Cellular Retinoic Acid-binding Protein 2 Inhibits Tumor Growth by Two Distinct Mechanisms*

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Background: CRABP2 delivers RA to its cognate nuclear receptor RAR and regulates gene expression by cooperating with HuR in stabilizing mRNAs.

Results: In conjunction with HuR, CRABP2 regulates the expression of multiple cancer-related genes and suppresses tumor growth.

Conclusion: The anticarcinogenic activities of CRABP2 are mediated by both HuR and RAR.

Significance: The data demonstrate a novel mechanism through which CRABP2 inhibits tumorigenesis.

Cellular retinoic acid-binding protein 2 (CRABP2) potently suppresses the growth of various carcinomas, but the mechanism(s) that underlie this activity remain incompletely understood. CRABP2 displays two distinct functions. The classical function of this protein is to directly deliver retinoic acid (RA) to RA receptor (RAR), a nuclear receptor activated by this hormone, in turn inducing the expression of multiple antiproliferative genes. The other function of the protein is exerted in the absence of RA and mediated by the RNA-binding and stabilizing protein HuR. CRABP2 directly binds to HuR, markedly strengthens its interactions with target mRNAs, and thus increases their stability and up-regulates their expression. Here we show that the anticarcinogenic activities of CRABP2 are mediated by both of its functions. Transcriptome analyses revealed that, in the absence of RA, a large cohort of transcripts is regulated in common by CRABP2 and HuR, and many of these are involved in regulation of oncogenic properties. Furthermore, both in cultured cells and in vivo, CRABP2 or a CRABP2 mutant defective in its ability to cooperate with RAR but competent in interactions with HuR suppressed carcinoma growth and did so in the absence of RA. Hence, transcript stabilization by the CRABP2-HuR complex significantly contributes to the ability of CRABP2 to inhibit tumorigenesis. Surprisingly, the observations also revealed that HuR regulates the expression of multiple genes involved in nuclear pore formation and is required for nuclear import of CRABP2 and for transcriptional activation by RAR. The data thus point at a novel function for this important protein.

The vitamin A metabolite retinoic acid (RA) regulates gene transcription by activating several members of the nuclear receptor family of transcription factors: the classical RA receptors (RARs) (1, 2) and peroxisome proliferator-activated receptor β/δ (PPAR/β/δ) (3, 4). The partitioning of RA between these receptors is regulated by two intracellular lipid-binding proteins: cellular retinoic acid-binding protein 2 (CRABP2), which has a high affinity for the hormone and shuttles it to RARs, and fatty acid-binding protein 5 (FABP5), which has a lower affinity for RA and delivers it to PPAR/β/δ. CRABP2 and FABP5 are cytosolic in the absence of their ligand, but upon binding of RA, they undergo a conformational change that activates their nuclear localization signals and results in their mobilization to the nucleus (5–8). In the nucleus, these binding proteins associate with their cognate receptors to form a complex through which RA is directly “channeled” to the receptor (9). CRABP2 and FABP5 thus markedly enhance the transcriptional activities of RAR and PPAR/β/δ, respectively (3, 5, 7, 10, 11). Consequently, RA activates RARs in cells that highly express CRABP2 but functions through PPAR/β/δ when FABP5 predominates. As RAR and PPAR/β/δ regulate the expression of distinct cohorts of genes, RA displays different and sometimes opposing biological activities in cells where due to a high CRABP2/FABP5 ratio it activates RAR, and in cells where this ratio is low, it results in activation of PPAR/β/δ. For example, in many carcinoma cells, RA up-regulates genes that trigger differentiation, apoptosis, and cell cycle arrest (12–18), whereas PPAR/β/δ induces the expression of genes that promote proliferation, angiogenesis, and survival (3, 19–22). Consequently, RA inhibits the growth of carcinoma cells that express CRABP2 (11–13, 23, 24) but promotes oncogenic activities in FABP5-expressing cells (3, 25, 26).

