Two Isoforms of the RNA Binding Protein, Coding Region Determinant-binding Protein (CRD-BP/IGF2BP1), Are Expressed in Breast Epithelium and Support Clonogenic Growth of Breast Tumor Cells*

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Background: The RNA binding protein, coding region determinant-binding protein (CRD-BP), is expressed by tumor cells and protects key mRNAs.

Results: This study identifies a novel variant of CRD-BP and finds that CRD-BP is required for breast tumor cell clonogenicity.

Conclusion: CRD-BP has tumorigenic activity and is ubiquitously expressed in breast epithelium.

Significance: Under-reporting of CRD-BP isoforms suggests that published studies may be incomplete.

The RNA binding protein, coding region determinant-binding protein (CRD-BP)2, was originally isolated as the protein responsible for stabilizing mRNA for the oncogene, c-Myc, by binding to a sequence in the coding region of the transcript (1). Since then, CRD-BP overexpression has been shown to be sufficient for mammary tumor induction in transgenic mice (2). Knockdown studies have shown that CRD-BP is essential for survival of many different types of tumor cells (3–6), and CRD-BP is highly expressed in melanoma, breast, ovarian, and colorectal cancers (reviewed by Bell et al. (7), among others). By way of a molecular explanation, CRD-BP has been shown to regulate many mRNAs encoding cancer-associated genes, including Gli1, PTEN, βTrCP1, MAPK4, MDRI, IGF2, H19, c-myc, CD44, and β-catenin mRNAs (1, 8–16). However, the remarkably universal requirement for CRD-BP expression by such disparate tumor types is not yet understood.

CRD-BP is also known as IGF2BP1, ZBP1, and IMP1. The variety of names ascribed to the same protein illustrates the fact that investigators from various fields have identified distinct activities for the same molecule. Furthermore, CRD-BP is a member of the highly conserved family of RNA binding proteins known as VICKZ proteins (17), which are structurally composed of two RNA recognition motifs at the N terminus and four K homology (KH) domains at the C terminus. Target mRNAs bind CRD-BP KH domains via combinatorial interactions through a looped tertiary structure with short consensus sequences. This interaction makes the mRNAs difficult to predict a priori (18, 19), but experimental results using overexpressed CRD-BP suggest there may be as many as 300–900 different mRNA species in CRD-BP-associated granules, which are ~100–300 nm in diameter (20, 21). Through its mRNA
binding activity, CRD-BP has been shown to affect RNA stability (C-MYC and BTRC), translatability (IGF2), and/or localization (ACTB) (1, 10, 12, 22) and thus regulate cell survival, migration, and chemoresistance.

Here we find that the typical oncofetal expression pattern reported for CRD-BP is not observed in breast tissue. Instead, all adult breast epithelial cells express CRD-BP. Interestingly, although CRD-BP expression is retained in breast tissue, it is not exclusively the canonical, full-length CRD-BP previously described. Instead, we identify and describe a protein variant (ΔN-CRD-BP) as a common and sometimes predominant isoform in breast epithelial cells. The mRNA transcript for this protein variant is missing N-terminal exons encoding two conserved RNA binding domains. Because some anti-CRD-BP antibodies are directed to N-terminal epitopes, previous studies may have under-reported CRD-BP protein expression. Furthermore, in adult tissues, CRD-BP mRNA is expressed at only 0.1–1% of the levels found in embryonic tissues, and this has also led to under-reporting of mRNA expression. Despite this relatively low expression level, we show that CRD-BP is required for clonogenic growth and that either the full-length or N-terminally truncated isoform of CRD-BP can rescue clonogenicity of CRD-BP knockdown breast cancer cells.

Experimental Procedures

Cell Culture—Mouse mammary EP and EN cell lines were derived by flow cytometric purification from parental HC11 cells (HC11 cells were a gift from Nancy Hynes (Friedrich Miescher Institute, Basel, Switzerland)).3 EP and EN cells were cultured as for HC11 cells (23). MCF7, MDA-MB-231, HS578T, BT474, and SKBr3 cells (obtained from the ATCC) were cultured in DMEM (high glucose; Life Technologies) supplemented with 10% FBS (Harlan) and 100 units/ml penicillin/streptomycin. MCF10A cells (also from ATCC) were cultured as for HC11 cells (23). MCF7, MDA-MB-231, H929T, and H9262 cells were a gift from Nancy Hynes (Emory University). Embryos from 12.5-day postcoital female mice (crossed with CRD-BP+/− males) were processed as precontrast X-rays. Mouse embryonic fibroblasts (MEFs) and HEK293T cells were maintained in DMEM (low glucose; Life Technologies) supplemented with 5% FBS (Harlan) and 100 units/ml penicillin/streptomycin. MEFs were propagated in low oxygen chambers (1% O2 content). Primary mammary epithelial cells were harvested and maintained as previously described (24).

Plasmids and Reagents—Expression constructs were created for mouse and human ΔN-CRD-BP and ΔN-CRD-BP in the pHIV-MCS-IRES-dTomato backbone (Addgene plasmid 21374). Full-length mouse CRD-BP sequence was obtained by digesting pSPORT1-mLGF2BP1 (Open Biosystems, clone 30008106). Human full-length CRD-BP was obtained by digesting pcDNA3.1-hIGF2BP1 (25). Expression constructs for mouse and human ΔN-CRD-BP were generated using PCR products from WT MEF and HEK293T cDNAs for mouse and human, respectively, using the following primers: SmaI-AGCGGGAAGGGGTAGTACCAGTTGAAGTGA, and BamHI-ACGGGAAGGGGTAGTACCAGTTGAAGTGA; and BamHI-ACGGGAAGGGGTAGTACCAGTTGAAGTGA, and BamHI-ACGGGAAGGGGTAGTACCAGTTGAAGTGA. Human CRD-BP-specific and scrambled shRNA constructs in the pSilenceTM 1.0-U6 backbone were described in previous publications (10).

