Expression of CtBP family protein isoforms in breast cancer and their role in chemoresistance

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Background information. CtBPs [C-terminal (of E1A) binding protein] have roles in the nucleus as transcriptional co-repressors, and in the cytoplasm in the maintenance of vesicular membranes. CtBPs are expressed from two genes, *CTBP1* and *CTBP2*, mRNA products of which are alternatively spliced at their 5′-ends to generate distinct protein isoforms. Extensive molecular and cellular analyses have identified CtBPs as regulators of pathways critical for tumour initiation, progression and response to therapy. However, little is known of the expression or regulation of CtBP isoforms in human cancer, nor of the relative contributions of *CTBP1* and *CTBP2* to the tumour cell phenotype.

Results. Expression of CtBP proteins and *CTBP1* and *CTBP2* mRNA splice forms in breast cancer cell lines and tumour tissue was examined. CtBP1 proteins are identifiable as a single band on Western blots and are ubiquitously detectable in breast tumour samples, by both Western blotting and immunohistochemistry. CtBP1 is present in six of six breast cancer cell lines, although it is barely detectable in SKBr3 cells due to reduced *CTBP1* mRNA expression. In the cell lines, the predominant *CTBP1* mRNA splice form encodes CtBP1-S protein; in tumours, both major *CTBP1* mRNA splice forms are variably expressed. CtBP2 proteins are ubiquitously expressed in all lines and tumour samples. The predominant *CTBP2* mRNA encodes CtBP2-L, although an alternatively spliced form that encodes CtBP2-S, previously unidentified in humans, is expressed at low abundance. Both CtBP2-L and CtBP2-S are readily detectable as two distinct bands on Western blots; here we show that the *CTBP2-L* mRNA is translated from two AUG codons to generate both CtBP2-L and CtBP2-S. We have also identified an autoregulatory feedback mechanism whereby CtBP protein abundance is maintained in proliferating breast cancer cells through the post-transcriptional regulation of CtBP2. This feedback is disrupted by UV-C radiation or exposure to cisplatin. Finally, we demonstrate that CtBP1 and CtBP2 both have p53-dependent and -independent roles in suppressing the sensitivity of breast cancer cells to mechanistically diverse cancer chemotherapeutic agents.

Conclusions. These studies support recent evidence that CtBP family proteins represent potential targets for therapeutic strategies for the treatment of cancer in general, and breast cancer in particular.

Introduction

The oncogenic CtBPs [C-terminal (of E1A) binding protein] were discovered through their interaction with the C-terminus of the E1A protein from adenovirus (Boyd et al., 1993). They have since begun to generate interest as potential therapeutic targets for the treatment of cancer (Bergman and Blaydes, 2006; Chinnadurai, 2009). Invertebrates such as *Drosophila* possess one CtBP-encoding locus (*dCtBP*), whereas vertebrate species possess two loci: *CTBP1* and
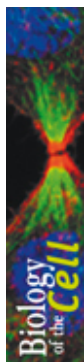
CTBP2 (Chinnadurai, 2002; Bergman and Blaydes, 2006). In humans these map to chromosomes 4 and 10 respectively. Several homologous proteins are encoded by mammalian CtBP-encoding genes: *CTBP1* encodes CtBP1-L and CtBP1-S, whereas *CTBP2* codes for CtBP2-L, CtBP2-S and RIBEYE (Spano et al., 1999; Schmitz et al., 2000; Verger et al., 2006), summarized in Bergman et al. (2006). The CtBPs are highly conserved between species and the vertebrate CtBP1 and CtBP2 proteins share 78 % amino acid homology. The two proteins exhibit a conserved N-terminal amino acid sequence, important in protein—protein interactions, and a central dehydrogenase domain necessary for NADH-dependent dimerization (Chinnadurai, 2002; Nardini et al., 2003). In animal models the CtBPs have been shown to be widely expressed during numerous developmental processes, as well as in adult cells (Chinnadurai, 2002; Hildebrand and Soriano, 2002). Indeed homozygous knockout *Drosophila* ($dCtBP^{-/-}$) exhibit lethal phenotypic abnormalities (Poortinga et al., 1998). Similar effects have been demonstrated in mouse embryo models (Hildebrand and Soriano, 2002): $ctbp1^{-/-}$ animals are small and have a significantly shorter life expectancy, whereas $ctbp2^{-/-}$ embryos die *in utero*. In addition, the phenotypic defects seen in the neurological, cardiovascular and musculoskeletal systems of $ctbp2^{-/-}$ embryos are worsened if they are also $ctbp1^{-/-}$, indicating that the two proteins, despite having distinct roles, may share considerable functional overlap.

Through their interaction with adenovirus E1A, the function of CtBPs was identified as transcriptional co-repressors (Chinnadurai, 2002). CtBPs associate with chromatin-modifying enzymes including histone deacetylases to form a chromatin-modifying complex, which is recruited to chromatin via the association of CtBPs with DNA-binding transcription factors (Shi et al., 2003). Most of these transcription factors, as well as E1A, are characterized by the presence of a consensus CtBP-binding motif, PxDLS, which associates with the N-terminal protein-binding domain of CtBPs (Chinnadurai, 2002). Formation of the chromatin-modifying complex is dependent on dimerization of CtBPs, which is promoted by elevated levels of NADH in the cytosol and nuclei of cells. Hence, CtBP-dependent transcriptional repression is sensitive to perturbations in cellular metabolism, such as hypoxia (Zhang et al.,

2002; Fjeld et al., 2003) and increased glycolysis (Zhang et al., 2007). Interestingly, the interaction of CtBPs with certain proteins is reduced by NADH, notably MDM2 (Mirnezami et al., 2003) and the p300 histone acetyl transferase (Kim et al., 2005), and hence rather than simply promoting the transcriptional repressor function of CtBPs, NADH binding may toggle CtBPs between two functional forms in response to cellular metabolic status. CtBPs function as co-repressors for a substantial number of transcriptional repressors and consequently regulate diverse cellular processes. For example, CtBP-regulated cellular phenotypes of relevance to tumour initiation and development include epithelial—mesenchymal transition, apoptosis and cell cycle progression, migration and invasion, angiogenesis, senescence and mitotic fidelity (e.g. Chen et al., 2008; Mroz et al., 2008; Bergman et al., 2009; reviewed in Bergman and Blaydes, 2006; Chinnadurai, 2009). Both CtBP1 and CtBP2 have demonstrated co-repressor activity, indeed the two proteins heterodimerize. The more severe phenotype associated with loss of CtBP2 compared with CtBP1 in some models (Hildebrand and Soriano, 2002; Paliwal et al., 2006) may in part be due to the presence of a nuclear localization sequence in CtBP2-L, whereas the nuclear localization of CtBP1 proteins requires that they associate with other molecules including CtBP2-L (Bergman et al., 2006; Verger et al., 2006; Zhao et al., 2006). In addition to their role as transcriptional repressors, CtBPs were independently identified as cytoplasmic proteins with a key role in the regulation of Golgi architecture and endocytosis (Gallop et al., 2005; Corda et al., 2006; Colanzi et al., 2007). To date this activity has been primarily ascribed to the CtBP1-S form [which is generally termed CtBP3 or BARS (Brefeldin A ADP-ribosylated substrate) in this context].

Early experiments indicated a role for CtBPs in the regulation of cellular transformation: E1A lacking its C-terminal CtBP-interacting domain being more effective than full-length E1A in promoting cellular immortalization, as well as metastatic transformation, when in cooperation with mutant *RAS* (Boyd et al., 1993). Recently, dysregulated control of the abundance of CtBP1 has been identified as a key initiating step in the formation of colorectal tumours: CtBP degradation is dependent on APC (adenomatous polyposis coli) in these cells,

and mutation of APC leads to elevated CtBP1 protein abundance with resultant changes in intestinal cell fate and differentiation that initiate adenoma formation (Nadauld et al., 2006; Phelps et al., 2009). Conversely, in melanoma, reduced CtBP1 expression is correlated with disease progression, due to loss of CtBP-dependent repression of MIA (melanoma inhibitory activity) gene expression in this cell type. A recent genome-wide association study in prostate cancer identified a single-nucleotide polymorphism in *CTBP2* as highly associated with this tumour type, although the functional consequences of this polymorphism are unknown. In general, however, results on the expression of CtBP proteins in cancer are rather limited; its expression in breast cancer has not been reported, and analysis of the major CtBP protein isoforms has not been described in any tumour type.

In addition to roles in the initiation and progression of tumorigenesis, CtBPs have also been implicated in the cellular responses to anticancer chemotherapeutic agents. Immortalized mouse embryo fibroblasts from $ctbp1^{-/-}ctbp2^{-/-}$ animals proliferate in culture apparently normally, but are hypersensitive to the topoisomerase II inhibitor, etoposide (Grooteclaes et al., 2003). In addition, certain forms of genotoxic agent induce the activity of signalling kinases, HIPK2 (homeodomain-interacting protein kinase 2) or JNK1 (c-Jun N-terminal kinase 1), which phosphorylate CtBP1-L on Ser-422 (Zhang et al., 2003; Wang et al., 2006; this site is conserved on CtBP2), and promote its proteasome-dependent degradation. This signalling event is a measurable component of the mechanism of cancer cell killing by cisplatin (Wang et al., 2006). Full details of the mechanism of this process are yet to be fully characterized; however, the tumour suppressor p14^{ARF} (ARF is alternative reading frame) has been demonstrated to be required for stress-induced degradation of CtBP proteins (Paliwal et al., 2006). Chemo-sensitization through reduction in CtBP abundance involves an enhanced apoptotic response, which can be due to loss of CtBP-dependent repression of BH3 domain (Bcl-2 homology domain 3)-only protein expression (Kovi et al., 2010). We have also recently shown that, in both breast cancer cell lines and non-transformed human fibroblasts, siRNA (small interfering RNA)-mediated knockdown of CtBP protein expression results in a loss of mitotic fidelity; CtBP knockdown also induces the activation of a p53-dependent cell cycle

arrest response, which, in cells where this pathway is functional, suppresses the proapoptotic response to CtBP loss (Bergman et al., 2009). As other studies of the CtBP-dependent suppression of chemosensitivity have focused on cells in which the p53 response is functionally compromised, a role of this p53 response in modifying CtBP-dependent chemosensitivity has not yet been established.

