3 together with 2.5 μg pEGFP used as a transfection marker, and apoptotic cell death was assessed by TUNEL assay and DAPI staining. The percentage of apoptotic cells among the transfected (EGFP positive) cells was calculated 24, 48, and 72 hours after transfection. The maximum effect, shown in the graphs was achieved 72 hours after transfection. *Columns,* mean values of three independent experiments for all breast cell lines except HMEC (see below); *bars,* SD. In each experiment, a minimum of 1,000 EGFP-positive cells were counted for all breast cancer cells and due to a lower transfection rate (<10%), a minimum of 50 EGFP-positive cells were counted for HMEC. Experiments using HMEC cells were therefore repeated 10 times to obtain reliable data □, TUNEL; ■, DAPI. *B, top,* CTCF levels are reduced in apoptotic cells. CAMA1 cells were transfected with 2.5 μg of pCTCF-AS plasmid and analyzed by immunofluorescence staining with the anti-CTCF antibody (FITC, green fluorescence). Apoptotic death was assessed by TUNEL staining (TMR, red fluorescence). Cells were visualized using phase contrast microscopy. *Merge,* overlay of the CTCF and TUNEL staining. Transfected apoptotic cells (*arrows*). *Middle,* EGFP expressing cells cotransfected with pCTCF-AS are TUNEL positive. T47D cells were cotransfected with 2.5 μg pCTCF-AS and 2.5 μg pEGFP and analyzed for the presence of EGFP marker (green fluorescence). Apoptotic cell death was assessed by TUNEL staining (TMR, red fluorescence). Cell nuclei were visualized with DAPI staining (blue). *Merge,* overlay of EGFP fluorescence, TUNEL, and DAPI staining. Transfected apoptotic cells (*arrows*). *Bottom,* CTCF and EGFP are coexpressed in cells cotransfected with pCTCF-S and pEGFP. ZR75-1 cells were cotransfected with 2.5 μg pCTCF-S and 2.5 μg pEGFP and analyzed for the presence of EGFP (green fluorescence) and for CTCF overexpression by immunofluorescence staining with the anti-CTCF antibody (TRITC, red fluorescence). Cell nuclei were visualized with DAPI staining (blue). *Merge,* overlay of EGFP fluorescence, the CTCF and DAPI staining. *C,* assessment of CTCF levels in ZR75-1 cells transfected with pcDNA3, pCTCF-S, and pCTCF-AS by Western blot analysis. Cellular extracts were prepared from 5×10^6 cells cotransfected with 2.5 or 5 μg of the above plasmids, together with 0.5 μg of pEGFP. Equal amounts (40 μg) of total protein were loaded onto SDS-PAGE, samples were electrophoretically separated, blotted, and probed with the anti-CTCF antibody. The same membrane was reprobed with the anti-α-tubulin antibody, which served as an internal control for protein loading. Transfection rates assessed by the EGFP marker (*below each lane*).