Transient Transfections, Generation of Lentiviral Particles, and Viral Transductions—pGIPZ lentiviral mouse CRD-BP-specific and control shRNA vectors (Open Biosystems; clones V2LMM_66224, V2LMM_64131, and V2LMM_77760) were transfected along with vesicular stomatitis virus G-protein and viral polymerase/core protein constructs into HEK293T cells (ATCC) using Lipofectamine LTX with Plus reagent (Life Technologies) according to the manufacturer’s instructions. Virus particle-containing medium was harvested at 48 and 72 h post-transfection, and filtered using 0.45-μm filters (Thermo Fisher Scientific) prior to use in transduction in the presence of 6 μg/ml Polybrene (Sigma-Aldrich).

Immunofluorescence Staining and Confocal Microscopy—Cells were plated in 4-well chamber slides (Thermo Fisher Scientific) and then fixed/permeabilized using ice-cold methanol for 4 min, followed by ice-cold acetone for 2 min. Cells were blocked in 10% normal goat serum (Jackson Immunoresearch) for 2 h, incubated with 10% antibodies at 4 °C overnight, washed three times for 5 min in TBS-Tween (0.05%), and incubated with 20% antibodies for 2 h at room temperature. The 10% antibodies and dilutions used were: anti-CRD-BP (Abcam catalog no. ab82968; Cell Signaling catalog no. 8482; VS (25)) all at 1:100. The 20% antibodies and dilutions used were: anti-mouse-Alexa 488 (Life Technologies) 1:200; anti-rabbit-Alexa 488 (Life Technologies) 1:200. Imaging was performed using a confocal microscope (Bio-Rad MRC1024).

Western Blotting—Cells were lysed in “Gutschner” buffer (26) (50 μm Tris-HCl, pH 8.0, 150 μm NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) with freshly added protease and phosphatase inhibitors (Thermo Fisher Scientific). Lysates were then sonicated for 10 pulses at 4–5 W and cleared by spinning at 5,000 × g for 10 min at 4 °C. Protein concentration was determined using Bradford reagent (Sigma-Aldrich). Lysates were analyzed by SDS-PAGE followed by transfer to PVDF membranes. Membranes were blocked in 5% milk in TBS-Tween and incubated with the 10% antibodies at 4 °C overnight and 20% antibodies for 1 h at room temperature. The 10% antibodies and dilutions used were: anti-CRD-BP (Abcam catalog no. ab82968; Cell Signaling catalog no. 8482; Sigma: Sigma-Aldrich catalog no. HPA021367; gift from Jeff Ross; kind gift from David Herrick) all at 1:1000 and anti-vinculin (Millipore catalog no. 05–368) for 1:5000. The 20% antibodies and dilutions used were: anti-mouse-HRP (Jackson Immunoresearch) 1:5000 and anti-rabbit-HRP (Life Technologies) 1:5000.

3 S. Kim and C. Alexander, manuscript in preparation.
**TABLE 1**

| Oligonucleotide                        | Sequence (5' → 3')       |
|----------------------------------------|--------------------------|
| m/hHPRT1-Fwd                           | CCCCAGCCGGGTTTTATATTT    |
| m/hHPRT1-Rev                           | GGTTGAGCTGGCTTTGAGCTTT   |
| m/h-Whaz-Fwd                           | CGTTGAGCTGGCTTTGAGCTTT   |
| m/h-Whaz-Rev                           | GGTTGAGCTGGCTTTGAGCTTT   |
| mTBP-Fwd                               | AGTTGAGCTGGCTTTGAGCTTT   |
| mTBP-Rev                               | AGTTGAGCTGGCTTTGAGCTTT   |
| hTBP-Fwd                               | AGTTGAGCTGGCTTTGAGCTTT   |
| hTBP-Rev                               | AGTTGAGCTGGCTTTGAGCTTT   |
| hCRD-BP-total-Fwd                      | AGTTGAGCTGGCTTTGAGCTTT   |
| hCRD-BP-total-Rev                      | AGTTGAGCTGGCTTTGAGCTTT   |
| hCRD-BP-FL-Fwd                         | AGTTGAGCTGGCTTTGAGCTTT   |
| hCRD-BP-FL-Rev                         | AGTTGAGCTGGCTTTGAGCTTT   |
| hCRD-BP-N1-Fwd                         | AGTTGAGCTGGCTTTGAGCTTT   |
| hCRD-BP-N1-Rev                         | AGTTGAGCTGGCTTTGAGCTTT   |
| hCRD-BP-N2-Fwd                         | AGTTGAGCTGGCTTTGAGCTTT   |
| hCRD-BP-N2-Rev                         | AGTTGAGCTGGCTTTGAGCTTT   |
| hCRD-BP-N3-Fwd                         | AGTTGAGCTGGCTTTGAGCTTT   |
| hCRD-BP-N3-Rev                         | AGTTGAGCTGGCTTTGAGCTTT   |
| hCRD-BP-3'-UTR-Fwd                     | AGTTGAGCTGGCTTTGAGCTTT   |
| hCRD-BP-3'-UTR-Rev                     | AGTTGAGCTGGCTTTGAGCTTT   |