The aim of the present study, therefore, was to establish the patterns of expression and localization of CtBP1 and CtBP2, as well as their major isoforms, in a common human malignancy, breast cancer. We have examined how CtBP protein expression is regulated in breast tumour-derived cells, and furthermore, how CtBPs and p53 coordinately modulate the response of these cells to cancer chemotherapeutic agents with diverse mechanisms of action.

Results and discussion

CtBP proteins are expressed in breast cancer

We first used Western blotting to determine the expression of CtBP proteins in a panel of six widely used breast cancer-derived cell lines (Figure 1A). Using a monoclonal antibody (E12, Santa Cruz Biotechnology) that was raised against human CtBP1, which we have previously characterized for its specificity towards CtBP1 and not CtBP2 (Bergman et al., 2009), a single band of approx. 47 kDa was detected in all of the lines, although one line, SkBr3, was characterized by barely detectable levels of CtBP1. Of the other lines there were more modest (<2-fold) variations in CtBP1 abundance, MCF-7 cells having slightly lower levels than the remaining lines. A CtBP2-specific antibody that was raised against amino acids 361−445 of murine CtBP2 (BD-16/CtBP2, BD Biosciences), and binds the extreme C-terminus of human CtBP2, recognizes a clear double band in all the cell lines over the range of 47−49 kDa. The abundance of these CtBP2 forms varied by less than 2-fold across the panel of cell lines. The intensity of the higher-molecular-mass band was consistently the greater of the two. We also compared representative breast cancer lines with two non-transformed human cell lines: hTERT-immortalized MRC-5 fibroblasts and the non-transformed breast epithelial cell line, MCF-10A. Expression of CtBP1 and CtBP2 in MCF-7 and MDA-MB-231 was comparable with that in MRC-5

Figure 1 | Expression of CtBP proteins in breast cancer cell lines and breast tumour samples

CtBP1 and CtBP2 expression was determined by Western blotting in (**A**) cell lines and (**B**) breast tumour samples. In (**A**), relative quantification of CtBP1 (solid bars) and CtBP2 (open bars) is shown, normalized to actin ('ND' indicates not done, due to multiple actin bands in this cell line). (**C**) Representative samples were probed with antibodies E12 or BD-3, both raised against CtBP1. bc-2 and bc-9 are two of the breast tumour samples from (**B**). CtBP1mh represents lysates from HEK-293 cells transfected with a plasmid expressing CtBP1 with a 3 kDa C-terminal mychis tag; less protein was intentionally loaded in this lane compared with the others.
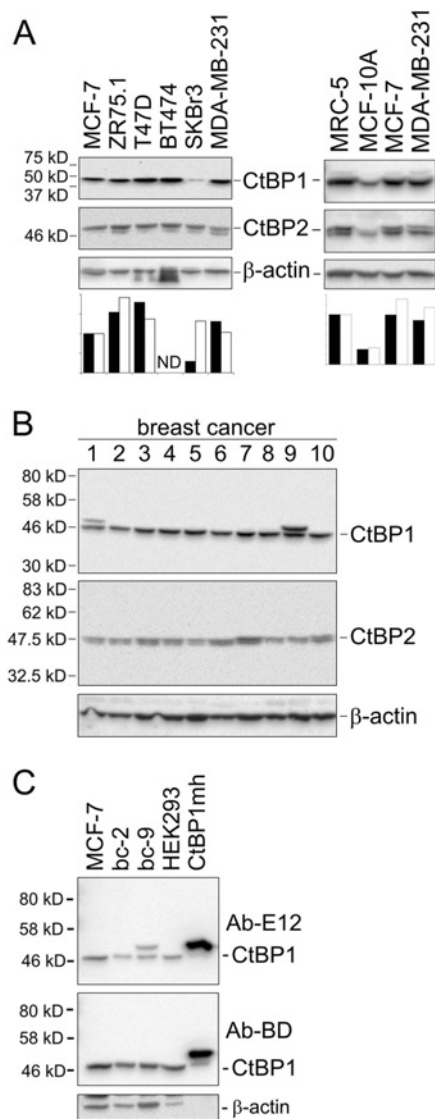
cells, but approx. 3-fold higher than that in MCF-10A.

We then performed comparable blots on samples from a randomly selected series of ten fresh-frozen breast cancer samples from the University of Southampton Cancer Sciences Division tissue bank (Figure 1B). The single CtBP1 band was present in all the samples at approximately equal abundance, none of these breast cancers appearing to lose CtBP1 expression. Interestingly, two of the samples also contained marked amounts of an apparent CtBP1 form with a reduced electrophoretic mobility. This mobility shift is equivalent to an increase of ∼3 kDa, as it runs level on the gel with CtBP1-L with a C-terminal tag of this size (Figure 1C). This band in breast cancer sample number 9 was not, however, recognized by an antibody raised against the C-terminus of murine CtBP1 (BD-3/CtBP1, amino acids 345−441), even though the lower CtBP1 band was detected by this antibody (Figure 1C). Therefore this higher-molecular-mass band may potentially represent either a protein that cross-reacts with E12 in these samples, a previously undescribed splice form of CtBP1 that lacks the C-terminus, or CtBP1 with a post-translational modification that masks the epitope for the C-terminal-directed antibody. No novel bands were detected in the breast cancer samples with the anti-CtBP2 antibody, a doublet being observed in all samples similarly to the cell lines.

We next examined the intra- and inter-cellular distribution of CtBP proteins in the breast cancer cells. Subcellular distribution in the MCF-7 line was assessed using immunofluorescence analysis of paraformaldehyde-fixed cell monolayers. Both CtBP1 and CtBP2 were predominantly nuclear, although some CtBP1 was also faintly detected in the cytoplasm, whereas CtBP2 was not (Figure 2A). We then performed immunohistochemical analysis of CtBP1 and CtBP2 expression in 22 breast tumour samples, using formalin-fixed, paraffin-embedded archival samples from the Cellular Pathology Department at Southampton General Hospital. The primary antibody E12 was used to stain for CtBP1, and separate sections were stained with two different CtBP2 primary antibodies: BD-16 and E16 goat polyclonal (Santa Cruz Biotechnology). Both CtBP2 antibodies produced similar staining patterns to each other (results not shown), but the staining achieved with the BD-16 antibody was generally more

Figure 2 | Inter- and intra-cellular distribution of CtBP proteins in breast cancer cell lines and breast tumour samples
(**A**) Cultured MCF-7 cells were analysed by immunofluorescence for CtBP1 (E12 antibody) or CtBP2 (BD-16 antibody). Green indicates CtBP proteins. Blue indicates DAPI (4′,6-diamidino-2-phenylindole) staining for DNA. Scale bar, 10 μM. (**B**) Representative sections of benign breast tissue, and ductal carcinoma of the breast, stained for CtBP1 (E12 antibody) or CtBP2 (BD-16 antibody). The upper panel shows images from sections that contained no tumour tissue, the tissue having been removed from a patient with a grade 2 ductal carcinoma. Scale bar, 100 μM.
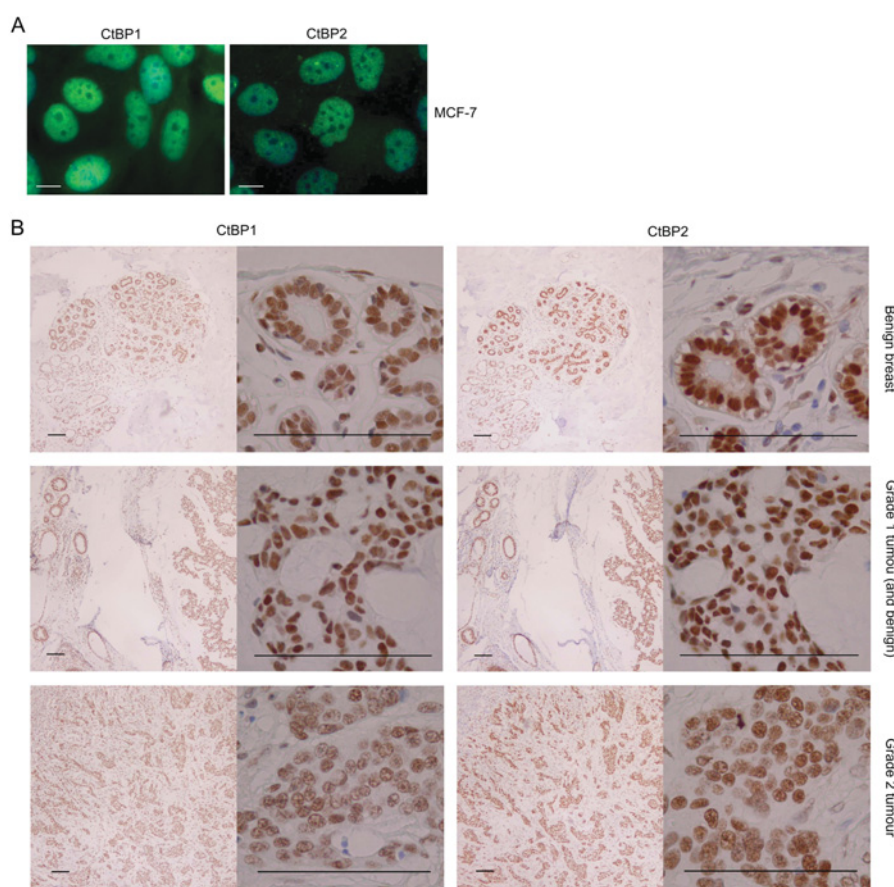
intense and less patchy and therefore it was these sections that were used for subsequent analysis. Both proteins were expressed within the nuclei of benign breast ducts and lobules (Figure 2B, upper panel); expression was identified within epithelial and myoepithelial cells but was sometimes less intense within the latter (Figure 2B, upper panel). Both proteins were also expressed within the nuclei of endothelial cells and stromal cells. In addition, CtBP1 was expressed, usually less intensely, by lymphocytes. Both CtBP1 and CtBP2 were widely expressed within the nuclei of invasive and *in situ* carcinoma cells (Figure 2B, middle and lower panels, *in situ* carcinoma