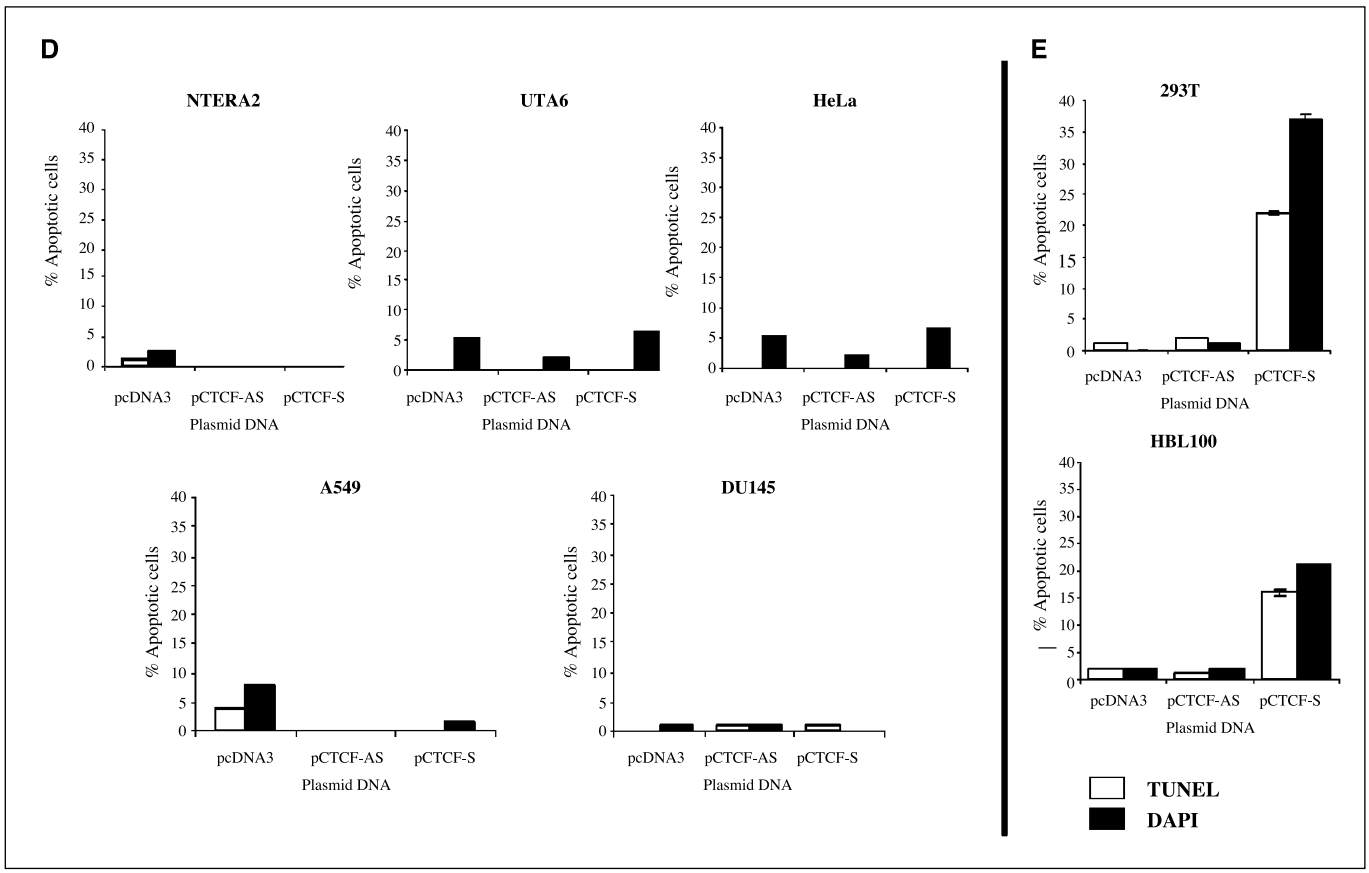

Figure 3. *Continued. D,* CTCF knockdown does not induce apoptosis in non–breast cells. Cells were transiently transfected with 2.5 μg pCTCF-S, pCTCF-AS or pcDNA3 together with 2.5 μg pEGFP used as a transfection marker, and apoptotic cell death was assessed by TUNEL assay and DAPI staining. The percentage of apoptotic cells among the transfected (EGFP positive) cells was calculated 24, 48, and 72 hours after transfection. The maximum effect, shown in the graphs, was achieved 72 hours after transfection. *Columns,* mean values of three independent experiments; *bars,* SD. In each experiment, a minimum of 1,000 EGFP positive cells were counted. *E,* CTCF knockdown does not induce apoptosis, whereas CTCF overexpression induces apoptosis in HBL100 and 293T cell lines. Cells were transiently transfected with 2.5 μg pCTCF-S, pCTCF-AS, or pcDNA3 together with 2.5 μg pEGFP used as a transfection marker, and apoptotic cell death was assessed by TUNEL assay and DAPI staining. The percentage of apoptotic cells among the transfected (EGFP positive) cells was calculated 24, 48, and 72 hours after transfection. The maximum effect, shown in the graphs, was achieved 72 hours after transfection for HBL100 cells and 24 hours after transfection for 293T cells. *Columns,* mean values of three independent experiments; *bars,* SD. In each experiment, a minimum of 1,000 EGFP positive cells were counted.

Results

CTCF levels are elevated in breast cancer cell lines and breast tumors. Our preliminary experiments aimed to assess CTCF expression in breast cell lines and tumors. We found that the levels of *CTCF* mRNA were highly variable in the breast cell lines and tumor samples, with no correlation to the ER status of the cells or levels of *c-Myc* or *BRCA1* mRNA.[6] Western blot analysis of CTCF protein in breast cell lines revealed that all cell lines contained the 130-kDa band characteristic for CTCF (Fig. 1). The lowest levels of CTCF were observed in the immortalized, nontumorigenic cell lines, HB4a and HBL100. In contrast, there was at least 2-fold elevation of CTCF protein in >90% of breast cancer cell lines compared with HB4a (Fig. 1; Supplementary Table 2).

Immunofluorescent staining of all the above mentioned cell lines (Supplementary Fig. A) and immunohistochemical staining of selected cell lines (Fig. 2*A*) confirmed elevated levels of CTCF in all of the breast cancer cell lines compared with HB4a.

To lend further support to our observations, we analyzed the levels of CTCF in breast cell lines with finite life span. The immunohistochemical staining of HMEC, obtained from two individuals (HMEC1 and HMEC2) and Western blot analysis with anti-CTCF antibodies revealed that CTCF levels in these cells were noticeably lower compared with the breast cell line HB4a (Fig. 2*A* and *B*).

To extend our observations on breast cancer cell lines, we determined the levels of CTCF in breast tumors. Immunostaining of these tissues using anti-CTCF antibodies revealed significant overexpression of CTCF ($P = 0.004$) in the great majority of tumors compared with very low CTCF levels detected in normal tissues derived from breast reductions or paired peripheral tissues to tumor (examples are shown in Fig. 2*C*; all data are summarized in Supplementary Table 1). Of note, a predominantly nuclear localization of CTCF in breast cancer cells was observed (see comments to Supplementary Fig. A), whereas in HMEC, HB4a and reduction tissues very weak staining seemed both nuclear and cytoplasmic.

Down-regulation of CTCF in breast cancer cells with high and moderate levels of CTCF leads to apoptotic cell death. The high levels of the candidate tumor suppressor CTCF (4, 5) seen in breast cancer cell lines and tumors was surprising. However, examples of increased levels of proteins with tumor suppressive

[6] A. Robinson, unpublished data.

functions in cancer cells have been previously reported (e.g., BRCA1 and BRCA2; refs. 28, 29). Furthermore, the considerably elevated levels of the retinoblastoma protein, pRB, in colorectal carcinomas have been associated with an antiapoptotic function of pRB (30).

We aimed to investigate whether up-regulation of endogenous CTCF might also be linked to suppression of apoptosis. To test this hypothesis, we chose several breast cell lines with varying levels of CTCF: high in MCF7, MDA453, and T47D; moderate in CAMA1 and ZR75-1; and low in HMEC1, HMEC2, and HBL100. These cell lines were transiently transfected with plasmids carrying CTCF cDNA in the sense (pCTCF-S) and antisense (pCTCF-AS) orientations and also the empty vector (pcDNA3). The pEGFP plasmid served as a transfection marker and the number of EGFP-positive cells and their apoptotic status, using TUNEL and DAPI staining, was determined. As shown in Fig. 3*A*, in all breast cancer cell lines, the number of apoptotic cells was significantly increased following transfection with pCTCF-AS. In comparison, a very low percentage of apoptotic cells was detected after cells were cotransfected with either pCTCF-S or pcDNA3. In identical experiments, no apoptosis was observed in HMEC1 and HMEC2 expressing low levels of CTCF, which were similarly treated (data for HMEC2 not shown as an identical result was obtained).

These results were verified by immunofluorescence analysis of transfected cells. We observed that following transfection with pCTCF-AS, only TUNEL-positive apoptotic cells contained significantly lower levels of CTCF (Fig. 3*B, top*). It was also confirmed that in cells cotransfected with pCTCF-AS and pEGFP, TUNEL-positive apoptotic cells also expressed the EGFP marker (Fig. 3*B, middle*). Finally, the coexpression of CTCF and EGFP was confirmed following cotransfection with pCTCF-S and pEGFP (Fig. 3*B, bottom*).

Expression from the transfected plasmids (pCTCF-S, pCTCF-AS, and pcDNA3) was additionally assessed by Western assay. An increase in levels of CTCF protein was observed in cells transfected with pCTCF-S compared with pcDNA3, and a reduction in CTCF levels was noticed following transfection with pCTCF-AS (Fig. 3*C*).

To establish whether the observed effects were characteristic for breast cancer cells, we also used 12 non–breast cancer cell lines of different origins. The results of transfection assays using five representative cell lines (NTERA2, UTA6, HeLa, A549, and DU145) showed no significant response to transfected plasmids (Fig. 3*D*). The other cell lines tested (LNCaP, J82, T24, HepG2, G361, and RD) reacted similarly (data not shown).

