**Epigenetic CRBP downregulation appears to be an evolutionarily conserved (human and mouse) and oncogene-specific phenomenon in breast cancer**

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**Abstract**

**Background:** The cellular retinol binding protein I gene (CRBP) is downregulated in a subset of human breast cancers and in MMTV-Myc induced mouse mammary tumors. Functional studies suggest that CRBP downregulation contributes to breast tumor progression. What is the mechanism underlying CRBP downregulation in cancer? Here we investigated the hypothesis that CRBP is epigenetically silenced through DNA hypermethylation in human and mouse breast cancer.

**Results:** Bisulfite sequencing of CRBP in a panel of 6 human breast cancer cell lines demonstrated that, as a rule, CRBP hypermethylation is closely and inversely related to CRBP expression and identified one exception to this rule. Treatment with 5-azacytidine, a DNA methyltransferase inhibitor, led to CRBP reexpression, supporting the hypothesis that CRBP hypermethylation is a proximal cause of CRBP silencing. In some cells CRBP reexpression was potentiated by co-treatment with retinoic acid, an inducer of CRBP, and trichostatin A, a histone deacetylase inhibitor. Southern blot analysis of a small panel of human breast cancer specimens identified one case characterized by extensive CRBP hypermethylation, in association with undetectable CRBP mRNA and protein. Bisulfite sequencing of CRBP in MMTV-Myc and MMTV-Neu/NT mammary tumor cell lines extended the rule of CRBP hypermethylation and silencing (both seen in MMTV-Myc but not MMTV-Neu/NT cells) from human to mouse breast cancer and suggested that CRBP hypermethylation is an oncogene-specific event.

**Conclusion:** CRBP hypermethylation appears to be an evolutionarily conserved and principal mechanism of CRBP silencing in breast cancer. Based on the analysis of transgenic mouse mammary tumor cells, we hypothesize that CRBP silencing in human breast cancer may be associated with a specific oncogenic signature.

**Background**

Retinol has been implicated in the regulation of adult epithelial cell differentiation in addition to being essential for normal embryonic development, vision and...
reproduction [1-3]. Most of the biological effects of retinol are secondary to its metabolism to retinoic acid (RA) and RA activation of members of the RA receptor (RAR) and retinoid X receptor (RXR) family of ligand-dependent transcription factors. As such, the study of RARs and RXRs has gained widespread attention. However, a number of other genes play fundamental roles in regulating RXR-RAR function by regulating retinol and RA metabolism. Among these genes is cellular retinol-binding protein I (CRBP), which has been shown to be indispensable for life when vitamin A levels in the diet are limiting. Thus, CRBP null mice are unable to maintain adequate retinol liver stores and survive only when dietary vitamin A is abundant, a condition that is artificial and does not reflect the dietary habits of mammals in the wild [4].

In addition to being expressed in stellate cells of the liver, CRBP is expressed in many other organs and cell types including epithelia [5-7]. Several observations, including the finding that CRBP expression during mouse embryogenesis overlapped that of RARβ and RARγ, led to the suggestion that the function of CRBP is to store and release retinol where high levels of RA are developmentally required [8]. mCRBP expression is regulated by RA through a RA response element located ~1 kb upstream of the transcription start site, suggesting the existence of a positive feedback mechanism for regulating retinoid metabolism and action [9]. Glucocorticoids, cAMP, transforming growth factor β and serum factors have also been shown to impinge on CRBP expression [10-13]. The proximal hCRBP promoter region has been cloned, the transcription start site identified, and functional NF1 sites at approximately -250 and +70 demonstrated [14,15] but beyond these earlier studies there have been no functional promoter analyses.

We have recently shown that CRBP expression is downregulated in a subset of human breast cancers [7]. In follow-up studies of the potential significance of this finding, we found that the downregulation of CRBP blocks the differentiation and promotes the growth of SV40-transformed breast epithelial cells ([16] and Farias et al., manuscript submitted). The differentiation-promoting, growth-arresting effect of CRBP, witnessed in both human and mouse mammary epithelial cells, was assigned to CRBP regulation of retinol storage and RAR activation (Farias et al., manuscript submitted). These results suggested that CRBP downregulation is a contributing cause rather than a consequence of breast cancer and led us to investigate the mechanism underlying CRBP downregulation. We report here that CRBP is epigenetically silenced as a result of promoter region hypermethylation in both human and mouse breast cancer cells. Furthermore, we identify one exception to this rule and provide data that suggests that CRBP silencing is an oncogene-specific event at least in the context of mouse mammary cancer. These results extend those published by Esteller et al. while this work was in progress [17].