not shown). CtBP1 and CtBP2 expression was detected within the invasive tumour cells in all the samples, with clear nuclear labelling in most of the tumour cells in each case. With neither the CtBP1 nor CtBP2 antibody did we detect sufficient variations in labelling intensity or distribution to enable the development of a scoring system to distinguish between the tumours. Staining in the tumour cells was no more intense than that in benign breast tissue (e.g. see Figure 2B, middle panel).

As far as it is possible to make comparisons therefore, the data from an immunohistochemical analysis of breast tumour material are consistent with those

from Western blotting of cell lines; breast cancer cells express readily detectable levels of CtBP1 and CtBP2, which, with some exceptions (e.g. SKBr3 cells), do not show substantial variation between tumours. The abundance of CtBPs in tumour cells is comparable with that seen in normal breast luminal epithelial cells *in vivo*, as well as non-transformed fibroblasts, both *in vivo* and *in vitro* (i.e. MRC-5.hTERT.neo). Interestingly, we saw that expression in both normal luminal breast epithelial cells and tumour cells is higher than in the normal myoepithelial cells that, along with the basal cells, surround the normal ducts and lobules. Luminal and myoepithelial/basal cells have very distinct gene-expression profiles and phenotypes, and whereas most of the breast tumours have a luminal phenotype, cell lines such as MCF-10A that are derived from normal breast invariably display characteristics of the basal cell (Wynford-Thomas and Blaydes, 1998; DiRenzo et al., 2002). Together, these findings suggest that the increased abundance of Ct-BPs in most of the breast cancer cell lines compared with MCF-10A most likely reflects this phenotypic difference, rather than any cancer-associated change. It is noteworthy, however, that MDA-MB-231 cells, which are representative of 10−15% of the breast tumours that do have a basal-like phenotype, do have elevated CtBP1 and CtBP2 levels compared with MCF-10A.

CTBP1 and *CTBP2* mRNAs encoding both long and short forms of CtBP proteins are expressed in breast cancer cells

The two forms of *CTBP1* mRNA expressed in human cells that encode CtBP1-L and CtBP1-S/CtBP3/BARS proteins are distinguishable by the inclusion of an additional exon (1α) in the CtBP1-S encoding transcript (summarized in Bergman et al., 2006), the result of which is that CtBP1-L contains 11 more amino acids at the N-terminus compared with CtBP1-S. The consequences of this on the function of the protein are unclear; CtBP1-S has been extensively characterized as a cytoplasmic protein with roles in membrane fission and Golgi maintenance. CtBP1-L has been characterized primarily as a transcriptional repressor, but while CtBP1-L has been shown to function comparably to CtBP1-S in the cytoplasm, whether CtBP1-S has transcriptional repressor activity has not been determined. PCR analysis of mouse embryo fibroblasts has shown both

splice variants to be present in these cells; however, to our knowledge, no analysis of the relative expression levels of these two transcripts in human cell lines or tissues has yet been reported. We therefore performed RT−PCR (reverse transcription−PCR) analysis of the breast cancer cell line panel, as well as non-transformed MRC-5 and MCF-10A cells, amplifying transcripts containing exons 1−3 of *CTBP1* to generate products from *CTBP1-L* and *CTBP1-S* (248 and 443 bp respectively, Figure 3A). In all the cell lines, *CTBP1-S* was the predominant transcript present, with *CTBP1-L* being detectable at low levels only. With the exception of SKBr3 cells, in which *CTBP1* transcripts were barely detectable, all the cell lines appeared to express approximately equal amounts of *CTBP1-S* mRNA. However, as the PCR reactions were optimized for detection of all *CTBP* transcripts, rather than relative quantification between samples, quantitative PCR for *CTBP* mRNA was performed (Figure 3A, histogram). This revealed a greater degree of variation, although again the *CTBP1* transcripts were barely detectable in SKBr3. *CTBP1* mRNA abundance in MCF-10A was not lower than that in the cancer cell lines, indicating that the reduced CtBP protein abundance in these cells is due to differential regulation at the post-transcriptional level. We also examined the breast cancer samples for *CTBP1* transcript expression; again both transcripts were present in most of the samples, and while *CTBP1-S* was again the predominant transcript in some samples, this was not the case in all the tumour samples (Figure 3B). This may reflect variation between breast cancer cells derived from different patients, or possibly differential content of stromal and inflammatory cells in the tumours. Together, these results indicate that the single protein band observed in Western blots contains both CtBP1-L and CtBP1-S, with CtBP1-S presumably being the predominant form in the cell lines.

CtBP2 protein shows a clear doublet on Western blots that, on the basis of conclusions made by Verger et al. (2006), are likely to consist of CtBP2-L and CtBP2-S that differ in size by 25 amino acids. The additional amino acids in CtBP2-L contain a basic KVKRQR motif, which may well contribute to the altered mobility of the two proteins in SDS/PAGE, but more importantly this region functions as a nuclear localization or retention sequence (Bergman et al., 2006; Verger et al., 2006; Zhao et al., 2006)

Figure 3 | Expression of *CTBP* mRNA transcripts in breast cancer cell lines and breast tumour samples

cDNA was derived from mRNA extracted from cell lines (**A**) or breast tumour samples (**B**) and amplified by PCR using primers specific for the 5′ regions of *CTBP1* and *CTBP2* mRNA to determine the expression of the mRNAs encoding the L- and S-forms of each CtBP protein. The histogram in (**A**) shows results from a TaqMan quantitative PCR analysis of CtBP1 (solid bars) and CtBP2 (open bars) in the cell lines.
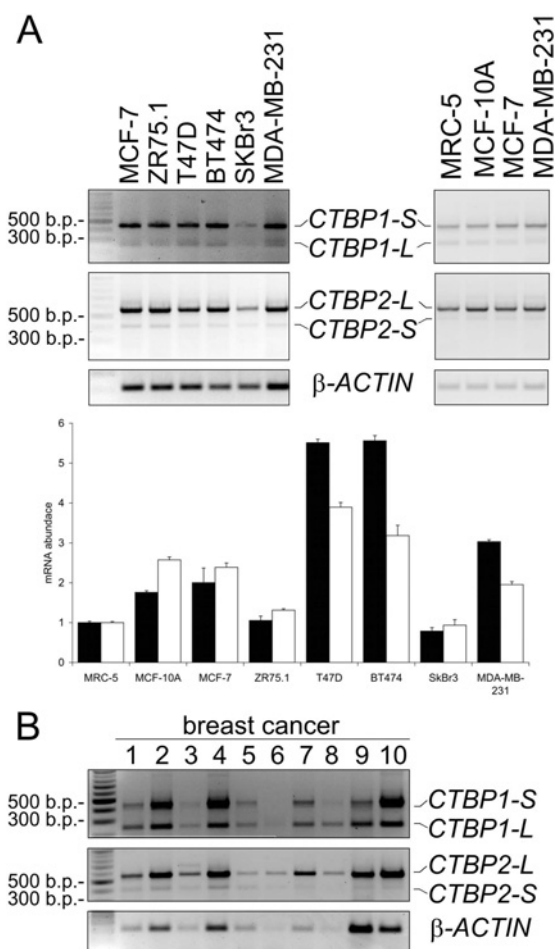

and as such potentially has a clear influence on the function of CtBP2-L as a transcription factor. Verger et al. (2006) used RT−PCR of murine foetal liver to demonstrate the presence of transcripts lacking the first coding exon of *ctbp2-L* that would be predicted to initiate transcription at an alternative ATG present in the next coding exon (exon 4) to generate CtBP2-S. As far as we are aware, however, this analysis has not

been performed in human cells, and the derivation of the two human isoforms of CtBP2 has not been determined. We therefore performed PCR-analysis of the 5′-region of *CTBP2* in the breast cancer cell lines, to amplify transcripts containing exons 2−6. *CTBP2-L* mRNA is predicted to generate a band of 571 bp in this assay, and this was indeed the predominant band obtained in all the breast cancer cell lines and tumour samples. However, a smaller band was also obtained in all the cell lines and in most of the breast cancer samples (Figures 3A and 3B). Both bands were isolated from RT−PCRs of MCF-7 cDNA, cloned and sequenced. The larger band corresponded to the predicted *CTBP2-L* sequences, and the smaller band was 471 bp and lacked exon 3, which is the first coding exon in the human *CTBP-L* coding sequence. Therefore, as is the case in the mouse, this transcript would be translated into CtBP2-S from the ATG in exon 4.