**TABLE 2**

| Oligonucleotide                        | Sequence (5' → 3')       |
|----------------------------------------|--------------------------|
| mCRD-BP-exon1-Fwd                      | ATGAAAGCTCTTTATATGATCT   |
| mCRD-BP-exon2-Fwd                      | ATGAAAGCTCTTTATATGATCT   |
| mCRD-BP-exon3-Fwd                      | ATGAAAGCTCTTTATATGATCT   |
| mCRD-BP-exon1a-Fwd                     | ATGAAAGCTCTTTATATGATCT   |
| mCRD-BP-exon15-Rev                     | ATGAAAGCTCTTTATATGATCT   |
| hCRD-BP-exon1-Fwd                      | ATGAAAGCTCTTTATATGATCT   |
| hCRD-BP-exon2-Fwd                      | ATGAAAGCTCTTTATATGATCT   |
| hCRD-BP-exon3-Fwd                      | ATGAAAGCTCTTTATATGATCT   |
| hCRD-BP-exon12-Fwd                     | ATGAAAGCTCTTTATATGATCT   |
| hCRD-BP-exon15-Rev                     | ATGAAAGCTCTTTATATGATCT   |

**Results**

**Identification and Characterization of the Novel ΔN-CRD-BP Isoform in Mouse and Human Cells**—We identified a discrepancy between the results of different primer sets designed to assess CRD-BP mRNA expression. To investigate this further, we performed 5’-RACE and found three separate transcripts, with differing 5’ ends, in the HC11-derived mouse mammary EN cell line. One of the transcripts includes the sequence for full-length CRD-BP, including all 15 exons (NCBI accession NM_009951.4). The sequences for the other two products include the annotated exon 3 but do not include exons 1 or 2; instead they include parts of intron 2 (21,609–21,721 and 23,093–23,174 bp, relative to the start of the annotated intron 2). This suggests there are alternate, shorter transcripts (start sites labeled F1a and F1b; Fig. 1A). In these shorter transcripts, we identified a candidate ATG that could initiate translation in exon 6, which is predicted to encode a CRD-BP isoform missing the N-terminal domain (amino acids 1–136), including the first two RNA binding domains (Fig. 1A, compare full-length and ΔN-CRD-BP structures). Interestingly, the 5’-RACE products we identified match CRD-BP alternative transcripts predicted by Gnomon gene prediction software (NCBI).

To further validate alternative CRD-BP mRNAs, RT-PCR primers were designed to test exon linkage for mRNA transcripts in several mouse and human cell lines. Amplification of products from PCRs with forward primers in exon 1 (F1), exon 2 (F2), or exon 3 (F3) (with a reverse primer in exon 15, R) showed that although MEFs and mammary epithelial cells expressed full-length CRD-BP, the mouse mammary cell lines (EP and EN cells) did not (Fig. 1B). Instead, these cells predominantly expressed an mRNA initiating in the ~25-kb second intron of CRD-BP (producing a ~1.3-kb PCR product).

The relative amounts of full-length and ΔN-CRD-BP for MEFs, mammary epithelial cells, and the mammary EP and EN
Cell lines are shown in Fig. 1 (knowing that the efficiency of primer pairs was approximately equal; data not shown). Embryonic cells expressed /H_1011100-fold more CRD-BP mRNA than cell lines from adult tissues.

To test the generality of this observation, we assayed a panel of human cell lines (Fig. 2). The human embryonic kidney epithelial cell line, 293T, has been a standard for investigating mRNA targets for CRD-BP (20, 21). PCR-based exon linkage analysis showed that 293T cells have the "embryonic" pattern of full-length CRD-BP mRNA expression, whereas the mRNA species that predominate in most breast tumor cell lines encode the truncated protein product (Fig. 2, B and C). Human tissues showed a pattern broadly similar to that observed for mouse embryonic and adult tissues. Indeed, 293T cells expressed 100-fold more CRD-BP mRNA than human breast cancer cell lines (as measured by quantitative PCR; Fig. 2C). The breast tumor cell lines examined included representatives of different breast cancer subtypes (Luminal type, MCF7; HER2 overexpressers, BT474 and SkBr3; basal type, HS578T; triple-negative breast cancer, MDA-MB-231). MCF10A cells, a nontumorigenic breast cell line, had low/undetectable levels of CRD-BP mRNA. When these breast tumor cell lines were compared with one another, the CRD-BP mRNA expression levels varied 40-fold (Fig. 2C).

The long (7-kb) 3'-UTR of CRD-BP mRNA contains six conserved miRNA binding sites for let7 family members (let7 miRNA binding sites are depicted as white circles in Figs. 1 and 2) that regulate the stability and translation of CRD-BP mRNA in embryonic tissues (30–32). The CRD-BP/IGF2BP1/IMP1 mRNA was one of the examples used to demonstrate the general shortening of 3'-UTRs in mRNAs expressed by tumor cell lines (33). Given the complexity of mRNA isoforms revealed by our current analysis, we re-examined the 3'-UTR shortening phenomenon in our panel of human breast cancer...
CRD-BP Is Required for Mammary Cell Clonogenicity

A. Human CRD-BP RNA (NM_006546.3)

Exon i2: [15,596]
CCATCGTCATCAGGGGCACTAAGGACCCGAGGACACGGGATGTCTGCCGGGTCTCTTCTG
GTTGCCGTTGAAAGCTTGCCGGAGACAGATTGCTTTGGAGGCTGGTTCTGGTTTCCCTCTCGGCTCT
GAGAGGCCACCTGCTCTGCACCAGAGAGGAGACCTGAGCTGTCTGTTTTCAAG [15,787]

B. 293T MCF7 HS578T BT474

C.

D.

E.