Although it is well established that CRABP2 suppresses carcinoma cell growth by delivering RA to RAR, it was noted previously that this binding protein also exerts biological activities independently of either RA or its receptor (27). It was thus reported that although expression of apoptotic peptidase-acti...
vating factor 1 (Apaf-1), the major protein in the apoptosome, is not controlled by either RA or RAR, ectopic expression of CRABP2 increases its level both in cultured carcinoma cells and in vivo (12, 24, 27). It was shown further that expression of CRABP2 in mammary carcinoma cells cultured in the absence of RA enhances the cleavage of several caspases, demonstrating that the protein exerts proapoptotic activities in the absence of its ligand (12, 27). These observations raise the possibility that the tumor-suppressive activities of CRABP2 may stem not only from its ability to activate RAR but also from an additional, RA- and RAR-independent function.

It was reported recently that CRABP2 devoid of RA (apo-CRABP2) functions in conjunction with HuR, one of the best characterized proteins involved in post-transcriptional regulation of gene expression in animals (28). HuR regulates various biological functions including RNA splicing, nuclear export, and transcript stabilization. It exerts the latter activity by binding to AU-rich elements in 3′-UTRs of target mRNAs, thereby protecting them against degradation and up-regulating their expression (29–32). CRABP2 cooperates with HuR in stabilization of certain mRNAs. It was thus shown that the binding protein directly interacts with HuR both in solution and when associated with some target transcripts and that it markedly increases the affinity of HuR for such transcripts. CRABP2 thus enhances the stability and increases the expression levels of such transcripts including mRNAs for the proapoptotic genes Apaf-1 and Casp7 and for HuR itself. Indeed, it was shown that CRABP2 can enhance apoptotic responses through its cooperation with HuR (27). The current work was undertaken to associate with some target transcripts and that it markedly increases the affinity of HuR for such transcripts. CRABP2 thus enhances the stability and increases the expression levels of such transcripts including mRNAs for the proapoptotic genes Apaf-1 and Casp7 and for HuR itself. Indeed, it was shown that CRABP2 can enhance apoptotic responses through its cooperation with HuR (27). The current work was undertaken to investigate whether its cooperation with HuR is involved in the anticarcinogenic activities of CRABP2 and to assess the relative contributions of CRABP2/RAR and CRABP2/HuR pathways in mediating these activities.

EXPERIMENTAL PROCEDURES

Cells—The M-2−/− cell line was generated from tumors that arose in murine mammary tumor virus-neu/CRABP2-null mice (24). MCF-7 cells were purchased from ATCC (Manassas, VA). Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 4.5 g/liter glucose, 4.5 g/liter l-glutamine, 10% fetal bovine serum (Invitrogen), 100 IU/ml penicillin, and 100 µg/ml streptomycin.

Reagents—RA was purchased from Calbiochem. Antibodies against HuR (3A2; sc-5261), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 6C5; sc-23233), actin (I-19; sc-1616), and RARβ (C-19; sc-552) were from Santa Cruz Biotechnology, Inc. Antibodies against Apaf-1 (8723) and poly(ADP-ribose) polymerase (9542) were from Cell Signaling Technology, Inc. Antibody against CRABP2 was a gift from Cecile Rochette-Egly (Institut Génétique Biologie Moléculaire Cellulaire, Strasbourg, France). Transfections were carried out using PolyFect (Qiagen).

Vectors—Mammalian expression vectors harboring cDNA encoding wild type (WT) or hCRABP2ANLS N-terminally tagged with EGFP (pEGFP-C2 vector) and vector encoding FLAG-tagged CRABP2 were described previously (27).

Lentiviral shRNA Production—pLKO.1 vectors harboring shRNAs (Elavl1, TRCN000012088; ELAVL1, TRCN0000017275; CRABP2, TRCN0000021373) were from Open Biosystems. pLKO.1 vectors harboring luciferase shRNA (SHC007) or non-targeting shRNA (SHC002) were from Sigma-Aldrich. Using pCMV packaging vector and pMD2.G envelope vector, lentiviruses were produced in HEK293T cells, and target cells were transduced according to standard protocols. Expression of Elavl1 and ELAVL1, encoding mouse HuR and human HuR, respectively, was reduced using respective shRNAs.