No effect from pCTCF-AS was seen in 293T cell line; however, increased apoptosis was observed upon overexpression of CTCF (Fig. 3*E*). A similar result was obtained for the breast cell line HBL100 (Fig. 3*E*). These observations will be discussed later (see Discussion).

The presence of endogenous CTCF in all non–breast cancer cell lines studied was verified by Western analysis (Supplementary Fig. B). Furthermore, a reduction in CTCF levels in non–breast cells was also observed after transient transfection with pCTCF-AS (data not shown).

To provide further evidence that down-regulation of CTCF in breast cancer cells can lead to apoptosis, we employed MCF7 cells, which express high amounts of CTCF protein, to generate the cell line producing the antisense *CTCF* mRNA in an inducible fashion. We observed that massive cell death occurred in the culture at day 3 after conditional knockdown of CTCF protein following stimulation with IPTG (Fig. 4*B* and *C*). To determine if cell death was due to apoptosis, cells at day 2 post-induction were stained for caspase-dependent proteolytic fragment of cytokeratin 18. As shown in Fig. 4*D*, the cytokeratin 18–positive cells (b, green) were observed in culture, whereas the number of proliferating BrdUrd-positive cells (a, red) was reduced dramatically. These effects were dependent on CTCF knockdown, because there were no indications of apoptosis in the MCF7/antisense cells not treated with IPTG, or in the control MCF/LAP5 cells treated with IPTG (data not shown).

CTCF overexpression protects breast cells from induction of apoptotic cell death. We then asked if overexpression of CTCF in breast cancer cells could rescue cells from induction of apoptosis. Expression of Bax alone, without any other death stimuli, is usually sufficient to induce apoptosis (31, 32). Thus, in this series of experiments, a Bax-expressing plasmid, pSFFV-Bax (or pBax), was transiently transfected alone or in combination with pCTCF-S into several breast cell lines with varying levels of the endogenous CTCF (high in MCF7 and T47D, moderate in CAMA1, and very low in HMEC). As shown in Fig. 5*A*, ectopically overexpressed CTCF could partially overcome apoptosis induced by exogenous Bax in all cell lines tested. However, apoptosis was not totally abrogated and the response to overexpression of CTCF varied in different cell lines.

To further show that overexpression of CTCF could protect against apoptosis, we induced apoptotic death by treatment with sodium butyrate (NaB) in cells with either high (MCF7) or moderate (MDA468) levels of endogenous CTCF. NaB was chosen because it induces apoptosis in many cell lines and the molecular mechanisms of NaB action in MCF7 cells are quite well understood (26, 33). As shown in Fig. 5*B*, overexpression of CTCF in breast cancer cells treated with NaB led to a considerable increase in survival of the transfected cells (EGFP positive) compared with the control plasmid (pcDNA3).

Immunofluorescent staining with the anti-CTCF antibody confirmed that following treatment with NaB, all the surviving (TUNEL negative) cells were overexpressing CTCF (Fig. 5*C*). In contrast, cells containing very low or undetectable levels of CTCF were undergoing apoptosis, as confirmed by positive TUNEL staining (Fig. 5*C*).

It was not possible to use the HMEC in this test as they showed resistance to NaB treatment.

Identification of proapoptotic Bax protein as a potential target for regulation by CTCF. To identify proteins whose cellular levels were significantly altered upon CTCF down-regulation, we used a proteomics approach based on two-dimensional PAGE and mass spectrometry. In this experiment, MCF7 cells were transfected with either pCTCF-AS or pcDNA3 and proteins obtained from these cell extracts were resolved on a two-dimensional gel. Proteins showing differential expression between the two proteomes were excised and subjected to the "in-gel" digestion and tryptic mixture mass mapping. As shown in Fig. 6*A*, one protein found to be up-regulated in MCF7 cells upon reduction of CTCF levels was the proapoptotic protein Bax (SwissProt protein data base accession number Q07812).

To further validate Bax as a potential CTCF target, a Western blot analysis of ZR75-1 cells transiently transfected with pCTCF-S, pCTCF-AS, and pcDNA3 was carried out using the anti-Bax antibody. As shown in Fig. 6*B*, CTCF overexpression led to a reduction of Bax levels, whereas CTCF down-regulation resulted in elevated expression of the protein.