Intron 2 mRNA start site
Cell lines. We designed three sets of qRT-PCR primers that span a region in the 3′-UTR lying between the two sets of three let7 miRNA binding sites (primer sequences listed under “Experimental Procedures”). Two of these primer sets, one of which corresponds to the sequence bound by the Agilent array probe, gave no signal for any of the adult tissues examined, suggesting that results of published assays based on the 3′-UTR could be compromised in general. Interestingly, however, data from one primer pair (with an amplicon at ~3.5–3.7 kb and the highest efficiency) showed that the proportion of CRD-BP mRNAs that include the 7-kb 3′-UTR varied between 0 and 60% for any given breast cancer cell line (Fig. 2D).

To test the exon usage of CRD-BP mRNAs in breast tumors, we analyzed the RNASeq data deposited in the Cancer Genome Atlas (29). The application of RNASeq analysis for this purpose has been reviewed recently (34). Our analysis confirmed that CRD-BP mRNA levels were elevated in breast tumors compared with normal breast tissue (Fig. 2E). Furthermore, we found that the expression of exons 1 and 2 was relatively depleted compared with exons 3–15 in breast tumors. Quantitation suggests that only 40% of the total transcripts (from all breast tumors) are likely to be full-length, and the remainder of mRNAs include exons 3–15. Another set of peaks implies that other transcripts are produced at this locus, although if these exons are linked together, they are predicted to be noncoding (exons 8, 9, and 12–14).

Each breast tumor subtype showed a characteristic expression level and exon usage for CRD-BP mRNA isoforms. Of interest, Luminal B type tumors (an estrogen receptor-positive but aggressive, treatment-resistant tumor type) showed higher CRD-BP mRNA expression compared with Luminal A type tumors (an estrogen receptor-positive and aggressive, treatment-resistant tumor type) (Fig. 2F). Variations in CRD-BP mRNA expression correlated with breast cancer subtype (Fig. 2F).

We utilized MEFs from the CRD-BP hypomorphic mouse strain to test the specificity of various antibodies used to characterize CRD-BP expression and subcellular distribution. The antibodies tested include two N-terminal epitope antibodies from Abcam and Cell Signaling, which we showed were highly specific for full-length CRD-BP by immunofluorescent staining of CRD-BP<sup>hi</sup>MEFs (Fig. 3C). A monoclonal antibody to the C terminus of CRD-BP (VS) showed decreased staining in CRD-BP<sup>hi</sup>MEFs compared with WT MEFs. We used this antibody for semiquantitative assessment of CRD-BP expression levels.

Evaluation of CRD-BP antibody specificity by Western blotting confirmed the immunocytochemical stains (Fig. 3D). Full-length CRD-BP, visualized as a 70kDa band, was undetectable in Western blots of lysates from CRD-BP<sup>hi</sup>MEFs. Bands at 70 and 50 kDa were detected using an antibody directed against a KH domain-derived epitope (Fig. 3D; pAb Sigma; epitope is indicated on protein scheme), which is predicted to be present in both full-length and ΔN-CRD-BP species (50 kDa is the predicted molecular mass of ΔN-CRD-BP). Expression of the shorter species was conserved in CRD-BP<sup>hi</sup>MEFs. An antibody to a distinct KH domain-derived epitope (pAb; kind gift from David Herrick) also detected 70- and 50-kDa species in human 293T cells, with amounts that correlate with the relative mRNA abundance (Fig. 2C). Similar 50-kDa species have been observed in various cells and tissues by other studies, although those studies suggested these species could be degradation products (13, 36–38). The C-terminally reactive VS mAb to CRD-BP used for immunostaining did not reliably detect endogenous levels of CRD-BP by Western blotting.

**Homologous Transcription Start Sites in Intron 2 of Mouse and Human CRD-BP Genes**—We searched for metadata to support the presence of a conserved start site for distinct CRD-BP mRNA species. We discovered highly conserved elements in intron 2 of both human and mouse CRD-BP gene loci, ~12 kb downstream of exon 2 (Fig. 4A). These elements are rich in CpG islands (often found near gene promoters) and in epigenetic marks associated with active transcription (H3K4 methylation).

Furthermore, the ENCODE ChIP-Seq data set (reported by the University of California, Santa Cruz cancer genome browser) showed extensive transcription factor binding/occupancy at the intron 2 candidate promoter, including occupancy by transcription factors such as POLR2A. Importantly, a pro-

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**FIGURE 2. The ΔN-CRD-BP transcript is expressed in human breast cancer cells.** A, a schematic diagram depicting CRD-BP gene structure as in Fig. 1A (based on NCBI accession NM_006546.3). The novel exon located in intron 2 is labeled Exon i2, and the location of the additional forward primer (F2) is shown. B, RNA was isolated from the human cell lines indicated, and RT-PCR analysis (as described for Fig. 1B) was performed using primers to an intronic region (orthologous to the mouse sequence) in the human CRD-BP gene (see also Fig. 4A). C, isoform-specific expression patterns of CRD-BP mRNAs were assayed by qRT-PCR analysis as described for Fig. 1C, and relative expression was compared with MCF7 breast cancer cell line. D, to evaluate whether the CRD-BP mRNA species detected express the long let7 miRNA regulated 3′-UTR, RNA preparations from the indicated cells/tissues were analyzed by qRT-PCR analysis. Results are shown comparing signals from a primer set amplifying a region in the 7-kb-long 3′-UTR to signals from a primer set amplifying total CRD-BP (amplicon located in exon 15). E, RNASeq data from the Cancer Genome Atlas was analyzed to determine the expression of CRD-BP exons 1–15 in normal tissue (purple line) compared with primary breast tumor tissues, divided by subtype. Tumor numbers: basal, n = 142; Her2, n = 67; Luminal A (Lum-A), n = 434; Luminal B (Lum-B), n = 194. Exons are numbered along the x axis, whereas the y axis shows relative abundance. Potential exon linkage products (based on their approximately similar abundance) are indicated with horizontal black arrows.
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A diagram designed to identify transcription start sites (Augustus (39)) independently predicted a CRD-BP transcript initiating in intron 2.