**Results**

**hCRBP expression in breast cancer cells varies widely**

We first analyzed a panel of human breast cancer cell lines for CRBP expression using a semi-quantitative RT-PCR Southern blot protocol. CRBP expression varied from robust (MDA-MB-468), to weak (MCF-7), to very weak or undetectable (ZR75-1 and MTSV1-7; Fig. 1A). Given that CRBP downregulation occurs with an estimated frequency of 24% in human breast cancer, i.e., it is not a universal event, the identification of cell lines, such as MDA-MB-468, in which CRBP was well expressed, should not be surprising. We wondered whether the intermediate level of CRBP expression in MCF-7 cells was a consequence of clonal heterogeneity and tested this idea by random clonal selection and analysis. In support of this notion, we were able to isolate subclones that mirrored the extremes of CRBP expression, with MCF-7 clone B resembling MDA-MB-468 and MCF-7 clone A resembling ZR75-1 (Fig. 1B, compare with Fig. 1A). After densitometry and normalization to a house keeping gene, we plotted the results for all cell lines on a log scale (Figs. 2A and 3A).

**hCRBP methylation, with one exception, is inversely proportional to hCRBP expression**

On the basis of preliminary Southern blot analyses that revealed no major hCRBP deletions or insertions in breast cancer cells (unpublished data), and taking into account the presence of a CpG island in the hCRBP regulatory region [14], we hypothesized that hCRBP downregulation might be secondary to gene hypermethylation. This hypothesis was tested by bisulfite sequencing of 26 CpG dinucleotides within the hCRBP locus (promoter and 5’-UTR region) in the cell lines whose CRBP expression we had characterized (Fig. 1). We found that all 26 sites were hypomethylated in MDA-MB-468 cells and 14 of the 26 sites were hypermethylated in ZR75-1 cells, providing the first evidence of an inverse association between CRBP methylation and expression (Fig. 2B). However, this notion was challenged by the finding that the same CRBP region was uniformly hypomethylated in MTSV1-7 cells (Fig. 2B). The bisulfite sequencing analysis of CRBP in MCF-7 parental cells and clones A and B proved very useful in that it demonstrated a very tight inverse association between CRBP methylation and expression. Thus, the intermediate level of CRBP expression in parental MCF-7 cells was associated with a mixed pattern of CRBP methylation (of 17 amplicons sequenced, 6 showed uniform hypermethylation and 10 showed uniform hypomethylation), and this mixed pattern resolved into uniform hyper or hypomethylation in clones A and B, respectively (Fig. 3B). These results suggest that, as a rule, CRBP hypermeth-
ylation is silencing, and that MTSV1-7 cells represent an exception to this rule.

**Treatment with a demethylating agent rescues hCRBP expression**

CRBP is a RA-inducible gene [9,18] and trichostatin A (TSA), a histone deacetylase inhibitor, has been shown to synergize with RAR induction of target gene transcription [19]. Cells in culture vary widely in their sensitivity to retinoids in the serum present in the culture medium; in the case of MDA-MB-468 cells (hypomethylated CRBP), there was robust CRBP expression in the absence of exogenous RA, whereas in the case of MCF-7 clone B cells [also hypomethylated CRBP] there was no CRBP expression unless exogenous RA was added and expression increased further in presence of TSA (Fig. 4A). This difference may relate to the fact that MDA-MB-468 cells are competent in RA synthesis from serum retinol whereas MCF-7 cells are not [20]. Based on the data for MCF-7 clone B cells (Fig. 4A), we predicted that treatment of MCF-7 clone A cells (hypermethylated CRBP) with 5-azacytidine (azaC), a DNA methyltransferase inhibitor, would result in some CRBP reexpression and that this effect would be potentiated by the addition of RA and TSA. We further predicted that in absence of azaC, RA and TSA would be ineffective. The data confirmed these expectations (Fig. 4B), thus supporting our hypothesis that hypermethylation is a proximal cause of CRBP silencing. In another cell line with hypermethylated CRBP (ZR75-1), reexpression could also be demonstrated to be critically dependent on azaC (Fig. 4A). Although we did not directly probe the effect of azaC on CRBP methylation, we showed earlier that the same protocol used here achieved partial demethylation of another hypermethylated gene in MCF-7 cells [21]. The work of Esteller et al. [17] suggest that our data for MCF-7 clone A is rather the exception than the rule in that azaC alone is generally sufficient to achieve CRBP reexpression when the gene is hypermethylated, suggesting that endogenous retinoids or other modulators of CRBP expression (Introduction) can drive CRBP transcription upon azaC-induced demethylation.