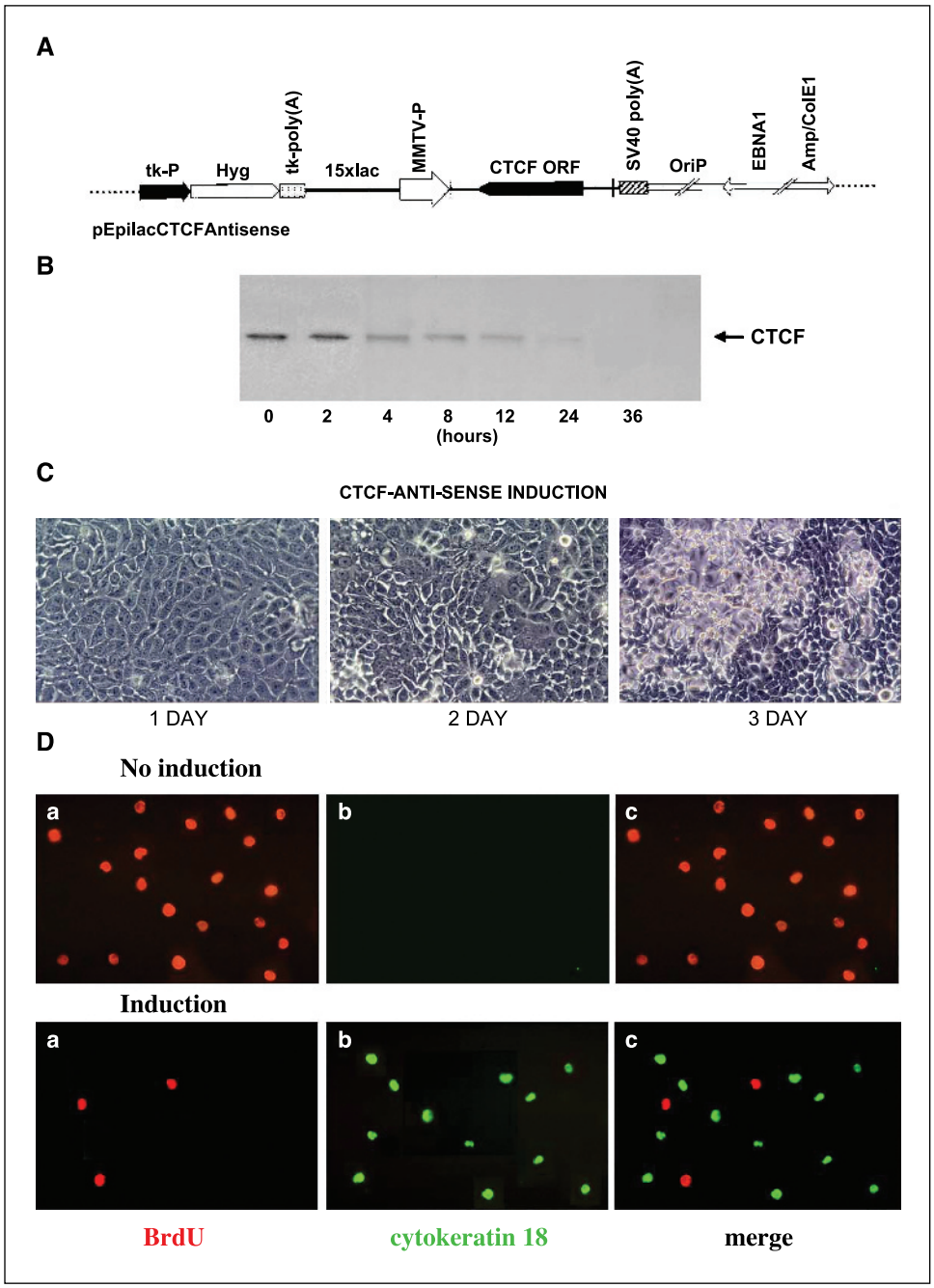

Figure 4. Conditional knockdown of CTCF in MCF7 leads to proliferation block and apoptotic cell death. *A,* schematic outline of the functional elements of the pEpiLac*CTCFanti* vector containing CTCF cDNA in the reverse orientation relative to the IPTG-inducible promoter (see Materials and Methods for detail). Abbreviations: *Hyg*, hygromycin B resistance gene; tk-P, thymidine kinase basic promoter; MMTV-P, mouse mammary tumor virus basic promoter; poly(A), poly(A +) signal; OriP, origin for plasmid replication of EB virus; EBNA1, EB virus nuclear antigen 1; Lac, lactose operator sequences; CTCF ORF, CTCF cDNA (open reading frame). *B,* inhibition of CTCF expression by IPTG. Western blot analysis of MCF7/LAP5/CTCFantisense cells after induction with IPTG. 1×10^6 MCF7/LAP5/CTCFantisense cells were plated in flasks and induced with 2 mmol/L IPTG. Cells were collected after 2, 4, 8, 12, 24, and 36 hours. Noninduced cells were used as control point 0. Cellular extracts were prepared and equal amounts (40 μg) of total protein were loaded onto SDS-PAGE. Samples were electrophoretically separated, blotted, and probed with the anti-CTCF antibody. *C,* active cell death occurs in MCF7 cells after conditional knockdown of CTCF protein. Phase contrast images of MCF7/LAP5/CTCFantisense cells cultured in the presence of 2 mmol/L IPTG for the indicated times. *D,* proliferation block and apoptotic cell death occurs in MCF7 cells after conditional knockdown of CTCF protein. MCF7/LAP5/CTCFantisense cells were cultured in the absence (*top*) or presence of 2 mmol/L IPTG (*bottom*) for 2 days, pulsed with BrdUrd for 3 hours, and stained with anti-BrdUrd antibodies (Texas, red fluorescence; *a*). Apoptotic cell death was assessed by staining using an antibody directed against the caspase-dependent proteolytic fragments of cytokeratin 18 (FITC, green fluorescence; *b*). *c,* merge of (*a*) and (*b*).

Discussion

CTCF expression and apoptotic cell death. The aim of this study was to investigate the levels of CTCF protein in breast cancer cell lines and primary tumors and a possible link between CTCF expression and resistance to apoptosis. We showed that CTCF levels were elevated in all breast cancer cell lines compared with very low levels of CTCF in HMEC. Our analysis also revealed that 88.2% (15 of 17 samples) of IDC tissues and all other tumors inspected had immunoreactivity scores (IRS) of >2, indicative of high CTCF expression. Of note, the two tumor samples (T51 and T138) with the IRS value of 2 were of low grade (Supplementary Table 1). This may be a significant finding because our analysis indicates that there is a positive correlation between CTCF expression and tumor grade.[7]

CTCF levels could not be attributed to differing rates of proliferation in normal and tumor cells. Indeed, immunofluorescent staining of nonsynchronized breast cells (Supplementary Fig. A) and immunohistochemical staining of tumor tissues and breast cells (Fig. 2*A* and *C*; data not shown) did not reveal any significant difference in the level of CTCF in individual cells. In addition, p53 and pRB status in breast cells lines had no correlation with CTCF levels (Supplementary Table 2).

It has been generally accepted that cancer cells are highly sensitized to apoptotic signals, whereas normal cells are more

[7] V. D'Arcy, unpublished data.

resistant to apoptotic challenges (34). We hypothesized that elevated levels of CTCF in cancer cells may have developed to counteract their hypersensitivity to apoptotic stimuli. Using both a transient transfection approach and an inducible system, we were able to manipulate the levels of endogenous CTCF. In our experiments, a reduction in CTCF levels led to apoptotic cell death in breast cancer cell lines. Overexpression of CTCF, on the other hand, rescued cells from apoptosis induced by either Bax or NaB. Interestingly, the effects of the CTCF knockdown seemed more pronounced in cells expressing the highest levels of CTCF such as MCF7 and T47D, than in other cells with lower CTCF levels. No apoptotic cell death was detected in HMEC in this assay. We also observed that ectopic CTCF conferred better protection from NaB-induced apoptosis in cells with moderate endogenous CTCF levels than in cells with high CTCF levels. The results of these experiments point to a possible link between CTCF expression and sensitivity to apoptosis; that is, higher levels of CTCF may be necessary to protect the more sensitive cancer cells from apoptotic stimuli. These findings may be relevant to the potential use of CTCF as a therapeutic target in breast cancers: reducing the levels of CTCF would then result in apoptotic cell death of cancer cells without affecting normal breast tissue, and the effect of CTCF down-regulation may be more dramatic in high grade breast tumors. However, these