However, we did note that the *CTBP2-S* mRNA was generally only expressed at very low levels compared with *CTBP2-L*, whereas the difference in the abundance of the two encoded protein isoforms was rather less marked (Figure 1). We therefore asked whether the *CTBP2-L* mRNA has the potential to be translated into both proteins, as it contains both ATGs at codons 1 (exon 3) and 26 (exon 4). We previously isolated a *CTBP2* clone from a HeLa cDNA library that contains 233 bp of 5′-UTR (5′-untranslated region) sequence, in addition to the complete coding sequence of *CTBP2-L*, and thus can be used to assess sites of translational initiation from the *CTBP2-L* splice form of the mRNA. This cDNA was cloned into the pCDNA3.1 expression vector and transfected into HEK-293 cells (human embryonic kidney cells). Endogenous CtBP2 in this cell line is present in the doublet form (Figure 4A), and transfection of increasing amounts of pcDNA3.1CtBP2 vector resulted in the abundance of both forms increasing, suggesting that both proteins are translated from a single cDNA (Figure 4B). To confirm this, vectors in which the two ATGs were individually mutated were transfected: loss of ATG1 (codon 1) clearly resulted in the synthesis of CtBP2-S only, whereas only CtBP2-L was synthesized when ATG2 (codon 26) was mutated (Figure 4C). Thus, in human cells, CtBP2-S may be synthesized as a result of both alternative splicing and the use of alternate translation start sites in the *CTBP2-L* mRNA.
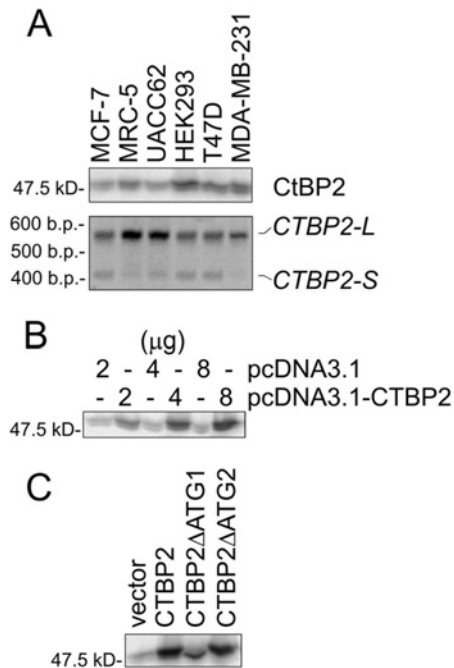
An autoregulatory feedback loop maintains CtBP protein abundance in proliferating breast cancer cells, and is disrupted in response to stress signalling

Using siRNA reagents to block the expression of both CtBP1 and CtBP2 together, we have previously demonstrated that CtBPs play an important role in the proliferation and survival of breast cancer cells (Bergman et al., 2009). However, because CtBP1 and CtBP2 potentially have some important functional differences, we wished to examine the effects of blocking the expression of each gene product independently. Figure 5(A) (Western blot and quantification) demonstrates the efficacy of the siRNAs to *CTBP1* and *CTBP2* in blocking the synthesis of the two proteins in MCF-7, MDA-MB-231 and

MRC5.hTERT.neo cells, either individually (CtBP1 or CtBP2) or in combination (CtBP1+CtBP2). CtBP1/2 is a single siRNA that targets the mRNAs derived from both CtBP-encoding genes. In doing these experiments, in addition to the expected knockdown of the target of the siRNA, we made the consistent observation that inhibition of CtBP1 synthesis alone results in an increase in the abundance of CtBP2 protein. This occurred in the two breast cancer cell lines we studied, as well as in the non-transformed fibroblast line, MRC5.hTERTneo. In contrast, *CTBP2* siRNA did not affect CtBP1 protein abundance. This indicates that cells posses a mechanism for maintaining homoeostasis of CtBP protein abundance through the control of CtBP2 protein levels. This appears to be a post-transcriptional effect on CtBP2, as CtBP1 siRNA does not up-regulate the abundance of *CTBP2* mRNA, as determined by quantitative RT−PCR (Figure 5B).

We have investigated this regulation of CtBP abundance further in the experiment described in Figure 5(C). MDA-MB-231 cells were transfected with an expression vector for a C-terminally tagged CtBP2 protein (CtBP2-TT), and stably expressing clones established. The CtBP2-TT vector has also had introduced synonymous mutations in the target region of the CtBP1/2 siRNA. As shown in Figure 5(C), CtBP2-TT protein was expressed at approximately identical abundance to endogenous CtBP2 in the selected clone (similar levels were observed in other clones). When endogenous CtBP protein expression was inhibited with CtBP1/2 siRNA, the expected decrease in endogenous CtBP2 was observed; however, there was a marked increase in the abundance of CtBP2-TT. This effect is consistent with our observations of endogenous CtBP2 when CtBP1 siRNA was used. It also confirms that this feedback regulation of CtBP2 protein abundance occurs at the post-transcriptional level, as CtBP2-TT expression is driven by the CMV (cytomegalovirus) promoter in the expression vector, which is unlikely to be under the same control as the endogenous *CTBP2* promoter. To determine whether this regulation involves proteasome-dependent degradation, the cells were transfected with control or CtBP1/2 siRNA and, at 27 h post-induction, cells were treated with the proteasome inhibitor MG132 (carbobenzoxy-L-leucyl-L-leucyl-leucinal; Figure 5D). In control siRNA-treated cells, this resulted in an increase in CtBP2-TT

Figure 5 | Evidence that CtBP2 protein abundance in cells is regulated by an auto-regulatory feedback loop
Legend on next page.
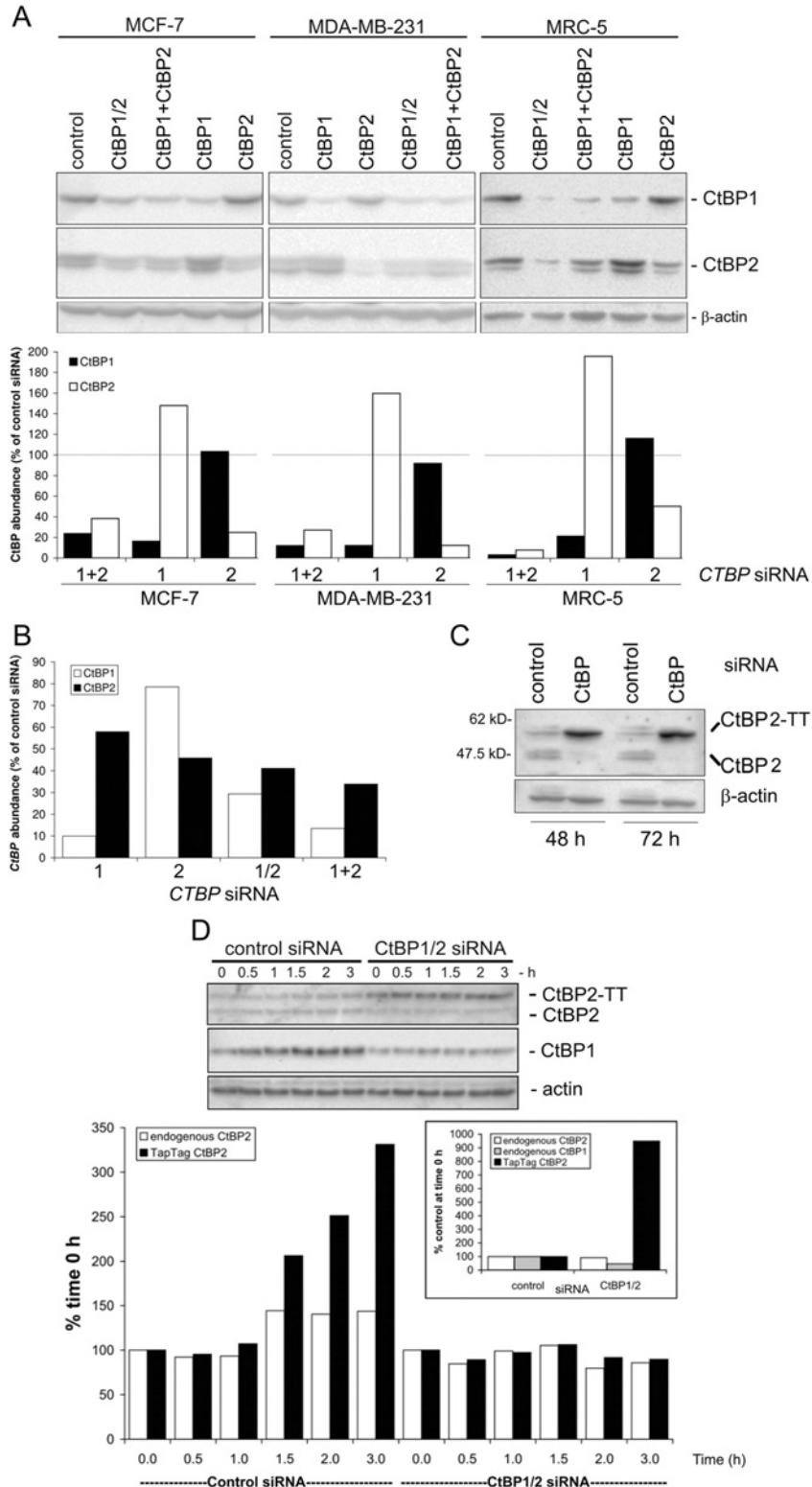
(A) Cells were transfected with siRNA reagents that either target both *CTBP1* and *CTBP2* together (CtBP1/2), independently (CtBP1 or CtBP2) or a combination of the two independently targeting siRNAs (CtBP1+CtBP2). Lysates were prepared for Western blotting 48 h after transfection. Quantification of Western blotting results is shown and is representative of 11 separate experiments in MCF-7 cells, in which eight showed a CtBP1 siRNA-induced increase in CtBP2 of up to 70 %. (B) MCF-7 cells were transfected with the indicated siRNA. Lysates prepared 72 h after transfection were analysed for expression of the *CTBP* mRNAs using quantitative RT−PCR. (C) MDA-MB-231 cells stably expressing tagged, CtBP1/2 siRNA-insensitive CtBP2 were transfected with CtBP1/2 siRNA and lysates were prepared for Western blotting at the indicated times post-transfection. E16 antibody was used to detect CtBP2, as the C-terminal tag obscures the epitope for BD clone 16 antibody. (D) MDA-MB-231 cells stably expressing tagged, CtBP1/2 siRNA-insensitive CtBP2 were transfected with either control or CtBP1/2 siRNA. At 27 h post-transfection, cells were treated with 50 μM MG-132 for the indicated times. E16 antibody was used to detect both the tagged and endogenous CtBP2.