**Extensive hCRBP hypermethylation in association with undetectable hCRBP in a human breast cancer specimen**

Esteller et al. demonstrated by methylation-sensitive PCR (MSP) that hCRBP is hypermethylated and silenced in a subset of human breast cancers [17]. MSP is a highly sensitive technique that will detect methylation of a gene of interest even when only a small percentage of cells within the tissue have methylated alleles [22]. We wished to probe CRBP methylation using a less sensitive technique to infer whether CRBP methylation was a rare event on a per cell basis or, alternatively, was a tissue-wide event. For this purpose we used a classical Southern blot analysis approach [23] that relied on the use of methylation-sensitive isoschizomers and knowledge of the specific CpG sites that are methylated in human breast cancer cells (Figs. 2B and 3B). To test the validity of this strategy, we first applied it to MDA-MB-468 and ZR57-1 cells. Southern blotting correctly identified CRBP hypo and hypermethylation in MDA-MB-468 and ZR57-1 cells, respectively (Fig. 5A). This strategy was then used to investigate CRBP methylation in a small panel of human breast cancers (n = 10) and the results obtained for 2 cases are
**Figure 2**

*hCRBP* hypermethylation explains some but not all cases of hCRBP silencing. 

**A.** Relative levels of hCRBP expression determined by RT-PCR Southern analysis (see Fig. 1). The limit of detection is represented by a relative log value of 1.5 that corresponds to film background after 24 h exposure. Note that hCRBP expression in MDA-MB-468 cells is greater than that in ZR75-1 or MTSV1-7 cells by about 3 logs.

**B.** hCRBP bisulfite sequencing results for the same cell lines as in A. Filled circles designate methylated and open circles designate unmethylated CpGs. Each row displays the methylation status of CpG sites across an individual amplicon; each "column" displays the methylation status of an individual CpG site across amplicons. The approximate position of each CpG site relative to the transcription start site is indicated in the abscissa.
Figure 3

**hCRBP methylation explains clonal variations in hCRBP expression.** A. Same as for Fig. 2A. Note that MCF-7 cells express hCRBP at a level that is intermediate to the extremes represented by MCF-7 clones A and B, which resemble in this regard ZR75-1 and MDA-MB-468 cells, respectively. B. hCRBP bisulfite sequencing results for the same cell lines as in A. Filled circles designate methylated and open circles designate unmethylated CpGs (see legend to Fig. 2B for further details).
shown in Fig. 5B. One breast cancer specimen (tumor B) exhibited frank \textit{CRBP} hypermethylation in association with low levels of \textit{CRBP} mRNA, whereas tumor A did not show evidence of \textit{CRBP} methylation by Southern blotting and this correlated with easily detectable \textit{CRBP} mRNA levels. Based on a densitometric evaluation of the yield of the 205 bp fragment, we estimate that >50% of the cells in tumor B had at least one hypermethylated \textit{CRBP} allele. When viewed in the context of the other data presented here and the work of Esteller et al. [17], this finding is of interest because it suggests that in some breast cancers there is clonal expansion of a cell population in which the \textit{CRBP} gene is hypermethylated. Fig. 5B also demonstrates that there is good agreement between \textit{CRBP} expression at the mRNA and protein levels. Analysis of the remaining 8 human breast cancer specimens did not identify other examples like tumor B in Fig. 5, either because of the small sample size, the low sensitivity of Southern blotting, or the low cellularity of the tumor specimen (data not shown).