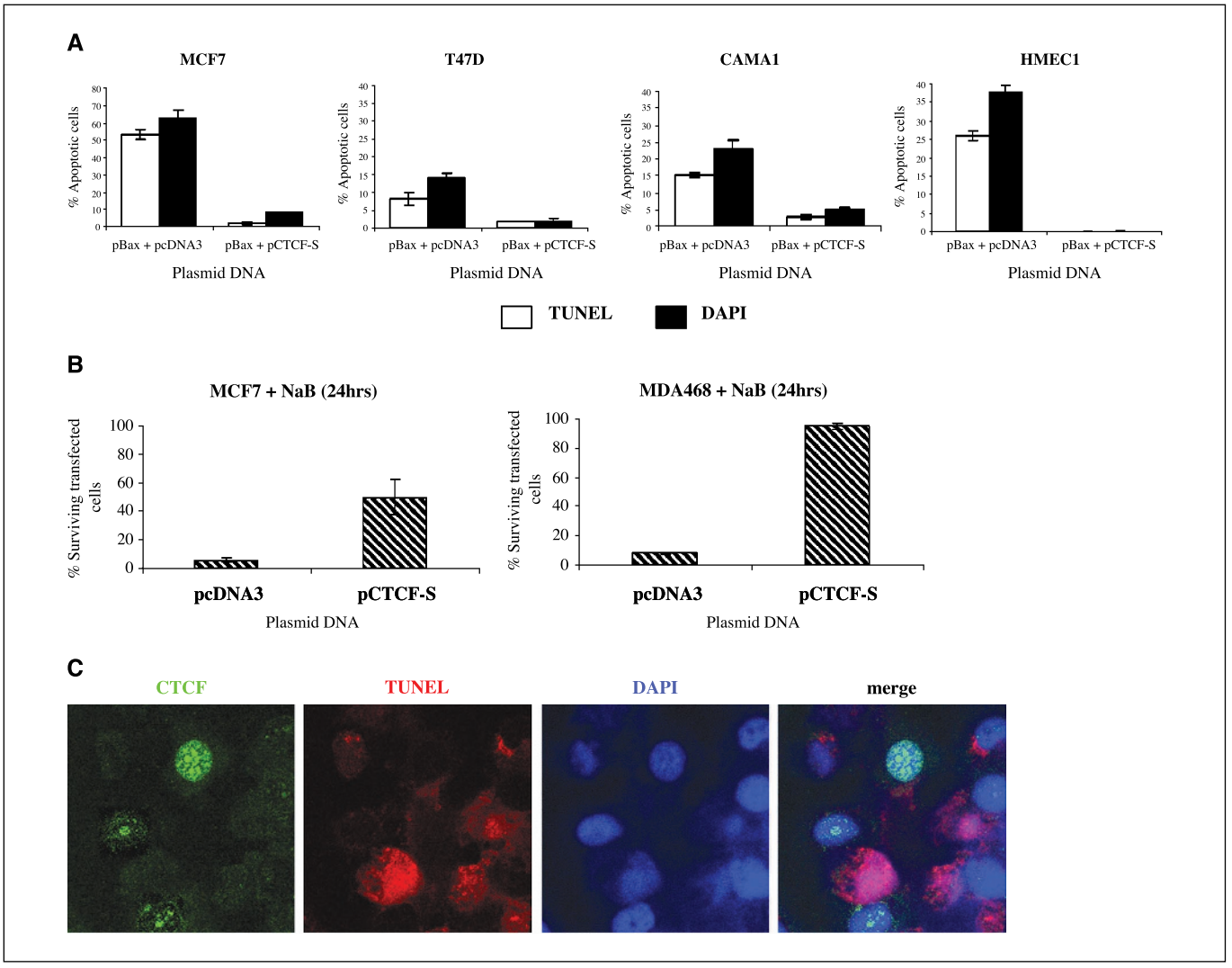

Figure 5. CTCF overexpression can protect breast cancer cells against apoptosis induced by Bax and NaB. *A,* overexpression of CTCF can overcome apoptotic effects caused by Bax. Cells were transfected with 2 μg of pSFFV-Bax (or pBax), 2 μg of pCTCF-S, or 2 μg of pcDNA3 (empty vector control); 2 μg of pEGFP (transfection marker) were included in each experiment. Apoptotic cell death was assessed by TUNEL assay and DAPI staining. The percentage of apoptotic cells among the transfected (EGFP positive) cells was calculated 24, 48, and 72 hours after transfection. The maximum effect, shown in the graphs, was achieved 72 hours after transfection. *Columns,* mean values of three independent experiments for MCF7, T47D, and CAMA1 breast cells lines and of 10 experiments for HMEC (see below); *bars,* SD. In each experiment, a minimum of 1,000 EGFP positive cells were counted for breast cancer cells and, due to a lower transfection rate (<10%), a minimum of 50 EGFP-positive cells were counted for HMEC. Experiments using HMEC were therefore repeated ten times to obtain reliable data. *B,* overexpression of CTCF alleviates apoptotic cell death induced by NaB. Cells were transfected with 2 μg of pCTCF-S or 2 μg of pcDNA3 (*control*) together with 2 μg of pEGFP used as a transfection marker; 5 mmol/L NaB were added 24 hours after transfection. The percentage of surviving and morphologically normal cells, as assessed by DAPI staining, among transfected (EGFP positive) cells was calculated 24 hours after addition of NaB. *Columns,* mean values of three independent experiments; *bars,* SD. A minimum of 1,000 EGFP-positive cells were counted for each experiment. *C,* typical image of MCF7 cells treated with NaB and rescued by CTCF overexpression. MCF7 cells were transfected with 2.5 μg of pCTCF-S, treated with NaB for 24 hours and analyzed by immunofluorescence staining with the anti-CTCF antibody 48 hours post-transfection (FITC, green fluorescence). Apoptotic cell death was assessed by TUNEL staining (TMR, red fluorescence). Cell nuclei were visualized with DAPI staining (blue). *Merge,* overlay of the CTCF, TUNEL, and DAPI staining.