protein abundance of over 3-fold within 3 h, indicating that the protein is subject to proteasome-dependent degradation. Endogenous CtBP2 and CtBP1 protein abundance also increased. In CtBP1/2 siRNA-treated cells, endogenous CtBP1 and CtBP2 did not increase in response to MG-132, as their synthesis was blocked by the siRNA. CtBP2-TT abundance was elevated compared with control siRNA-transfected cells (see the inset to Figure 5D), but was not further increased by MG-132. This demonstrates that inhibiting the synthesis of the endogenous CtBP proteins results in a loss of proteasome-dependent degradation of the exogenous CtBP2-TT.

The post-transcriptional regulation of CtBP protein abundance is known to be important in both tumour initiation (Phelps et al., 2009) and the response to genotoxic stress and cancer chemotherapeutics (Zhang et al., 2003; 2005; Paliwal et al., 2006; Wang et al., 2006). In the main, these studies have focused on CtBP1; CtBP2 is also known to be degraded in response to UV radiation damage (Paliwal et al., 2006) although the reported effects of this stress on CtBP2 abundance appear to be less than those reported for CtBP1 (Zhang et al., 2003), albeit the experimental systems used in the separate studies not being directly comparable. To our knowledge, the relative effect of genotoxic stress on the abundance of CtBP1 compared with CtBP2 has not been directly investigated in the literature. We therefore wished to determine whether the autoregulatory mechanism controlling CtBP2 abundance might function in response to stress-induced CtBP1 degradation, and result in a differential effect of stress on the expression of these two proteins. Figure 6(A) shows that, in normally proliferating MCF-7 cells, blockade of

protein synthesis by cycloheximide for 16 h does not decrease the abundance of CtBP1 or CtBP2. This is consistent with the abundance of CtBP proteins being tightly regulated at the post-transcriptional level such that their levels do not decrease rapidly when their synthesis is blocked. Therefore any effects of genotoxic stress on the abundance of CtBPs within this time scale should involve increased rates of protein degradation, rather than inhibition of protein synthesis. MCF-7 and MDA-MB-231 cells were then exposed to increasing doses of UV-C radiation and examined by Western blotting 16 h later. In MCF-7 cells, 5 J/m^2 UV-C induced the stabilization of p53 protein, confirming the activation of stress-responsive signalling pathways at this dose (Figure 6B). Higher doses appeared to attenuate this response, presumably due to non-specific cell damage. Both CtBP1 and CtBP2 protein abundance was markedly reduced in MCF-7 cells in response to 5 J/m^2 UV-C, as well as higher doses (Figure 6B). In MDA-MB-231 the same doses of UV-C had a negligible effect on the abundance of CtBP1 or CtBP2 (Figure 6C; p53 protein abundance was also not affected, this being due to the presence of a stable mutant p53 protein in these cells). Comparable experiments were then performed using cisplatin, the cells being continuously exposed to the drug for 16 h. In MCF-7 cells, p53 stabilization was observed at 25 μM cisplatin; a decrease in CtBP2 abundance also occurred at this concentration (Figure 6D), and 50 μM cisplatin caused a marked reduction in both CtBP1 and CtBP2. As was the case for UV-C, cisplatin essentially failed to promote CtBP degradation in MDA-MB-231 cells (Figure 6E). Finally, we wished to determine whether a broader range of chemotherapeutic drugs affected CtBP

Figure 6 | Regulation of the abundance of CtBPs by genotoxic and chemotherapeutic agents in breast cancer cells
(**A**) MCF-7 cells were exposed to 100 μg/ml cycloheximide to inhibit protein synthesis for the indicated time periods before cell lysis for Western-blot analysis. (**B**) MCF-7 cells were irradiated with the indicated dose of UV-C and harvested 16 h later for Western blotting. (**C**) MDA-MB-231 cells were irradiated with the indicated dose of UV-C and harvested 16 h later. (**D**) MCF-7 cells were exposed to the indicated concentrations of cisplatin for 16 h. (**E**) MDA-MB-231 cells were exposed to the indicated concentrations of cisplatin for 16 h. (**F**) MCF-7 cells were incubated for 24 h with the following compounds at their IC_{50} concentration: 5-fluorouracil (12.5 μM), Taxol (4.13 nM), etoposide (1.56 μM) and cisplatin (15.5 μM) before analysis of CtBP abundance by Western blotting.
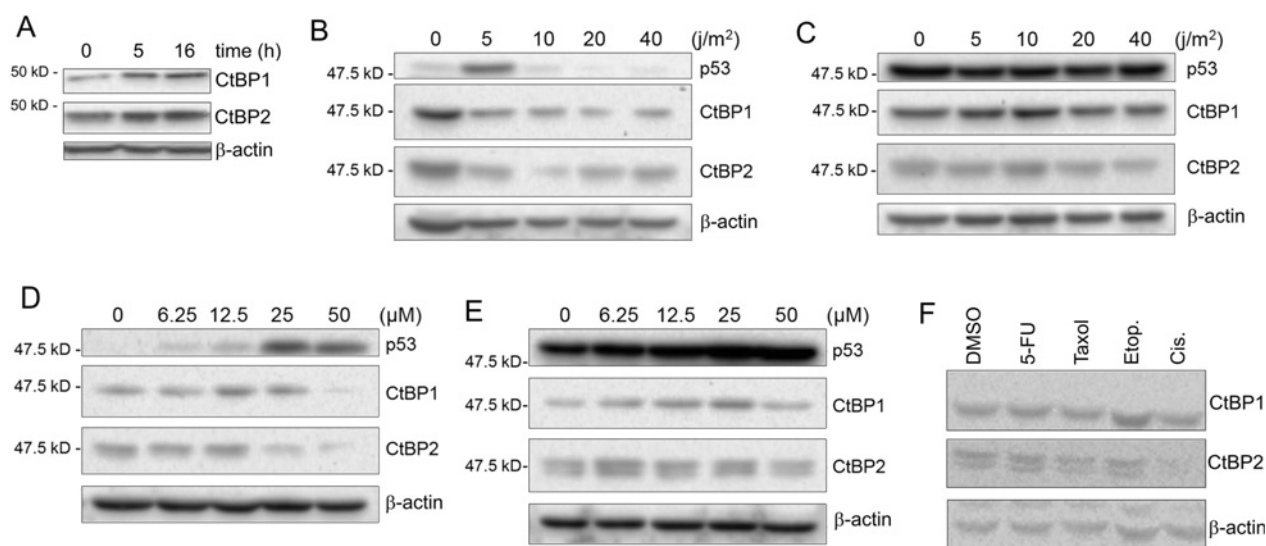

protein abundance. Cell proliferation assays were used to determine the IC_{50} values for the drugs in MCF-7 cells (results not shown and Figure 8), which were then exposed for 24 h to IC_{50} concentrations before Western blotting (Figure 6F). Cisplatin (15.5 μM) caused a reduction in CtBP2 (note that while the concentration is lower than the minimum required for observing an effect in Figure 6D, the treatment time of 24 h is longer). At IC_{50} concentrations, none among 5-fluorouracil, Taxol and etoposide promoted the degradation of either CtBP1 or CtBP2, indicating that induced CtBP protein degradation is not a general response to all forms of genotoxic stress.

Together, these data demonstrate that both UV radiation and cisplatin, which have previously been shown to promote the degradation of CtBP proteins in other experimental models, also do so in the MCF-7 breast cancer cell line. At least in the case of cisplatin, these effects do occur at concentrations within the range of the IC_{50} and so could potentially contribute to the antiproliferative activity of the drug.