\textbf{Myc but not Neu leads to mCRBP hypermethylation and silencing in mouse mammary epithelial cells}

Morrison and Leder have shown that \textit{CRBP} expression in transgenic mouse mammary tumors is a function of the initiating oncogene, with Myc-induced tumors lacking and Neu-induced tumors displaying \textit{CRBP} expression [24]. We hypothesized that this observation might be
In some human breast cancers, hCRBP hypermethylation is widespread. A. Sketch outlining the Southern blot strategy used to evaluate hCRBP methylation in human breast cancer specimens. The strategy was tested in MDA-MB-468 and ZR75-1 cells whose hCRBP methylation status was known beforehand. B. BH and BM stand for, respectively, Bsu36I digestion alone, Bsu36I digestion followed by Hpall digestion, and Bsu36I digestion followed by MspI digestion (MspI is the methylation-insensitive isoschizomer of Hpall). In agreement with the bisulfite sequencing data, Southern blotting revealed hCRBP undermethylation in MDA-MB-468 and hypermethylation in MCF-7 clone A cells (note loss of the large Bsu36I fragment after BH digestion of MDA-MB-468 genomic DNA and retention of this fragment in the case of MCF-7 clone A). A hCRBP restriction fragment length polymorphism (an extra Bsu36I site within the 205 bp fragment) accounts for the displaced band migration pattern in MDA-MB-468 cells. B. Southern, RT-PCR and Western blotting results for 2 human breast cancer specimens. The yield of the 205 bp product was decreased by >50% after BH digestion relative to BM digestion. We infer from this that >50% of the cells in the tumor displayed hCRBP hypermethylation. The protein blot was post-stained with Ponceau S and a major band used as loading control.
explained by Myc but not Neu promotion of CRBP hypermethylatation. To test this, we obtained two mammary tumor cell lines established from Myc and two others established from Neu-induced tumors [25] and, as a first step, confirmed that CRBP was expressed in the latter but not the former cell lines (Fig. 6A). We then analyzed CRBP methylation in the same cell lines. As predicted by our hypothesis, bisulfite sequencing of CpG dinucleotides within the mCRBP promoter and 5'-UTR region revealed extensive to moderate CRBP hypermethylation in MMTV-Myc as opposed to virtually no methylation in MMTV-Neu mammary tumor cells (Fig. 6B). As shown in Fig. 6C, treatment with azaC rescued CRBP expression in MMTV-Myc (16MB9a) cells. Thus, CRBP hypermethylation and silencing appears to represent an evolutionarily conserved event in mammary carcinogenesis. The data in Fig. 6 sets the stage for additional work investigating whether CRBP hypermethylation in human breast cancer can be associated with a specific oncogenic signature.

Discussion
Our data extend the work of Esteller et al. [17] in several respects. First, it provides an analysis of the pattern of CpG methylation across the CRBP promoter region. One interesting feature revealed by the bisulfite sequencing of CRBP in ZR75-1 and MCF-7 clone A cells, both of which express virtually no CRBP, is that 2 alternative methylation patterns are equally suppressive. Thus, in ZR75-1 cells, CRBP methylation showed intra-allelic variegation, with alternating methylated and non-methylated CpG sites (Fig. 2B). Contrast this with the uniform methylation of all CpG sites in MCF-7 clone A cells (Fig. 3B). As shown in Fig. 4, both of these patterns contributed to CRBP down-regulation, suggesting that uniform methylation is not required for silencing, which is consistent with the demonstrated ability of MeCP2 to bind single methylated CpG sites [26]. CRBP methylation in MMTV-Myc mammary tumor cells defined yet another methylation pattern characterized by both intra- and inter-allelic variegation and different degrees of methylation penetrance (Fig. 6B). The conditions that lead to the establishment of these variant methylation patterns in the first place are not known.
in MTSV1-7 cells (Fig. 2B) argues against our idea that c-myc overexpression underlies CRBP silencing in MTSV1-7 cells. However, hypermethylation has been suggested to represent a mechanism for the maintenance not the initial induction of transcriptional silencing [30]. Therefore, it is conceivable that the hCRBP locus in MTSV1-7 cells is repressed in a c-myc-dependent manner and is susceptible to but has not yet undergone hypermethylation.