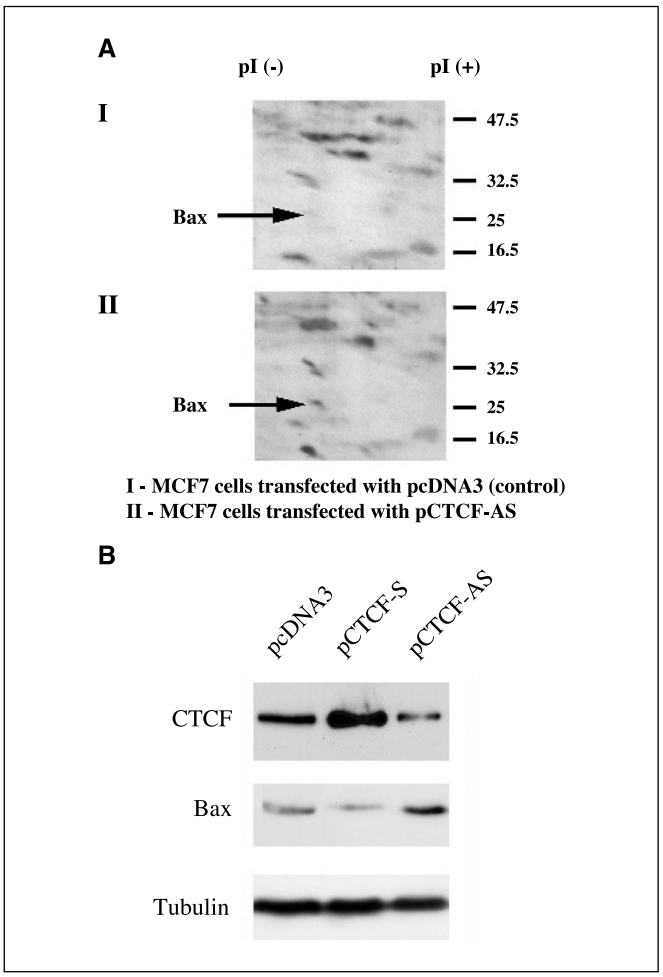

Figure 6. Expression of a proapoptotic protein, Bax, depends on the levels of expression of CTCF. *A,* comparison of proteome profile sections of MCF7 cell line transfected with empty vector and pCTCF-AS. Cells (3×10^6) were transfected with 30 μg pCTCF-AS or 30 μg pcDNA3, cell lysates prepared and resolved on a two-dimensional gel. Proteins showing differential expression were digested by trypsin and analyzed by mass spectrometry to establish their identity. Spots corresponding to Bax (*arrows*) indicate that Bax protein is up-regulated in cells transfected with pCTCF-AS (*II*). *B,* Bax levels are increased following CTCF down-regulation and decreased following CTCF up-regulation. Western blot analysis of ZR75-1 cells transiently transfected with pCTCF-S, pCTCF-AS, and pcDNA3. Cells (1×10^6) were transiently transfected with 2.5 μg of pCTCF-S, pCTCF-AS, and pcDNA3, cellular extracts prepared and equal amount (40 μg) of total protein loaded onto SDS-PAGE. Samples were electrophoretically separated, blotted, and subsequently probed with the anti-CTCF and anti-Bax antibodies. The same membrane was reprobed with the anti-α-tubulin antibody, which was used as an internal control for protein loading.

observations will require further study because only limited information has been obtained from HMEC.

No significant apoptotic cell death was observed following transfection of pCTCF-S, pCTCF-AS, or pcDNA3 (control vector) into 12 randomly selected non–breast cancer cell lines. These cell lines contained variable levels of endogenous CTCF, which was anticipated as CTCF seems essential for cell functions and its expression has been reported in all cell lines tested thus far (1, 5, 21).[8] This result rules out a trivial explanation that absence of the effects from the pCTCF-AS plasmid could be due to lack of CTCF in these cells and implies that antiapoptotic mechanisms involving CTCF function may have developed specifically in breast tumors. We also noticed that the overall levels of CTCF in non–breast cells were lower than in breast cells lines, which may be another factor contributing to the difference in response to CTCF knockdown between breast and non–breast cells. It is conceivable that CTCF in breast cells may, for example, be involved in the regulation of a subset of genes specific for mammary gland development and function.

We observed, however, that CTCF overexpression in the immortalized breast cell line HBL100 and kidney cell line 293T resulted in an increase in apoptosis. Apoptotic cell death from CTCF overexpression was also reported by Qi et al. in WEHI 231 immature B cells (18). Although these results apparently contradict our proposed model, many factors, such as cellular environment and genetic background, have to be taken into consideration in these cases. For instance, the presence of SV40 T antigen in 293T and HBL100 may interfere with CTCF function. This possibility deserves further investigation.

Our findings introduce CTCF into a wider debate concerning the controversial role for tumor suppressors in apoptosis, and a close link between proliferation and apoptosis further adds to the complexity (8, 35). Cellular environment, cell type, genetic background, and many other variables play important roles in making the decision to commit to apoptosis. A combination of these influences, which can often have conflicting effects, makes it difficult to predict the exact functional outcome of any combination. For example, depending on appropriate cellular milieu, pRB, p21, and WT-1 may behave either as antiapoptotic or as proapoptotic factors (30, 36–40).

CTCF, a candidate tumor suppressor, is involved in the regulation of genes controlling proliferation, such as *c-Myc* and *p14/p19 ARF* (1, 2), and has documented growth suppressive properties in various cell types (4) including breast cells.[9] Although these observations seem in conflict with the proposed function of CTCF in apoptosis, studies of c-Myc, pRB, WT-1, and p53 have revealed their seemingly conflicting roles in the regulation of both apoptosis and proliferation. There is experimental evidence these proteins transcriptionally regulate apoptotic genes (34, 41–45) and their ability to trigger apoptosis may be independent from their control of proliferation (a proposed "dual signal" model; refs. 34, 41, 44, 45). We therefore suggest a similar "dual signal" role for CTCF.

Explanation of this complex behavior will undoubtedly require a better understanding of regulatory networks and control mechanisms associated with a particular TSG and a combination of these influences may be unique for a given cell type. From this point of view, the increased levels of CTCF in breast cancer cells may be one of the factors in developing resistance from apoptosis. CTCF functions can also be modulated by post-translational modifications (20, 46) and interactions with protein partners (22, 47, 48), which adds yet other layers of complexity to this model.

Molecular mechanism(s) for protection of breast cancer cells against apoptosis by CTCF. As mentioned previously, we found no correlation between the status of p53 or pRB and CTCF expression in breast cell lines (Supplementary Table 2). Furthermore, the protein expression levels of p53 and pRB were unchanged upon ectopic overexpression or down-regulation of CTCF in all breast cancer cell lines tested (data not shown). Finally, NaB-induced

[8] E. Klenova and V. Lobanenkov, unpublished data.

[9] S. Rai et al., in preparation.

apoptosis in MCF7 breast cancer cells is reported p53 independent (26), which also implies that CTCF apoptotic rescue pathways in MCF7 cells are p53 independent.

Using a direct proteomics approach we identified the proapoptotic protein Bax as a potential target for regulation by CTCF. The initial finding was further verified by Western blot analysis showing that endogenous Bax was down-regulated following CTCF overexpression and conversely up-regulated upon CTCF down-regulation. Our recent data also indicated that the Bax promoter can be regulated by CTCF in reporter assays.[10] Bax expression is reported decreased in breast tumors, although the molecular mechanisms responsible for this phenotype are not known (49–51). These observations and our findings suggest a model in which CTCF directly regulates Bax through a transcriptional mechanism. High CTCF levels may cause repression of Bax and inhibition of apoptosis. Conversely, lowering levels of CTCF may result in activation of Bax, leading to apoptosis. In addition, our preliminary data suggest that the antiapoptotic protein, Bcl-2, is also regulated by CTCF, as the Bcl-2 promoter can be activated by CTCF in a reporter assay.[10] In contrast to Bax, the levels of Bcl-2 in breast cancer cells are high (49–51). It is conceivable that CTCF may regulate Bcl-2 through a transcriptional mechanism, but in a positive manner. Work is now in progress to explore this possibility.