UV-C affected the abundance of both CtBP1 and CtBP2 proteins to a comparable extent, whereas cisplatin appeared to down-regulate CtBP2 at slightly lower concentration than was required for its effect on CtBP1. This suggests that the feedback mechanism whereby CtBP2 protein abundance is maintained or increased in response to decreased CtBP protein synthesis does not function in response to stress signalling-induced degradation of CtBPs. Neither UV-C nor cisplatin had any substantial effect on the abundance of CtBP proteins in the MDA-MB-231 cell line, even at doses severalfold higher than were effective in MCF-7. There are many phenotypic and genetic differences between these two cell lines, and hence it is not possible to directly infer any mechanistic details from this difference; however, we have performed a characterization of some of the known key regulators of CtBP protein abundance in the cell lines (Figure 7). HIPK2 is expressed in both MCF-7 and MDA-MB-231 cells (Figure 7A), and hence the lack of UV-C-induced CtBP protein degradation in

Figure 7 | Status of known regulators of CtBP degradation in breast cell lines

(**A**) The abundance of HIPK2 and p14^{ARF}-encoding mRNAs was determined by RT−PCR (left panel) and HIPK2 protein by Western blotting (right panel). (**B**) APC protein was detected by immunofluorescence microscopy: green, APC; blue, DAPI staining of DNA. *APC* mRNA transcripts derived from promoter A or B were determined by RT−PCR.
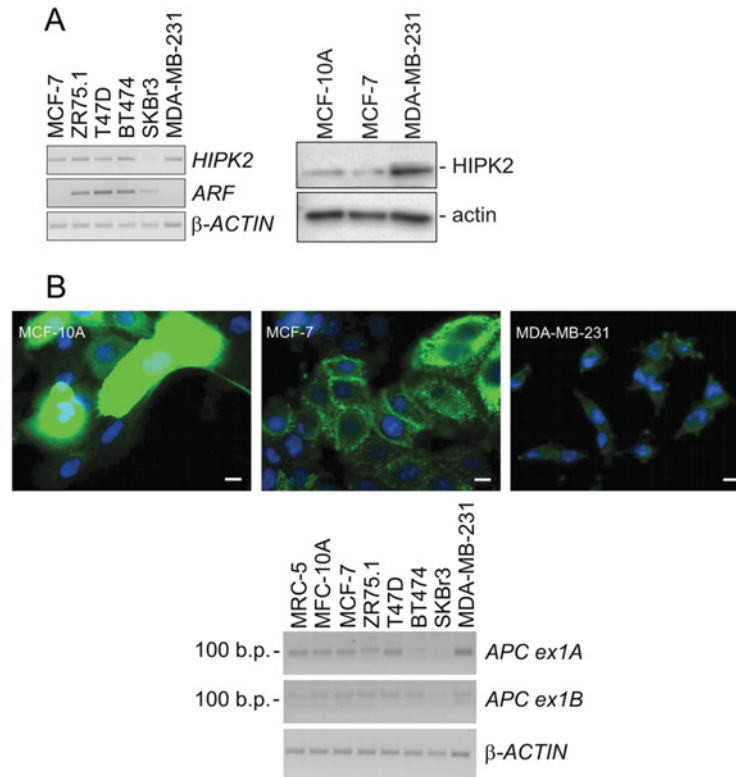
MDA-MB-231 cells cannot be attributable to loss of this signalling kinase. Like HIPK2, p14^{ARF} can also be required for UV-radiation-induced CtBP2 degradation (Paliwal et al., 2006); however, MCF-7 cells express neither p14^{ARF}-encoding mRNA nor p14^{ARF} protein (Stott et al., 1998; Saadatmandi et al., 2002) (see Figure 7A for confirmation of these previous reports). Thus stress-inducible down-regulation of CtBP abundance does not appear to require p14^{ARF} in the MCF-7 breast cancer cell line. APC is known to promote the proteasome-mediated degradation of CtBP1 (Nadauld et al., 2006) and APC mutation correlates with an increase in CtBP1 abundance in early-stage colorectal tumours (Phelps et al., 2009), although a role of APC in stress-inducible degradation has not been described. Both MCF-7 and MDA-MB-231 cells are known to express functional APC protein (Smith et al., 1993); however, we did find the abundance of APC protein in MDA-MB-231 cells to be markedly lower than that in MCF-7 (Figure 7B). Interestingly, the abundance of APC in both lines was very much lower than that in MCF-10A. The expression of APC may be down-regulated in cancer cells due to promoter methylation (Tsuchiya et al., 2000); however, RT−PCR analysis indicated that this was not the cause for the differential APC protein abundance in these three lines (Figure 7B). These results suggest that the low levels of CtBP proteins in MCF-10A cells could potentially be due to high levels of APC-dependent degradation. It is also possible that the differential response of CtBPs to genotoxic stress between MCF-7 and MDA-MB-231 may relate to differences in their APC levels; in both cases further work will be required to clarify the interplay between APC and CtBPs in breast epithelial cells.

Inhibition of CtBP expression sensitizes breast cancer cells to diverse chemotherapeutic agents

Previous studies have demonstrated that loss or reduction in CtBP family proteins can sensitize some cell lines to cell killing by certain chemotherapeutic agents, specifically etoposide and cisplatin having been examined (Grooteclaes et al., 2003; Wang et al., 2006). We wished to extend these studies to breast cancer-derived cells treated with a wider range of chemotherapeutics with distinct mechanisms of action. Given recent data on the functional differences between CtBP1 and CtBP2, as well our present results demonstrating feedback regulation of CtBP2, we also wished to compare the effects of reagents targeting either CtBP1 or CtBP2 individually in these assays. Finally, the pro-apoptotic effects have been demonstrated to occur independently of p53; however, we have recently demonstrated that loss of CtBP1 and CtBP2 does lead to p53 activation, and this can protect against CtBP siRNA-induced apoptosis, and hence we examined the role of p53 in CtBP siRNA-induced chemosensitization in breast cancer cells.

MCF-7 cells were transfected with combinations of control, CtBP1, CtBP2, CtBP1/2 and p53-specific siRNAs such that the total amount of siRNAs transfected was identical in each case. Western blotting of cell lysates 72 h post-transfection confirmed that CtBP- and p53-targeting siRNAs retain their efficacy when applied in combination and with a chemotherapeutic agent (Figure 8A). As we have shown previously, p53 protein abundance increased in response to the combined knockdown of CtBP1 and CtBP2, using either the CtBP1/2 siRNA or a combination of CtBP1 and CtBP2 siRNAs. Knockdown of either CtBP1 or CtBP2 in isolation did not affect p53 abundance (results not shown). The effects of the siRNA combinations on overall cell proliferation in the absence of additional chemotherapeutics were then determined by a 96-well plate MTS {3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium] proliferation assay. At 7 days after cell transfection, equal numbers of cells are plated on the wells 2 days post transfection (Figure 8B). The MTS assay measures cellular reducing potential to determine changes in the numbers of metabolically active cells and thus integrates the combined effects of inhibition of cell division and induction of cell death over the treatment period. CtBP1 depletion alone caused a small reduction in MCF-7 cell proliferation to 92.6 % of control siRNA-treated cells. CtBP2 siRNA had a greater effect, proliferation being reduced to 76.4 %. Given the Western blotting results, this differential response to CtBP1 and CtBP2 siRNAs is unlikely to be due to any differential efficacy of the siRNAs on their respective targets and suggests that CtBP2 is more critical than CtBP1 in the maintenance of proliferative capacity in these cells. This finding is consistent with the results from knockout mouse studies (Hildebrand and Soriano, 2002), as well as a previous study looking at apoptosis in colon cancer cell lines induced by CtBP1 versus CtBP2 siRNA (Paliwal et al., 2006). Inhibition of expression of both CtBPs reduced proliferation to 68.0 % or 66.0 % depending on whether the single CtBP1/2 siRNA or the CtBP1 and CtBP2 siRNA combination was used. Inhibition of p53 expression on its own caused a small reduction in proliferation, and also reduced proliferation in the presence of CtBP1/2 siRNAs, to 60 % of untreated cells. These confirm our previous findings that loss of both CtBPs has anti-proliferative effects in MCF-7 cells, which are not dependent upon p53, and that loss of p53 further sensitizes the cells to loss of CtBPs. We showed previously that the antiproliferative effects of CtBP 1 and 2 knockdown in these cells is primarily through cell cycle arrest, with a switch to an apoptotic response occurring when p53 is lost. Because the MTS assay used here does not distinguish between these two mechanisms of reduction in the numbers of viable cells, we also performed time-lapse video-microscopy of cells, which again confirmed this (see Supplementary Movies 1—4 at http://www.biolcell.org/boc/103/boc1030001add.htm). Cells were treated as per the Western blot in Figure 8(A), and after 72 h, the medium was replaced and video-microscopy commenced. Combined knockdown of CtBP1 and CtBP2 resulted primarily in cytostasis, with an increase in the proportion of the remaining mitoses that are aberrant (Supplementary Movie 2). p53 siRNA on its own had little effect (Supplementary Movie 3), but when combined with knockdown of both CtBP proteins, reduced the cytostatic effect of CtBP loss, and increased cell death (Supplementary Movie 4).