Transcriptome Analyses—MCF-7 cells were transduced with lentiviruses harboring the indicated shRNAs. 4 days post-transduction, cells were harvested, and RNA was extracted using RNeasy columns (Qiagen). Samples were amplified, labeled, and hybridized on Affymetrix® Human Gene 2.1 ST Arrays (Affymetrix) by the Gene Expression and Genotyping Facility of the Case Comprehensive Cancer Center of Case Western Reserve University. Raw data files were analyzed using Affymetrix Expression Console and Transcriptome Analysis Console. Signal intensities were normalized using the robust multichip average method. t test analyses were used to select genes differentially expressed in cells in which either ELAVL1 or CRABP2 was knocked down versus luciferase with -fold change and p value cutoffs fixed at 1.2 and 0.01, respectively. Venn analysis was used to identify the overlapping genes between the 2 groups. The list of overlapping genes was further analyzed for known functions and pathways using Ingenuity Pathway Analysis (Ingenuity Systems).

Real time quantitative PCR (qPCR)—Real time qPCR was performed using a StepOnePlus Real Time PCR System with TaqMan probes: Apaf1, Mm01223702_m1; HuR/Elavl1, Mm00516012_m1; Rarb, Mm01319677_m1; Bcra1, Mm0129840_m1; Bcra2, Mm01218747_m1; Casp7, Mm00432324_m1; Casp9, Mm00516563_m1; Btg2, Mm00476162_m1; BRCA1, Hs01556193_m1; BRCA2, Hs00609073_m1; ELAVL1, Hs00169152_m1; HuR/ELAVL1, Hs00171309_m1; CRABP2, Hs00275636_m1; 18S, 4352930E (Applied Biosystems). Levels of mRNAs were normalized to 18 S ribosomal RNA using the ΔΔCt method (Applied Biosystems Technical Bulletin Number 2).

Transactivation Assays—Transactivation assays were carried out as described previously (10). Briefly, cells were cultured in delipidated medium for 48 h and co-transfected with a luciferase reporter driven by a DR-5 RAR response element and a vector encoding β-galactosidase used as a transfection control. Cells were treated with RA (1 µM) for 16 h and lysed, and expression of luciferase was measured and corrected for encoding β-galactosidase.

Confocal Fluorescence Microscopy—M-2−/− cells cultured in DMEM containing 10% charcoal-treated FBS were transfected with pCMV-3′Tag-1 encoding FLAG–CRABP2. Cells were fixed in 4% paraformaldehyde, PBS; blocked; and permeabilized with PBS containing 0.2% Triton X-100 and 1% BSA (room temperature for 1 h). FLAG-tagged CRABP2 was visualized by immunostaining using antibodies against FLAG (Sigma-Aldrich, F1804). Nuclei were visualized by DAPI staining. Cells were mounted with Fluoromount-G (SouthernBiotech) and imaged using a LSM510 confocal microscope (Leica).

Animal Studies—9-week-old NCrnu/nu nude female mice were purchased from the Athymic Animal and Xenograft Core Facility of the Case Comprehensive Cancer Center and housed at the Case Western Reserve University School of Medicine.
Animal Facility in accordance with the regulations of the American Association for the Accreditation of Laboratory Animal Care. 3 × 10^6 cells in 100 µl of serum-free DMEM were injected subcutaneously. Tumor growth was measured with calipers, and tumor volumes were calculated using the following formula: (length × width^2)/2.

**Histology**—Tumors were excised, fixed in 4% paraformaldehyde, embedded in paraffin, sectioned, mounted on glass slides, and stained with hematoxylin and eosin (H&E) by the Tissue Procurement, Histology, and Immunochemistry Core Facility of the Case Comprehensive Cancer Center. Immunohistochemistry was performed using the EXPOSE rabbit-specific HRP/diamino-benzidine detection immunohistochemistry IHC kit (Abcam, ab80437). Antigen retrieval was achieved by boiling slides in 10 mM sodium citrate, pH 6.0 for 10 min. Sections were incubated with antibody against phosphorylated histone H3 (Cell Signaling Technology, 9701) at a 1:200 dilution (16 h at 4 °C). Slides were imaged using a Leica DM6000 and Volocity Acquisition software at the Imaging Core Facility of the Department of Genetics and Genome Sciences at Case Western Reserve University.