Of two in-frame start codons that could serve as the translation initiation site for ΔN-CRD-BP, only one had a high consensus, proximal Kozak sequence based on a prediction tool.

Mouse CRD-BP RNA (NM_009951.4)

A.

B.

C.

N-terminal Antibodies

C-terminal Antibody

D.

E.
ATGpr (40). Translation of ΔN-CRD-BP, predicted to start at Met-137 of full-length CRD-BP, generates a protein comprising 441 amino acids (compared with the 577 amino acids for the full-length protein; Fig. 4B).

CRD-BP Is Highly Expressed and Ubiquitous in Human Breast Tumors—Transgenic expression of CRD-BP has been demonstrated to be oncogenic in the mouse mammary gland (2), and studies have suggested links between CRD-BP locus amplification or increased CRD-BP mRNA expression levels and mammary tumorigenesis (41, 42). Given the previously reported functional links between CRD-BP expression and tumorigenicity for many tumor types, we evaluated the expression of CRD-BP in human breast tumors. A human breast tumor tissue microarray (243 distinct breast cancer samples, together with four normal breast samples) was stained with the C-terminally reactive VS mAb to CRD-BP, to evaluate total CRD-BP expression (including both the full-length and ΔN isoforms). Breast tumor samples were predominantly (96%) obtained from Caucasian women with a mean age of 55 years. 80% of tumors on the microarray were estrogen receptor/progesterone receptor-positive, 12% were HER2-positive, and 8% were triple negative.

Interestingly, CRD-BP was ubiquitously expressed in normal breast tissues (although enriched in luminal breast epithelial cells) and also expressed in all breast tumors (examples of the stains are shown for various tumor types and normal tissues in Fig. 5A). There was no obvious heterogeneity of protein expression in tumors when evaluated for distinct cell types, proximity to hypoxic zones, stromalized areas, margins, or tumor subtype (data not shown).

Thus, both RNAseq and histological data suggest that the expression of CRD-BP isoforms is ubiquitous in breast epithelial cells. These sources were corroborated by mRNA expression data from Agilent microarrays, published by the Cancer Genome Atlas database. This analysis confirmed that CRD-BP mRNA was expressed by all tumor types and that the HER2 cohort of breast tumors showed the highest expression levels of CRD-BP (predicted based on co-amplification of the 17q21 amplicon containing the HER2 gene) (Fig. 5B).

Using our purpose-built, isoform-specific primers, we performed quantitative PCR analysis to determine the expression of full-length and ΔN-CRD-BP mRNAs in a limited cohort of breast tumors and normal breast tissue (Fig. 5C). The results also showed that relative CRD-BP mRNA expression was elevated in tumors and demonstrated that whereas some tumors expressed only one variant (either full-length CRD-BP or ΔN-CRD-BP), other tumors expressed both. Finally, we found that CRD-BP transcripts could be spliced to either include or exclude the let7-regulated long 3′-UTR. Thus, tumor mRNAs that include the long 7-kb 3′-UTR comprised 20–96% of total CRD-BP transcripts detected (Fig. 5D).

CRD-BP Is Required for Clonogenic Growth of Mouse and Human Breast Epithelial Cells—Previous studies have shown that not only is CRD-BP overexpressed in cancer cells, but also that CRD-BP is important for tumor cell survival, as summarized by Bell et al. (7). However, a previous study suggested that CRD-BP/IMP1 had no effect on breast cancer cell growth; instead it was ascribed tumor suppressor functions, because CRD-BP knockdown resulted in increased growth of metastatic cells and increased cell migration (43). To test the functionality of CRD-BP in breast tumor cells, we knocked down expression in both mouse (by stable transduction of shRNA constructs) and human (by transient transfection of shRNA constructs) mammary cell lines and found that although CRD-BP was not required for growth in mass culture, it was essential for the clonogenic activity of mammary cell lines. Thus, 50–90% knockdown of CRD-BP mRNA (Fig. 6, A and C) resulted in a corresponding decrease in clonogenic growth for both human and mouse breast tumor and nontumorigenic cells (Fig. 6, B–D). Most of these cell lines express majority ΔN-CRD-BP mRNA. For a nontumorigenic mouse epithelial cell line (EP cells), expressing very low amounts of CRD-BP (only 0.1× expression compared with tumorigenic EN cells; Fig. 1C), 60–90% knockdown did not affect clonogenicity (Fig. 6D).

Re-expression of Either Full-length or ΔN-CRD-BP Rescues Clonogenicity—To the best of our knowledge, all previous studies investigating the effect of CRD-BP expression on clonogenicity and cell survival have only tested the function of full-length CRD-BP (for summary see review by Yisraeli (17)). We have shown that CRD-BP is expressed in at least two distinct forms, where the ΔN-CRD-BP variant can predominate in breast tumor cells in vitro and in vivo. For example, this is the main mRNA species expressed by MCF7 cells (Fig. 2C). To compare the functionality of ΔN-CRD-BP compared with full-length CRD-BP, we knocked down expression of CRD-BP in MCF7 human breast cancer cells using a human CRD-BP-specific shRNA (or a scrambled shRNA control) and rescued expression with mouse full-length or ΔN-CRD-BP. Knockdown and overexpression were assessed by immunofluorescent staining, Western blotting, and qRT-PCR (Fig. 7, A–C). Colony formation assays performed on MCF7 cells showed that either full-length or ΔN-CRD-BP mouse proteins, when expressed at comparable levels, partly rescued the decrease in clonogenicity observed following CRD-BP knockdown (Fig. 7D). We conclude that either full-length or ΔN-CRD-BP show similar activity with respect to their support of breast tumor cell clonogenicity.