To assess the chemo-sensitizing effects of the siRNA combinations, cells were transfected with

Figure 8 | Depletion of CtBPs enhances the sensitivity of MCF-7 cells to cancer chemotherapeutics
(**A**) Cells were transfected with the indicated siRNAs (50 nM total siRNA concentration). After 48 h, 5-fluorouracil was added where indicated, and lysates were prepared for Western blotting a further 24 h later. (**B**) The effects of the indicated siRNAs on cell survival were determined by MTS assay 7 days after transfection ($n = 16$ for each data point). (**C−F**) Effect of the indicated siRNAs on the IC_{50} of the cells for 5-fluorouracil (**C**), Taxol (**D**), etoposide (**E**) and cisplatin (**D**). Results in each graph represent the means for three independent experiments.
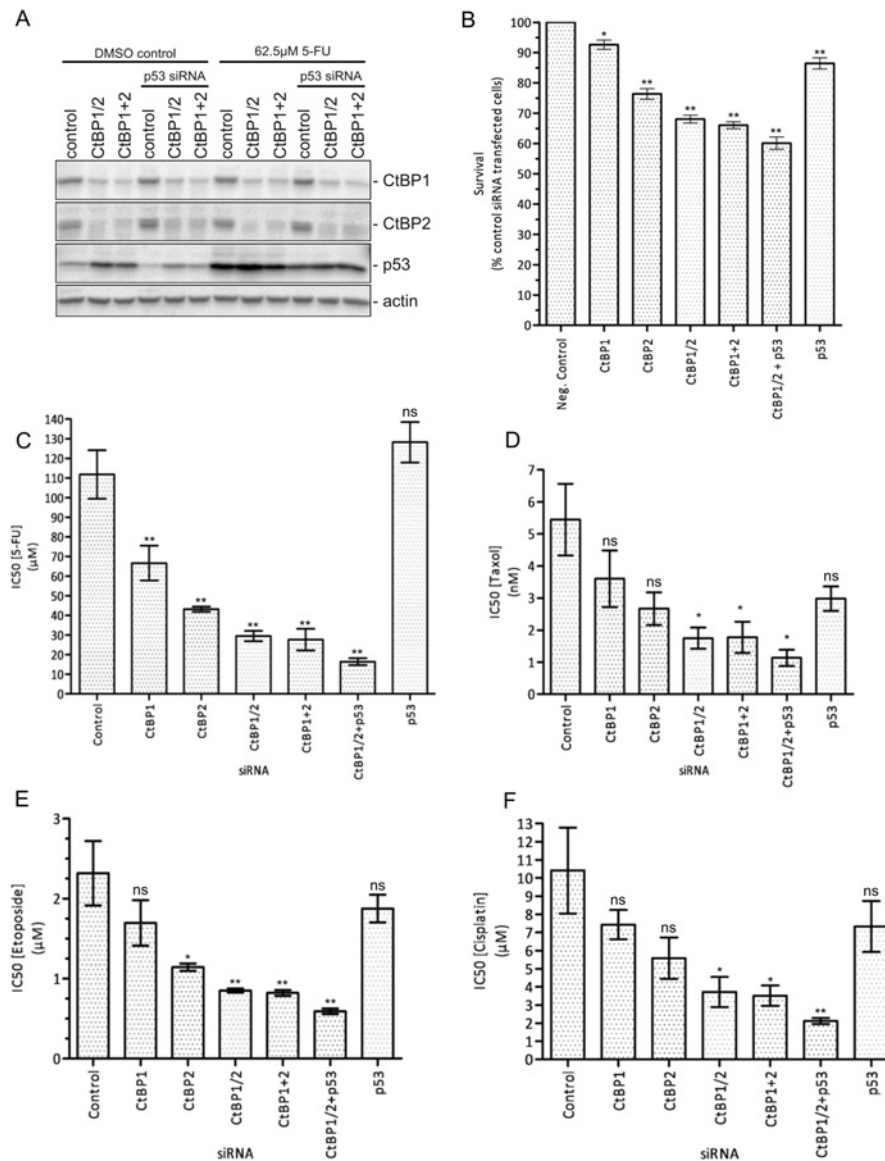
siRNA, and transferred to 96-well plates 2 days later; then 1 day later the drug was applied and cells were left for 1 day, after which the cells were cultured in a fresh medium for a further 3 days. Independent IC_{50} values for the drugs for each siRNA combination were then calculated for these experimental conditions. The anti-metabolite 5-fluorouracil (Figure 8C) had an IC_{50} of 118 μM in control siRNA-transfected cells. Despite having a relatively modest effect on untreated cells, CtBP1 siRNA significantly reduced

the IC_{50} for 5-fluorouracil to 66.7 μM, the effect of CtBP2 siRNA on IC_{50} being somewhat greater than this (decreased to 43.3 μM). The IC_{50} was further reduced to 29.5 or 27.6 μM when both CtBP1 and CtBP2 expression was inhibited by the CtBP1/2 or CtBP1&2 siRNAs respectively. When transfected on its own, siRNA to p53 increased the IC_{50} slightly, although not significantly. In contrast to this effect under conditions when CtBPs were expressed normally, when CtBP1 and CtBP2 expression was inhibited, loss of p53 further sensitized the cells to 5-fluorouracil ($IC_{50} = 16.4$ μM). To examine these effects in more detail, we again performed time-lapse video-microscopy, cells being treated with 5-fluorouracil for 24 h before commencement of the imaging period. In the absence of CtBP knockdown, 5-fluorouracil was primarily cytostatic (see Supplementary Movie 5 at http://www.biolcell.org/boc/103/boc1030001add.htm), this cytostatic effect being somewhat reduced by p53 knockdown (see Supplementary Movie 7 at http://www.biolcell.org/boc/103/boc1030001add.htm). In CtBP-depleted cultures, 5-fluorouracil increased cell death (Supplementary Movie 6 at http://www.biolcell.org/boc/103/boc1030001add.htm); clearly, this effect was most pronounced in the absence of p53 (compare Supplementary Movie 4 with Supplementary Movie 8 at http://www.biolcell.org/boc/103/boc1030001add.htm).

The microtubule-stabilizing agent Taxol (Figure 8D) has an entirely different mechanism of action to 5-fluorouracil, and hence it was of interest to establish whether the effects of CtBP siRNA were similar. In contrast to 5-fluorouracil, p53 siRNA slightly sensitized cells to Taxol, reducing the IC_{50} from 5.45 to 2.99 nM. Again this effect was not significant, although such an effect would be consistent with published results demonstrating that wild-type p53 is protective for microtubule-targeting drugs in cancer cells (Stewart et al., 1999). Inhibiting the expression of the CtBP proteins demonstrated a similar pattern of effects on the IC_{50} as was seen in 5-fluorouracil-treated cells, individual siRNA to CtBP2 alone having a greater effect than CtBP1 siRNA, although in this case experimental variability was higher than that obtained with 5-fluorouracil, and neither effect of the individual siRNAs was significant. Combined CtBP knockdown had greater and significant effect, and loss of p53 reduced the IC_{50}

further still. An essentially similar pattern was seen with the genotoxic agents cisplatin and etoposide (Figures 8E and 8F respectively), in each case the IC_{50} in the presence of combined inhibition of CtBP1 and CtBP2 expression being 2.7−2.8-fold lower than in control treated cells, and lower still when p53 expression was blocked.

Overall, while we have shown that a substantial reduction in the abundance of CtBPs is not a general occurrence in response to all forms of genotoxic stress, the chemo-sensitizing effects of suppressing CtBP protein expression do occur in response to treatment with chemotherapeutic agents that have diverse mechanisms of action. This suggests that, rather than specifically modulating the pathways induced in response to the chemotherapy, the role of CtBPs in chemo-resistance may be through pathways with broad effects on cell proliferation and survival, such as suppression of pro-apoptotic gene expression (Grooteclaes et al., 2003; Kovi et al., 2010), and the maintenance of mitotic fidelity (Bergman et al., 2009). Additionally, CtBP1 has been shown to promote drug resistance by increasing expression of the *MDR1* (multi-drug resistance 1) gene (Jin et al., 2007); this provides an additional mechanism whereby siRNAs to CtBPs may increase chemosensitivity. It is potentially interesting, however, that the greatest effect was observed in response to 5-fluorouracil. This was particularly apparent in cells in which p53 expression was inhibited, in which combined CtBP1 and CtBP2 knockdown reduced the IC_{50} for 5-fluorouracil by 7.8-fold. The mechanistic basis of this is currently unclear, although it is of interest that, compared with other genotoxic agents, the antiproliferative affects of 5-fluorouracil can be shown to be highly dependent on the presence of a functional p53 response pathway (Bunz et al., 1999). It is clear from our data that, particularly in cells that cannot undergo p53-dependent apoptosis or cell cycle arrest, CtBPs play an important role in protecting cells from the pro-apoptotic effects of 5-fluorouracil.

Notwithstanding the requirement for further work to fully understand the mechanistic basis of CtBP-regulated chemosensitivity, this effect of CtBP siRNA on the cellular response to 5-fluorouracil in particular clearly represents a substantial sensitizing effect of a chemotherapeutic agent that is widely used in the therapy of cancer, including the therapy of breast cancer. Thus factors that affect CtBP

protein activity in cells, particularly those in which p53 function is lost by *p53* gene mutation, could potentially influence the response of tumours to this treatment. Such factors that exist within a tumour environment include hypoxia and enhanced aerobic glycolysis, which both increase intracellular NADH and thus promote the formation of CtBP dimers and CtBP chromatin-modifying complexes (Zhang et al., 2002, 2007). That these complexes can be recruited to chromatin to suppress the expression of pro-apoptotic BH3-only genes (Kovi et al., 2010) suggests that hypoxia-induced CtBP protein dimerization would be chemo-protective under these conditions. It is important to note, however, that our siRNA experiments will clearly deplete cells of both monomeric and dimeric CtBP proteins and so do not formally exclude a role for monomeric CtBPs in the chemo-resistance mechanism.