Third, based on a small sample of 10 human breast cancer specimens, we succeeded in identifying one instance of widespread CRBP hypermethylation and silencing. Thus, hypermethylation was easily detected in total tumor DNA using a low sensitivity technique (Southern blotting) and this correlated with the lack of any detectable CRBP mRNA and protein in total tumor RNA and tumor lysates, respectively. Although only a single such tumor was identified, it seems reasonable to extrapolate, based on the strength of the combined evidence ([17] and this report), that a small subset of human breast cancers are characterized by pervasive CRBP methylation.

Fourth, we have found that mCRBP methylation is associated with Myc but not Neu-induced mammary tumors, suggesting that mCRBP silencing may be a requirement for MMTV-Myc tumorigenesis. We plan to test this hypothesis by monitoring CRBP expression and methylation in situ hybridization and MSP, respectively, over the period of time during which premalignant changes accumulate in MMTV-Myc mammary tissue. We expect to find that CRBP downregulation precedes or coincides with the appearance of preneoplasia. We further expect that ectopic CRBP will prevent or delay the onset of mammary tumorigenesis. One theoretical argument against our hypothesis is that MMTV-Myc may transform a subpopulation of cells that do not express CRBP to begin with. We find this unlikely because CRBP mRNA is uniformly expressed in both layers of human breast ducts, i.e., the epithelium itself and the underlying myoepithelium [7]. Interestingly, MMTV-Myc tumors were recently identified as having both epithelial and myoepithelial components [31], suggesting that MMTV-Myc transforms a pluripotent cell population. For the reason just stated above, we hypothesize that this population expresses CRBP.

Finally, our study of CRBP methylation in mouse mammary tumor cell lines derived from Myc and Neu-induced tumors provides a hypothetical explanation for why CRBP hypermethylation is characteristic of only a subset of human breast cancers. It should be noted that CRBP is not unique in this regard, i.e., several other genes shown to be epigenetically silenced in breast cancer are silenced in a subset of, not all breast cancers [32]. The data in Fig. 6 suggests that subset-specific CRBP silencing might be explained by the nature of the initiating oncogenic event. Specifically, we propose that most human breast cancers in which c-myc is amplified and/or overexpressed might be characterized by CRBP hypermethylation and, conversely, most breast cancers with c-neu amplification and/or overexpression might be characterized by CRBP hypomethylation. Interestingly, the incidence of c-myc amplification and CRBP hypermethylation in breast cancer have both been estimated at 16% [17,33]. If further work were to validate this notion, then it would be of interest to understand how c-myc overexpression leads to CRBP silencing and how c-neu bypasses the anti-tumor function of CRBP. The finding of other defects in retinol and RA metabolism in breast cancer as well as alterations in RAR isomser expression suggest that several bypassing mechanisms may exist [34-37].

Conclusions
We conclude that: (i) CRBP hypermethylation is, as a rule, a proximal cause of CRBP silencing in a subset of human and mouse breast cancers; (ii) exceptions to this rule may exist in some human breast cancers, as indicated by our results for the MTSV-1-7 cell line, in which CRBP is silenced despite being hypomethylated; (iii) the intracellular pattern of CRBP hypermethylation may vary and tumors may consist of mixtures of cells with hyper and hypomethylated CRBP, but in at least some cases most tumor cells (>50%) display CRBP hypermethylation; (iv) at least in the context of mouse mammary cancer, CRBP hypermethylation appears to be an oncogene-specific event. The significance of these findings should be viewed against the background of our functional analyses of CRBP, which suggest that CRBP silencing is a contributing cause rather than a consequence of breast carcinogenesis [16].

Methods
Cells and reagents
Human breast cancer cell lines, with the exception of MTSV1-7 cells [16,28], were obtained from the American Type Culture Collection (Rockville, MD) and grown as recommended. 16MB9a and M1011 (MMTV-Myc) and SMF and NAF (MMTV-Neu/NT) mammary tumor cells were cultured as described [24]. Gel purified primers were obtained from Genelink (Hawthorne, NY). RA, TSA and azaC were obtained from Sigma (St.Louis, MO). Sodium metabsulfite was obtained from BDH, England.