Discussion

Our investigation of discrepancies arising between analyses of CRD-BP mRNA and protein expression has revealed that transcriptional regulation of the CRD-BP gene is more complex...
FIGURE 4. Homologous alternative transcription start sites in intron 2 for mouse and human CRD-BP genes. A, the human and mouse CRD-BP alleles are illustrated, together with summary data for detection of the active histone mark (H3K4Me3), summed transcription factor binding activity (from ENCODE ChIP-Seq data), and distribution of CpG islands often associated with active promoters. The bottom panel shows the sequence conservation across intron 2 for human and mouse CRD-BP, as well as filtered ChIP-Seq data exclusively showing RNA pol2 (POLR2A) binding. B, Kozak sequence analysis identified the predicted start codon for translation from the ΔN-CRD-BP isoform with the alternative transcription initiation site in intron 2. Amino acid sequences for full-length and ΔN-CRD-BP are shown.
than previously understood. An internal promoter drives expression of a short isoform (ΔN) lacking the N terminus in adult normal and tumor tissues. Although the full-length protein is characteristic of embryonic tissues (and is the target of several commercially available antibodies), the short form is widely expressed. Indeed, we observe CRD-BP staining (using an antibody directed to the C-terminal domain predicted to react with all known isoforms) in adult tongue, liver, lung, and colon tissue sections (Fig. 5 and data not shown). Importantly, the antibody stains we report are validated by the use of cells with a hypomorphic CRD-BP locus, as well as cells transfected with CRD-BP shRNA \textit{in vitro}.

![Diagram](image)

**FIGURE 5. Expression of CRD-BP in human breast tumors.** A, immunohistochemical analysis of a breast tumor microarray and control tissue sections (liver and colon) stained for CRD-BP using the VS mAb to CRD-BP (diaminobenzidine/brown stain), targeted against the C-terminal domain of CRD-BP, and counterstained with hematoxylin (pink). Representative stains of each subtype of breast tumor along with a normal breast tissue section are shown. Stains of normal human colon and liver are shown for comparison. Scale bars, 50 μm for low magnification images (left column) and 5 mm for high magnification images (right column), mag., magnification. B, the relative levels of CRD-BP mRNA were assessed from expression arrays associated with the Cancer Genome Atlas data set (670 human breast tumors; probe sets target 3'-UTR (53)) divided by tumor subtype, defined by their PAM50 signature (as indicated, including normal-like tumors). CRD-BP transcript levels are expressed as log2 fold change over the median CRD-BP expression level across tumor types. The boxes enclose the 25th to 75th percentile data points, with the horizontal bars showing the median values. The whiskers demark the 10th and 90th percentiles. *, p < 0.05.

C, RNA isolated from primary human normal (N1–N2) and tumor (T1–T9) breast tissue was analyzed by qRT-PCR with the same primers used for Fig. 2C to assess the relative expression of CRD-BP isoforms. D, qRT-PCR analysis was performed as for Fig. 2D to assess the expression of the long 7-kb 3'-UTR of CRD-BP mRNA in primary human normal and tumor breast tissue.
pattern is supported by a published biochemical study that showed, using Nycodenz gradient centrifugation, that overexpressed and endogenous CRD-BP showed distinct fractionation patterns (20). As this study noted, altered localization of overexpressed CRD-BP may lead to the acquisition of novel functions.

To investigate whether short form ΔN-CRD-BP mRNA is expressed by human breast tissue in vivo, we interrogated RNASeq data from the Cancer Genome Atlas database. This analysis revealed that normal breast tissues express low levels of the full-length CRD-BP mRNA, whereas breast tumors express relatively elevated levels of CRD-BP transcripts, the majority of which are ΔN-CRD-BP mRNA species (exons 3–15). This broadly corresponds with results of exon-specific qPCR analysis of a small number of biopsy samples from breast tumors (Fig. 5C) and the expression patterns of breast tumor cell lines in vitro (Fig. 2C). We predict therefore that the principle CRD-BP protein expressed by the most malignant breast tumors in vivo in patients is the ΔN-CRD-BP short isoform that we have identified here.

Our immunohistochemical analysis of CRD-BP expression shows that this protein is ubiquitously expressed in normal breast epithelial cells and also in breast tumor cells in vivo. Although expression levels are lower in adult cells compared