The ability of CTCF to rescue cells from apoptotic death induced by overexpression of Bax (Fig. 5*A*) can not be explained solely by the above mechanism. This implies that regulation of apoptosis by CTCF also involves other mechanisms, such as direct effects on other transcriptional targets and indirect effects at the post-transcriptional level. Further investigation is needed to clarify the specific contributions from direct and indirect effects of CTCF to this regulation.

Taken together, our results support the hypothesis that the increased levels of CTCF protein can protect breast cancer cells from apoptosis. This effect is likely to reflect direct and indirect influences of CTCF on the function of antiapoptotic proteins (such as Bcl-2, Bcl-XL, Mcl-1) and proapoptopic proteins (such as Bax, Bak, Bad, Bik, Bid, and Bok). Profiling of proteins and identification of the *in vivo* DNA targets of CTCF upon CTCF overexpression and knockdown will be needed to more precisely elucidate the molecular mechanisms by which CTCF renders breast cancer cells resistant to apoptosis. These directions may lead to development of breast cancer therapies based on selective reduction of CTCF in breast cancer cells.

[10] D. Farrar and F. Docquier, unpublished data.

Acknowledgments

Received 11/7/2003; revised 3/4/2005; accepted 3/31/2005.

Grant support: Medical Research Council Studentship (A.F. Robinson), Breast Cancer Campaign (F. Docquier), Association for International Cancer Research (I. Chernukhin), Breast Cancer Research Trust (H. Dorricot), BBSRC PhD studentship (V. D'Arcy), Essex University PhD studentship (D. Farrar), and Research Promotion Fund of the University of Essex and Essex Rivers Charitable Trust (F. Docquier, E. Klenova, and I. Chernukhin).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank F. MacNeill, P. Murray, K. Rooke, T. Dearson, M. Marshall, and R. Gooch for help with tissue collection and information; I. Seddon for specialist advice and expertise on breast tumor tissues; H.C. Morse III and M. O'Farrell for critically reading the article and valuable suggestions; M. Biba and G.-X. Kita for doing pilot experiments; P. O'Toole and I. Morrison for assistance with confocal microscopy; L. Lau and D. Pestov for MCF/LAP5 cells; S. Korsmeyer for the pSFFV-Bax plasmid; J. Baker and K. Malik for sharing technology and expertise in tissue sectioning and immunostaining; A. Gonzalez-Garcia and H. Paterson for advice on anti-Bax antibodies; and the reviewers for helpful comments.

References

1. Klenova EM, Morse HC III, Ohlsson R, Lobanenkov VV. The novel BORIS + CTCF gene family is uniquely involved in the epigenetics of normal biology and cancer. Semin Cancer Biol 2002;12:399–414.
2. Ohlsson R, Renkawitz R, Lobanenkov V. CTCF is a uniquely versatile transcription regulator linked to epigenetics and disease. Trends Genet 2001;17:520–7.
3. Klenova E, Ohlsson R. Poly(ADP-ribosyl)ation and epigenetics: is CTCF PARt of the plot? Cell Cycle 2005;4:96–101.
4. Rasko JE, Klenova EM, Leon J, et al. Cell growth inhibition by the multifunctional multivalent zinc-finger factor CTCF. Cancer Res 2001;61:6002–7.
5. Filippova GN, Lindblom A, Meincke LJ, et al. A widely expressed transcription factor with multiple DNA sequence specificity, CTCF, is localized at chromosome segment 16q22.1 within one of the smallest regions of overlap for common deletions in breast and prostate cancers. Genes Chromosomes Cancer 1998;22:26–36.
6. Filippova GN, Qi CF, Ulmer JE, et al. Tumor-associated zinc finger mutations in the CTCF transcription factor selectively alter tts DNA-binding specificity. Cancer Res 2002;62:48–52.
7. Bertram JS. The molecular biology of cancer. Mol Aspects Med 2000;21:167–223.
8. Evan GI, Vousden KH. Proliferation, cell cycle and apoptosis in cancer. Nature 2001;411:342–8.
9. Igney FH, Krammer PH. Death and anti-death: tumour resistance to apoptosis. Nat Rev Cancer 2002;2:277–88.
10. Stamps AC, Davies SC, Burman J, O'Hare MJ. Analysis of proviral integration in human mammary epithelial cell lines immortalized by retroviral infection with a temperature-sensitive SV40 T-antigen construct. Int J Cancer 1994;57:865–74.
11. Gaffney EV. A cell line (HBL-100) established from human breast milk. Cell Tissue Res 1982;227:563–8.
12. Bergstraesser LM, Weitzman SA. Culture of normal and malignant primary human mammary epithelial cells in a physiological manner simulates *in vivo* growth patterns and allows discrimination of cell type. Cancer Res 1993;53:2644–54.
13. Stampfer MR, Bodnar A, Garbe J, et al. Gradual phenotypic conversion associated with immortalization of cultured human mammary epithelial cells. Mol Biol Cell 1997;8:2391–405.
14. Filippova GN, Fagerlie S, Klenova EM, et al. An exceptionally conserved transcriptional repressor, CTCF, employs different combinations of zinc fingers to bind diverged promoter sequences of avian and mammalian c-*myc* oncogenes. Mol Cell Biol 1996;16:2802–13.
15. Chen C, Okayama H. High-efficiency transformation of mammalian cells by plasmid DNA. Mol Cell Biol 1987;7:2745–52.
16. Baim SB, Labow MA, Levine AJ, Shenk T. A chimeric mammalian transactivator based on the lac repressor that is regulated by temperature and isopropyl β-D-thiogalactopyranoside. Proc Natl Acad Sci U S A 1991;88:5072–6.
17. Li Y, Lau LF. IPTG-inducible episomal expression system for exogenous genes in primate cells. Biotechniques 2000;28:577–81.
18. Qi CF, Martensson A, Mattioli M, et al. CTCF functions as a critical regulator of cell-cycle arrest and death after ligation of the B cell receptor on immature B cells. Proc Natl Acad Sci U S A 2003;100:633–8.
19. Klenova EM, Nicolas RH, Paterson HF, et al. CTCF, a conserved nuclear factor required for optimal transcriptional activity of the chicken c-*myc* gene, is an 11-Zn-finger protein differentially expressed in multiple forms. Mol Cell Biol 1993;13:7612–24.
20. Klenova EM, Chernukhin IV, El-Kady A, et al. Functional phosphorylation sites in the C-terminal region of the multivalent multifunctional transcriptional factor CTCF. Mol Cell Biol 2001;21:2221–34.
21. Delgado MD, Chernukhin IV, Bigas A, Klenova EM, Leon J. Differential expression and phosphorylation of CTCF, a c-*myc* transcriptional regulator, during differentiation of human myeloid cells. FEBS Lett 1999;444:5–10.
22. Chernukhin IV, Shamsuddin S, Robinson AF, et al. Physical and functional interaction between two pluripotent proteins, the Y-box DNA/RNA-binding factor, YB-1, and the multivalent zinc finger factor, CTCF. J Biol Chem 2000;275:29915–21.
23. Harlow E, Lane D. Using antibodies: a laboratory manual. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 1999.
24. Shi SR, Key ME, Kalra KL. Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. J Histochem Cytochem 1991;39:741–8.
25. Martelli AM, Zweyer M, Ochs RL, et al. Nuclear apoptotic changes: an overview. J Cell Biochem 2001;82:634–46.
26. Chopin V, Toillon RA, Jouy N, Le Bourhis X. Sodium butyrate induces P53-independent, Fas-mediated apoptosis in MCF-7 human breast cancer cells. Br J Pharmacol 2002;135:79–86.
27. Beck T, Weikel W, Brumm C, et al. Immunohistochemical detection of hormone receptors in breast carcinomas (ER-ICA, PgR-ICA): prognostic usefulness and comparison with the biochemical radioactive-ligand-binding assay (DCC). Gynecol Oncol 1994;53:220–7.
28. Egawa C, Miyoshi Y, Taguchi T, Tamaki Y, Noguchi S.