RNA analyses
For RT-PCR, 2 µg total RNA (Purescript kit, Gentra, Minneapolis, MN) were reverse transcribed using the Superscript Preamplification System (Gibco BRL Life Technologies, Grand Island, NY). This RNA input was chosen after preliminary experiments demonstrated a dose-dependent increase in CRBP RT-PCR product within
the range of 0.5 to 4 µg total RNA. The PCR mixture consisted of 10X PCR Buffer, 1 mM MgCl₂, 200 µM dNTPs, 0.4 µM each primer, 10% cDNA, and 5U of Taq (Promega, Madison, WI) in a total volume of 50 µl. hCRBP cDNA (320 bp) was amplified with sense primer TGTT- GGCCAAACTGGCTCCA and antisense primer ACACT- GGACCTGTGCTCCGCT and the following cycling parameters: 94 C for 5 min; 26 cycles of 94 C for 1 min, 48 C for 1 min, 72 C for 1 min; 72 C for 5 min. 18S rRNA (489 bp) was amplified using the same protocol as above except that 5% cDNA, a 3:7 mix of primers:competitors (0.4 µM total), and an annealing temperature of 57°C were used (18S rRNA primers and competimers were from Ambion, Austin, TX). GAPDH RT-PCR was performed as described [38]. The PCR products were separated on 1% agarose, stained with ethidium bromide (18S rRNA) or blotted overnight and probed with full length CRBP cDNA or a commercial GAPDH cDNA fragment (Ambion). Northern blot analyses were performed using standard methods and a full length CRBP cDNA probe as described earlier [7].

**Bisulfite sequencing**

Genomic DNA was isolated using the Puregene kit (Genta Minneapolis, MN) and digested with Xbal. The bisulfite method established by Frommer et al. [39,40] was carried out on 5 µg genomic DNA digest with the following modifications. Digested DNA was denatured with 0.5 M NaOH at 75 C for 15 min, followed by deamination with freshly prepared 4.0 M, pH 5.0 sodium metabisulfite and incubated at 55 C for 8 hours in a total volume of 1.2 ml under mineral oil. The bisulfite-treated DNA was then desalted using the Wizard DNA clean-up system (Promega), desulfonated with 0.3 M NaOH at 37 C for 15 min, neutralized with 0.1 volume of 3 M NH₄AC and ethanol precipitated with 2 volumes of cold ethanol. The pellet was resuspended in 50 µl of sterile H₂O. A 780 bp hCRBP fragment was amplified using 2 rounds of fully-nested PCR, which in our experience was required to yield reproducible results. First round PCR was carried out in a 50 µl reaction mixture of 10X PCR buffer, 1.2 mM MgCl₂, 160 µM dNTPs, 0.4 µM each primer, 10 µl of bisulfite-treated DNA, and 5 U of Taq (Promega). The nested PCR reactions (rounds 2 and 3) were carried out in the same way using 5% of the product obtained in the preceding round as template. The primers used were as follows: 1st round PCR, GTTGAATTTTTAGT TTTTGTAGTTTACT (sense) and ACACAAAAAATTAACAAAAAAT (antisense); 2nd round, AGTTTTTAGATAAGGTTTGTAAGTGG (sense) and TCCCAAAAATACACACCCC (antisense); 3rd round, GTTTTTATATTTGAGTTGTAGT (sense) and ATCTACAACTAAAACACTACCTAAA (antisense). Cycling parameters were: 94 C for 5 min; 40 (round 1) or 20 cycles (rounds 2 and 3) of 94 C for 1 min, 45 C (rounds 1 and 3) or 50 C (round 2) for 1 min, 72 C for 1 min; 72 C for 5 min. The amplified PCR product was gel purified, subcloned into the TA cloning vector (Invitrogen, Faraday, CA), transformed into TOPO kit cells (Invitrogen) using a blue-white colony X-gal selection, and individual clones submitted for sequencing. mCRBP bisulfite sequencing was performed essentially as described above for hCRBP but using semi-nested PCR and the following primers and conditions: 1st round, TGAAGGATTTTTAAGGGAGTAAGG (sense) and AAATCTCATCAGCTCAATCTTCAATAC (antisense), 40 cycles, annealing at 52 C; 2nd round, ATCCACAAAAATTTTAAAAACACTA (sense) and same antisense primer as above, 42 cycles, annealing at 46 C.