**FIGURE 6. CRD-BP is required for clonogenic growth of breast tumor cell lines.** A, human breast cell lines were transfected with an shRNA construct targeting the human CRD-BP gene (CRD-BP shRNA) or scrambled shRNA control (Scr shRNA). Knockdown efficiency of total CRD-BP mRNA was assessed 48 h post-transfection by qRT-PCR analysis. Representative knockdown efficiencies for two cell lines are shown (experiments were repeated at least three times). B, human breast cell lines were subcultured 24 h post-transfection with CRD-BP shRNA (CRD-BP shRNA) or a scrambled shRNA control (Scr shRNA) either to clonal density (10^4 cells/10-cm^2 plate) for functional assay or by passaging (2-fold dilution) for assessment of knockdown efficiency (A). Colony formation was assessed by crystal violet staining ~1 week postsubculture (as detailed under “Experimental Procedures”). Representative images of this assay are shown (left panel), and results were quantified for each cell type (right panel). C, the clonogenicity of stable CRD-BP knockdown mouse mammary cells was measured (EP and EN cells stably transduced with lentiviral shRNA constructs). Knockdown efficiencies are shown, determined by qRT-PCR analysis. Two CRD-BP shRNA constructs were used, and results were normalized to the control shRNA-transduced condition. D, crystal violet staining was performed as for B in mouse mammary EP and EN cells stably transduced with one of two CRD-BP shRNA constructs (CRD-BP shRNA-1 or shRNA-2) or a control shRNA construct (Control shRNA). Representative images from colony formation assays are shown, and quantified at right.
FIGURE 7. Both full-length and ΔN-CRD-BP isoforms can rescue clonogenicity of CRD-BP knockdown breast cancer cells. A, MCF7 cells were transfected with human CRD-BP shRNA construct (or a scrambled shRNA construct as a control), fixed 48 h later, and stained with VS mAb to CRD-BP, illustrating the dynamic range of this visual assay. Representative images are shown. Scale bars, 10 μm. B, MCF7 cells were transfected with expression constructs encoding mouse full-length or ΔN-CRD-BP isoforms (or an empty vector as a control) and stained as for A. Higher magnification pictures show the subcellular distribution of CRD-BP. Scale bars, 50 μm for the top panel and 10 μm for the bottom panel. C, to test the rescue activity of the full-length and ΔN-CRD-BP species, combinations of specific or control shRNA to human CRD-BP, together with overexpressed mouse CRD-BP isoforms (or an empty vector control), were transfected into MCF7 cells. Overexpression of both isoforms was assessed by Western blot analysis using CRD-BP antibodies to the N-terminal domain (Cell Signaling) or the C-terminal domain (gift from Jeff Ross; left panel; see also epitope locations marked in Fig. 3C) and also by qRT-PCR analysis of total CRD-BP mRNA levels (right panel). D, MCF7 cells transfected with the indicated constructs were subcultured to clonal densities as for Fig. 5A (experiments were repeated at least three times). After ~1 week, colonies were stained with Crystal Violet dye (optical density quantified below). ***, p < 0.05; **, p = 0.06; *, p = 0.08.
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with embryonic sources, the CRD-BP gene locus cannot accurately be described as oncopetal. More accurately, the gene locus is regulated heterochronically in embryo and adult (32), with respect to both transcription start sites and overall expression levels. Also, the post-transcriptional regulation of CRD-BP/IMP1 mRNAs has been shown to depend at least in part upon binding of the let7 class of miRNAs (11, 30). We confirmed that embryonic 293T cells express CRD-BP transcripts that include the long form let7-regulatable 3′-UTR CRD-BP mRNA (Fig. 2D and Northern data not shown) but also show that breast cancer cell lines (Fig. 2D) and human tumor samples (Fig. 5D) contain significant amounts of mRNAs without this long 3′-UTR. This partly confirms data from Mayr and Bartel (33), who showed that alternative polyadenylation of mRNA species, leading to 3′-UTR shortening, was characteristic of a variety of cancer cell lines. RNA species with shorter 3′-UTRs were more stable and produced more cognate protein (10× more than mRNAs with long 3′-UTRs associated with normal cells). Specifically for CRD-BP/IGF2BP1, they showed that the 3′ truncated CRD-BP/IGF2BP1 mRNA was a more potent oncogene, leading to increased clonogenicity in NIH 3T3 cells overexpressing CRD-BP/IGF2BP1.

Other studies have shown that CRD-BP is significantly expressed in adult tissues. Thus, using reagents targeted toward total CRD-BP (including both the full-length and ΔN isoforms), Dimitriadis et al. (44) reported expression of CRD-BP in intestinal crypts and showed widely divergent expression of CRD-BP in different colorectal tumors. They also show that high CRD-BP expression levels correlated with poor patient outcomes. A recent study (26) employing an Agilent microarray platform showed that CRD-BP/IGF2BP1 is expressed in adult liver. This study utilized PCR primers directed to sequences in exon 11 to confirm the gene expression changes observed for hepatocellular tumors; we predict that this assay would capture the majority of mRNA isoforms (equivalent to our total CRD-BP assay with qRT-PCR primers targeting a region in exon 15). We confirmed the expression pattern reported in this study (Fig. 5A).

Overall, expression of the CRD-BP locus is likely to have been significantly underestimated in previous studies, for two reasons: 1) antibody-reactive epitopes are lacking from the newly identified ΔN-CRD-BP isofrom, and 2) there are a variety of mRNA isoforms that exist, not all of which are detected by PCR primers directed to the canonical GenBank™ sequence.

Our results showed that expression of CRD-BP mRNA isoforms, as well as total CRD-BP expression, varied more between breast cancer cell lines in vitro than for tumors in vivo. Also, the amount of CRD-BP protein per cell was not strictly related to mRNA expression (data not shown), implying considerable post-transcriptional regulation. For example, we expected to observe higher protein expression of CRD-BP in HER2-positive breast cancer cells and tumors, given the previously described co-amplification of HER2 and CRD-BP (41). In fact, there was surprisingly little difference between CRD-BP protein expression levels in the tissue samples from various breast tumor subtypes (Fig. 5).

We find that CRD-BP protein is predominantly cytoplasmic in breast epithelial cells and is distributed in a granular pattern. This distribution has also been reported in other studies (20, 45). Thus, using a monoclonal antibody raised against full-length recombinant CRD-BP, Gutschner et al. (26) showed a cytoplasmic stain of both normal hepatocytes and hepatocellular carcinoma tumor cells that resembles the subcellular distribution we report. Knockdown of CRD-BP/IGF2BP1 in hepatocellular carcinoma cell lines resulted in reduced growth rate and increased rate of apoptosis and correlated with decreased rates of growth of xenografts in vivo (26).