The present study has also identified some important novel aspects of the regulation of CtBP protein expression that will be relevant to future studies of these molecules. First, our RNA analysis has demonstrated that the predominant form of CtBP1 expressed in a panel of commonly studied breast cancer cell lines is CtBP1-S, rather that the CtBP1-L form that was originally cloned from HeLa cells (Schaeper et al., 1995) and upon which all the mechanistic studies of the role of CtBP1 proteins as transcriptional repressors have been performed. Indeed, to date, functional studies on CtBP1-S have exclusively focused on its role in the cytoplasm as a regulator of vesicular membranes (Corda et al., 2006). CtBP1-L and CtBP1-S proteins only differ by 11 amino acids at their N-terminus, and are indistinguishable by standard Western-blot analysis. This region of the molecule is not known to have any functional role; however, this clearly warrants further analysis. As far as we are aware the only comparable analysis of CtBP1 isoform expression that has been published examined murine embryo fibroblasts, and also found the CtBP1-S encoding splice form to be somewhat more abundant than that encoding CtBP-L (Yang et al., 2006) in these cells.

We have also shown that the overall abundance of CtBP proteins in cells is controlled through what appears to be an auto-regulatory feedback loop that regulates CtBP proteins at the post-transcriptional level. The mechanism for this remains to be determined, although we have shown that it involves the regulation of the rate at which the protein is degraded.

The APC protein has been demonstrated to control CtBP protein turnover in other experimental systems and, given that it is expressed in a functional form in the cell lines we have examined, may potentially be involved in this auto-regulation of CtBP abundance. APC is known to regulate the abundance of CtBP1, whereas we only found an increase in CtBP2 abundance in response to CtBP1 siRNA, and not vice versa. This result suggests a CtBP2-selective regulatory mechanism, although a simpler explanation could be that CtBP1 may be more abundant in cells than CtBP2, so that knockdown of CtBP2 results in an undetectable relative increase in CtBP1 abundance to restore total CtBP protein levels. Whatever the mechanism, the presence of a pathway that maintains the homoeostasis of CtBP protein abundance in proliferating cells is perhaps not surprising, given the critical role of these proteins in so many pathways that define the cellular phenotype.

Materials and methods

Cell culture and reagents

Primary human breast cancer material was obtained from the Southampton Cancer Research UK tumour bank; the cohort of formalin-fixed, paraffin-embedded archival breast cancer tissue from 144 consecutive patients with available tissue block has been previously described (Cutress et al., 2003). A total of 22 consecutive blocks from this cohort were analysed: of these, 18 were from patients with ductal carcinoma; the remainder were lobular carcinoma. All human tissue was assayed with local ethics committee approval. Culture of the panel of human breast cancer lines has been described previously (Phelps et al., 2003). Medium for MCF-10A was supplemented with 500 ng/ml cortisol and 0.01 mg/ml insulin. MRC-5.hTERTneo are an immortalized derivative of MRC-5 fibroblasts (McSharry et al., 2001), and were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% (v/v) foetal calf serum. Transfection of plasmids into cells, as well as siRNA into MRC-5.hTERT.neo cells, was performed using Lipofectamine™ 2000 reagent (Invitrogen). Breast cancer cell lines were transfected with siRNA at a combined concentration of 50 nM using INTERFERin reagent (Polyplus Transfection). siRNAs reagents targeting *CTBP1/2*, *CTBP1*, *CTBP2* and *TP53* mRNAs, as well as control siRNAs, have been described previously (Bergman et al., 2009). CtBP1mh plasmid was generated by cloning the human *CTBP1* coding region into pcDNA3.1mychisA vector. To generate pcDNA3.1CtBP2, the human *CTBP2* cDNA was excised from pBSK-CtBP2 (Mirnezami et al., 2003) and ligated into pcDNA3.1. Derivative plasmids containing mutations of the ATGs at codon 1 and codon 26 were generated by site-directed mutagenesis. pcDNA3.1-CTBP2-TT expresses CtBP2 with a C-terminal tandem affinity purification tag; the plasmid also contains a synonymous mutation of the target region of the CtBP1/2 siRNA, which was introduced by site-directed

mutagenesis. Cells were exposed to UV-C radiation using an XL-1000 UV cross-linker (Spectronics).

Cell proliferation assay and statistical analysis

For cell proliferation assays, cells were plated at 2000 cells per well on 96-well plates; when combined with siRNA knockdown analysis, plating was 48 h after siRNA transfection. At 24 h after plating, the medium was replaced with 100 μl of a medium containing serial dilutions of a chemotherapeutic agent, the final concentration of the solvent DMSO was 0.5% in all wells. All conditions were assayed in duplicate. After 24 h the drugs were removed and a fresh medium was added; cells were cultured for a further 96 h before performing an MTS-based cell proliferation assay (CellTitre Aqueous One Cell Proliferation assay; Promega). IC_{50} values were generated from the results by using Prism software (GraphPad Software). Results of the drug response experiments are expressed as means $\pm$ S.E.M. of IC_{50}. Statistical significance was evaluated using the one-way ANOVA parametric test and Tukey's post hoc test. $*P < 0.05$, $**P < 0.01$. Time-lapse video-microscopy was performed as described previously (Bergman et al., 2009).

Protein analysis

Cells lines in culture were washed with PBS, pelleted by centrifugation at 1000 g for 5 min, snap-frozen and stored at $-80°$C. Sample lysis and immunoblotting were performed as described previously (Blaydes and Hupp, 1998). Membranes were probed for CtBP1 using either mouse monoclonal E12 [Santa Cruz Biotechnology; this was raised against amino acids of human CtBP1 and does not recognize human CtBP2 (Bergman et al., 2009), see also Figure 5A] or, where specifically stated, mouse monoclonal BD-3/CtBP1 (BD Biosciences; raised against amino acids 345−441 of mouse CtBP1). CtBP2 was detected using mouse monoclonal BD-16/CtBP2 (BD Biosciences), raised against either amino acids 361−445 of mouse CtBP2 or, where specifically stated, goat polyclonal E16 (Santa Cruz Biotechnology; raised against a peptide from near the C-terminus of human CtBP2). Other antibodies were used to detect p53 [DO-1 (Serotec)]; HIPK2 [ab57328 (Abcam)] and β-actin (Sigma). Images were captured and quantified using a Fluor-S MultiImager with Quantity One software (Bio-Rad) or ImageJ. Immunofluorescence analysis was performed as described previously (Bergman et al., 2009). False colours were applied to greyscale images using ImageJ software. Antibody to APC was obtained from Abcam. For immunohistochemistry analysis, formalin-fixed, paraffin-embedded tissue was stained by the Cellular Pathology Department at Southampton General Hospital, essentially as described previously, using microwave antigen retrieval (Cutress et al., 2003). Primary antibodies for the detection of CtBP1 and CtBP2 were E12 (1:250) and BD-16/CtBP2 (1:400) respectively, diluted in Tris-buffered saline (10 mM Tris/HCl, pH 8.0, and 150 mM NaCl). Optimum antibody dilutions were determined by titration using sections of breast tumour material. The specificity of E12 towards CtBP1, and not human CtBP2, was confirmed using HEK-293 cells that were transfected with pcDNA3.1CtBP2mh plasmid (Mirnezami et al., 2003) and then fixed and processed the same way as the breast tumour material (results not shown). Images of slides were captured using a Nikon Eclipse E600 microscope equipped with a Nikon Coolpix camera.

RNA analysis

RNA was extracted from cell lines and tissue samples using RNABee (Biogenesis). For semi-quantitative RT−PCR analysis of transcripts, 0.5−2 μg of RNA was reverse transcribed in a 20−25 μl volume using Superscript II RNAse H$^-$ reverse transcriptase (Invitrogen) and oligo(dT) primer. A 2 μl portion of cDNA product was used as the target in 50 μl of PCR reactions using GoTaq DNA polymerase (Promega). Primers for the 5′-ends of each of the *CTBP1* and *CTBP2* mRNAs were: CTBP1−172F 5′-AGCTCGCACTTGCTCAA-CA-3′; CTBP1−419R 5′-TCCTCCCTGGTGAGAGTGAT-3′; CTBP2L 5′-GAACTATAGAGTTTGTGGAC-3′; CTBP2R 5′-TGAGGATGTGGCAGATGGTA-3′. Primers for HIPK2 were 5′-GGCCTCACATGTGCAAGTTTTC-3′ and 5′-TT-GGTAGGTATCAAGGAGGCTC-3′; for p14^{ARF} encoding mRNA they were 5′-GGTTTTCGTGGTTCACATCCCG-CG-3′ and 5′-CAGGAGCCCTCCCGGGCAGC-3′. Primers for APC PCR have been described (Tsuchiya et al., 2000). Taq-Man quantitative PCR for *CTBP1* and *CTBP2* was performed using commercial primer/probe sets (Applied Biosystems; Assays on Demand Hs00972288_g1 and Hs00949547_g1 respectively) using the ABI PRISM 7900HT instrument and software (PerkinElmer Life Sciences). A standard curve was used to convert threshold cycle numbers into relative transcript numbers, which were then normalized to *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) transcript levels in the sample.

Authors' contributions

Rachel Harding, Gehan Soosaipillai, Trisha Halder and Ali Azim-Araghi were B.Sc. project students who each contributed to the experimental results. Matthew Darley performed PCR and Western blotting analyses for several of the Figures. Charles Birts undertook laboratory supervision of project students, helped write the manuscript and generated experimental results required for its completion. Ramsey Cutress provided the cohort of breast cancer samples and guidance on their analysis and data interpretation. Adrian Bateman is a consultant histopathologist and analysed the immunohistochemical staining of CtBPs. Jeremy Blaydes obtained the funding, coordinated the project and wrote the manuscript.

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