**Southern blot analysis**

Human breast carcinoma specimens that had been flash frozen within 1 h of surgery were obtained through the Cooperative Human Tissue Network under Institutional Review Board approval. All specimens were diagnosed as invasive breast carcinoma, with or without a carcinoma in situ component, except for one specimen which was an adenoid cystic breast carcinoma (not shown in Fig. 5). Approximately 0.2 mg of tissue were pulverized under liquid nitrogen and RNA and DNA isolated using Qia-gen's RNA/DNA kit according to manufacturer's instructions (Qiagen, Valencia, CA). Tumor DNA (45 µg) was digested with Bsu36I, ethanol precipitated, and divided into 3 parts that were either not digested further, digested with HpaII, or digested with MsPl (restriction enzymes were from New England Biolabs, Beverly, MA). After ethanol precipitation, Southern blots were prepared and hybridized to a 205 bp hCRBP probe amplified from a cloned hCRBP genomic fragment using CCGGTCTCCTCTCCCTTCTCTCTCTTGAGGG (sense) and GGGACAGGGGCTCCTGCAGGGG (antisense) primers.

**Immunoblot analysis**

A small amount of pulverized tissue was homogenized in 10 mM Tris.Cl pH 7.4, 1 mM EDTA, 7 µM beta-mercaptoethanol, the homogenate was centrifuged at 10,000 × g for 10 min, and the supernatant centrifuged further at 100,000 × g for 30 min to yield cytosol. Protein concentration was analyzed using the method of Bradford [41] with Bio-Rad reagents (Bio-Rad, Hercules, CA) and 50 µg protein were electrophoresed per lane in a 15% sodium dodecyl sulfate-polyacrylamide gel. After blotting, CRBP protein were electrophoresed per lane in a 15% sodium dodecyl sulfate-polyacrylamide gel. After blotting, CRBP protein was visualized by reaction with anti-CRBP antibody followed by secondary antibody and enhanced chemiluminescence detection. The anti-CRBP antibody was raised in rabbits against a keyhole limpet hemocyanin-conjugated CRBP-specific peptide [42] and was characterized elsewhere [16].
List of abbreviations
CRBP cellular retinol binding protein type I
MMTV mouse mammary tumor virus
RA retinoic acid
RAR RA receptor
RXR retinoid x receptor
RT-PCR reverse transcription-coupled polymerase chain reaction
ER estrogen receptor
TSA trichostatin A
azaC 5-azacytidine
MSP methylation-sensitive PCR

Authors’ contributions
AA established the bisulfite sequencing protocol for hCRBP, applied it to most of the human breast cancer cell lines tested, generated data toward Figs. 1 and 4, established the Southern blotting strategy used in Fig 5 and generated the data in Fig. 5A. SB performed the analysis of hCRBP methylation in MTSV1-7 cells, extended and improved the bisulfite sequencing method to mCRBP (Fig. 6), and generated data toward Figs. 1, 4, and 6C. YSK established the semi-quantitative CRBP RT-PCR method and contributed toward Fig. 1. SN generated the CRBP peptide antibody used in the experiment of Fig. 5. RML initiated and supervised all aspects of the project, helped perform some of the experiments, and wrote the manuscript.

Acknowledgements
We thank the following investigators for reagent gifts: Joyce Taylor-Papadimitriou (MTSV1-7 cells), Phil Leder (16MB9) and SFM cells), Pierre Chambon (mCRBP cDNA) and Winnie Eskild (hCRBP cDNA). Tissue samples were provided by the Cooperative Human Tissue Network which is funded by the National Cancer Institute. Other investigators may have received specimens from the same subjects. This work was supported by NCI grant CA54273 to RML, the Samuel Waxman Cancer Research Foundation, the Norman and Rosita Winston Foundation and the Chemotherapeutics Foundation.

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