Of note, the phenotype of the CRD-BP/IMP1 knock-out mouse, as reported by Hansen et al. (35), requires re-evaluation. This strain was produced by insertion of a gene trap cassette in intron 2, which effectively knocks out transcription of the full-length mRNA; however, the insertion point is ∼10 kb upstream of the putative internal entry promoter, thereby maintaining transcription of ΔN-CRD-BP (Fig. 3). These mice show progressive dwarfism that initiates during embryogenesis, after the peak of CRD-BP/IMP1 expression (from the annotated 5′ upstream promoter) at embryonic day 12.5. Half of newborn hypomorphic pups die, and this is associated with intestinal malformation. The phenotype of the total CRD-BP knock-out is therefore likely to be more severe. The promoter that drives embryonic expression of CRD-BP is a hot spot for transcription factor binding (Fig. 4) and is preferentially methylated in breast cancer cells (43) and in human mesenchymal stem cells (the latter by TET1/2) (46). A more select group of transcription factors (identified by ChIP analysis from the ENCODE database) is loaded onto the novel, internal promoter we describe here for adult normal and tumor tissues.

We show that loss of CRD-BP has a dramatic effect on clonogenicity of breast cancer cell lines, while not significantly affecting the growth of cells in mass cultures (another study using reagents targeted toward total CRD-BP also showed the latter result for MCF7 cells (3)). We found that loss of clonogenicity increases with higher efficiency of knockdown. We have shown that the short form of CRD-BP, ΔN-CRD-BP, can rescue clonogenic activity of breast cancer cells in vitro and propose therefore that the widely expressed ΔN isoform is able to substitute for the full-length CRD-BP isoform at least in the context of clonogenic activity. ΔN-CRD-BP lacks the two N-terminal RNA binding motifs. These motifs have been suggested to be a relatively recent evolutionary addition (47), and previous functional studies have suggested that the four KH domains dominate RNA binding activities of this protein (18, 19). Although one structure function study of CRD-BP suggests that the first two RNA recognition motifs control the targeting of CRD-BP/ZBP1-associated granules to the leading edge of fibroblasts (19), other results suggest that these motifs are largely dispensable for specific activities (48, 49).

Functional studies in breast epithelial cells have shown that CRD-BP is an oncogene with 100% penetrance when expressed in transgenic mice (albeit with a long latency of almost 12 months depending on the amount of CRD-BP expressed (2)). However, studies by Singer and co-workers (43, 50) have shown that CRD-BP/IGF2BP1 has tumor suppressor functions, such that loss of CRD-BP/IGF2BP1 increased growth and motility of breast cancer cells. This conclusion varies from our study and others. The functional aspects of the variant studies focused on the metastatic mammary tumor cell line, MTLn3, which we did
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not assay. Furthermore, some of the reagents described in that study, including the siRNA constructs used, were specific to full-length CRD-BP. To confirm the effects we observed, we performed the knockdown experiment using two different approaches, first using CRD-BP knockdown constructs together with puromycin selection (data not shown) and second without co-transfection of the selection marker or addition of puromycin (Fig. 6). Results obtained from both methods were concordant and showed a dramatic effect of CRD-BP knockdown on clonogenic growth for a wide variety of cell lines. We are currently investigating the molecular basis of the requirement of tumor cells for CRD-BP, knowing that clonogenicity has been related to various factors, including integrin activity, mitochondrial function, and growth factor production.

In summary, the description of CRD-BP as an oncofetal protein/gene glosses over crucial differences between embryonic and adult expression of the CRD-BP gene locus. The predominant mRNA transcribed in adult cells is likely to be induced by different transcription factors and epigenetic programs, and the predominant protein isoform expressed in adult tissues is different from that expressed in embryonic tissues. As far as we are aware, prior studies in vitro and in vivo have focused on the full-length canonical protein described prominently in the databases, and the current study is the first to identify and characterize expression of an N-terminally deleted short form of CRD-BP.

We do not eliminate the possibility that there are other splice variants expressed by this locus, perhaps several different polyadenylated versions of each mRNA, and potentially several distinct protein products. Indeed, there are precedents for such complexity. For example, the N-terminally truncated CRD-BP species resembles protein variants observed for another member of the IMP family, IMP2 (51), as well as another RNA binding protein, FMRP (fragile X mental retardation protein). Expression of the FMR1 gene locus shares other characteristics with CRD-BP. Thus, as many as 12 transcript isoforms are derived from the FMR1 locus, which vary in abundance at least 2 orders of magnitude between adult and embryonic mouse brain (52). These various isoforms have different functions and show specific regulation.

Despite low expression levels that make the protein(s) difficult to detect, we show that CRD-BP is important for clonogenic growth of breast cancer cells in vitro. The mRNA binding partners regulated by full-length and/or ΔN-CRD-BP to maintain clonogenicity of breast cancer cells are not yet characterized but are likely to be important to the expression of fully malignant characteristics of breast cancer cells in vivo.

Acknowledgments—We thank Jeff Ross for generously donating a polyclonal antiserum and for advice during this study. We thank Celia Bisbach for technical assistance and other lab members for their expertise and Gary Bassell for sharing CRD-BP mutant mice. We thank Sally Ann Drew and Thomas Pier (TRIP Laboratory, Department of Pathology, University of Wisconsin-Madison) for expert assistance in staining and evaluation of clinical specimens, the Translational Science BioCore Biobank of the University of Wisconsin Carbone Cancer Center, and Lance Rodenkirch (Keck Lab for Biological Imaging) who assisted with confocal microscopy.

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