Quantitative analysis of BRCA1 and BRCA2 mRNA expression in sporadic breast carcinomas and its relationship with clinicopathological characteristics. Jpn J Cancer Res 2001;92:624–30.
29. Zwahlen D, Tschan MP, Grob TJ, et al. Differential expression of p73 splice variants and protein in benign and malignant ovarian tumours. Int J Cancer 2000;88:66–70.
30. Yamamoto H, Soh JW, Monden T, et al. Paradoxical increase in retinoblastoma protein in colorectal carcinomas may protect cells from apoptosis. Clin Cancer Res 1999;5:1805–15.
31. Xiang J, Chao DT, Korsmeyer SJ. BAX-induced cell death may not require interleukin 1 β-converting enzyme-like proteases. Proc Natl Acad Sci U S A 1996;93:14559–63.
32. Zha H, Fisk HA, Yaffe MP, et al. Structure-function comparisons of the proapoptotic protein Bax in yeast and mammalian cells. Mol Cell Biol 1996;16:6494–508.
33. Mandal M, Kumar R. Bcl-2 expression regulates sodium butyrate-induced apoptosis in human MCF-7 breast cancer cells. Cell Growth Differ 1996;7:311–8.
34. Vousden KH, Lu X. Live or let die: the cell's response to p53. Nat Rev Cancer 2002;2:594–604.
35. Vermeulen K, Berneman ZN, Van Bockstaele DR. Cell cycle and apoptosis. Cell Prolif 2003;36:165–75.
36. Bowen C, Birrer M, Gelmann EP. Retinoblastoma protein-mediated apoptosis after γ-irradiation. J Biol Chem 2002;277:44969–79.
37. Detjen KM, Murphy D, Welzel M, et al. Down-regulation of p21(waf/cip-1) mediates apoptosis of human hepatocellular carcinoma cells in response to interferon-γ. Exp Cell Res 2003;282:78–89.
38. Tsao YP, Huang SJ, Chang JL, et al. Adenovirus-mediated p21(WAF1/SDII/CIP1) gene transfer induces apoptosis of human cervical cancer cell lines. J Virol 1999;73:4983–90.
39. Fraizer G, Leahy R, Priyadarshini S, et al. Suppression of prostate tumor cell growth *in vivo* by WT1, the Wilms' tumor suppressor gene. Int J Oncol 2004; 24:461–71.
40. Algar EM, Khromykh T, Smith SI, et al. A WT1 antisense oligonucleotide inhibits proliferation and induces apoptosis in myeloid leukaemia cell lines. Oncogene 1996;12:1005–14.
41. Mayo MW, Wang CY, Drouin SS, et al. WT1 modulates apoptosis by transcriptionally upregulating the bcl-2 proto-oncogene. EMBO J 1999;18:3990–4003.
42. Conzen SD, Gottlob K, Kandel ES, et al. Induction of cell cycle progression and acceleration of apoptosis are two separable functions of c-Myc: transrepression correlates with acceleration of apoptosis. Mol Cell Biol 2000;20:6008–18.
43. Moroni MC, Hickman ES, Denchi EL, et al. Apaf-1 is a transcriptional target for E2F and p53. Nat Cell Biol 2001;3:552–8.
44. Chau BN, Wang JY. Coordinated regulation of life and death by RB. Nat Rev Cancer 2003;3:130–8.
45. Prendergast GC. Mechanisms of apoptosis by c-Myc. Oncogene 1999;18:2967–87.
46. Yu W, Ginjala V, Pant V, et al. Poly(ADP-ribosyl)ation regulates CTCF-dependent chromatin insulation. Nat Genet 2004;36:1105–10.
47. Lutz M, Burke LJ, Barreto G, et al. Transcriptional repression by the insulator protein CTCF involves histone deacetylases. Nucleic Acids Res 2000; 28:1707–13.
48. Yusufzai TM, Tagami H, Nakatani Y, Felsenfeld G. CTCF tethers an insulator to subnuclear sites, suggesting shared insulator mechanisms across species. Mol Cell 2004;13:291–8.
49. Krajewski S, Thor AD, Edgerton SM, et al. Analysis of Bax and Bcl-2 expression in p53-immunopositive breast cancers. Clin Cancer Res 1997;3:199–208.
50. Reed JC. Balancing cell life and death: bax, apoptosis, and breast cancer. J Clin Invest 1996;97:2403–4.
51. Schorr K, Li M, Krajewski S, Reed JC, Furth PA. Bcl-2 gene family and related proteins in mammary gland involution and breast cancer. J Mammary Gland Biol Neoplasia 1999;4:153–64.

Heightened Expression of CTCF in Breast Cancer Cells Is Associated with Resistance to Apoptosis

France Docquier, Dawn Farrar, Vivien D'Arcy, et al.

Cancer Res 2005;65:5112-5122.