**RESULTS**

**CRABP2 and HuR Regulate a Common Cohort of Cancer-related Genes**—In some carcinoma cells that express CRABP2, RA inhibits proliferation by activating RAR and thereby inducing the expression of antiproliferative RAR target genes (11–13, 23, 24, 33). In accordance, treatment with RA

**FIGURE 1. CRABP2 and HuR regulate a common subset of genes.** A, MCF-7 cells were transduced with lentiviral particles harboring a non-targeting shRNA (shCtrl) or shRNA targeting CRABP2 (shCRABP2). Cells were selected with puromycin to generate cell lines stably expressing the respective shRNAs. Immunoblots demonstrate reduced expression of CRABP2. B, cells were cultured in delipidated medium for 48 h, treated with vehicle or RA (1 µM) for 4 days, and counted. Data are mean ± S.E. (n = 3). C and D, MCF-7 cells were transduced with lentiviral particles containing shRNAs targeting luciferase (shCtrl), CRABP2 (shCRABP2; C), or HuR (shHuR; D). Levels of mRNA for CRABP2 (C) or HuR (D) were assayed by qPCR. Data are mean ± S.E. (n = 3). *, p < 0.01 by two-tailed Student’s t test. E, Venn diagram depicting changes in gene expression in cells with reduced expression of CRABP2 and HuR. 135 genes were found to be regulated by both CRABP2 and HuR. F and G, expression profiles of genes commonly regulated by CRABP2 and HuR represented in a heat map clustering (F) or plotted as log_2(-fold change) of genes regulated by CRABP2 versus HuR (G). Error bars represent S.E.

**FIGURE 2. CRABP2 and HuR co-regulate cancer-related genes.** A, top 16 biological functions and diseases found to be significantly represented in the set of genes commonly regulated by CRABP2 and HuR. B and C, levels of denoted mRNAs in MCF-7 cells expressing shRNAs targeting luciferase (shCtrl), CRABP2 (shCRABP2; B), or HuR (shHuR; C) measured by qPCR. Data are mean ± S.E. (C, n = 3; D, n = 4). *, p < 0.01; ‡, p = 0.02 by two-tailed Student’s t test. Error bars represent S.E.
inhibited the growth of MCF-7 mammary carcinoma cells, which highly express CRABP2 (33), and decreased the expression of CRABP2 (Fig. 1A), diminishing the antiproliferative activity of RA (Fig. 1B). Interestingly, however, decreasing the expression of CRABP2 in these cells promoted cell proliferation even in the absence of RA (Fig. 1B). These observations suggest that, in addition to delivering RA to nuclear RAR, CRABP2 suppresses cell growth by an additional, RA-independent mechanism. In regard to this possibility, it was recently reported that apo-CRABP2 directly binds to the RNA-binding protein HuR, increases its affinity for some target transcripts, and thereby enhances the stability of these mRNAs and increases their expression (27). Transcriptome analyses were carried out to begin to examine whether the RA-independent growth-suppressive activity of CRABP2 may stem from its cooperation with HuR. The expression levels of CRABP2 or HuR in MCF-7 cells were reduced using respective shRNAs (Fig. 1C and D). Cells were depleted of retinoids by culturing them in charcoal-treated medium, and transcriptome analyses were carried out using Affymetrix Human Gene 2.1 ST Arrays. Decreasing the expression of CRABP2 or HuR altered the expression of 607 or 1678 mRNAs, respectively. Of these, 135 transcripts were found to be regulated in common by CRABP2 and HuR (Fig. 1E and supplemental Table S1). Array data were deposited in the Gene Expression Omnibus (GEO) database of the NCBI under accession number GSE62291. Notably, commonly regulated mRNAs were predominantly regulated in the same fashion: 93 mRNAs were down-regulated and 38 mRNAs were up-regulated in response to reducing the expression of either protein (Fig. 1F and G). Only four mRNAs were regulated by HuR and CRABP2 in opposite directions (Fig. 1F and G). Hence, a significant subset of HuR-regulated mRNAs is also targeted by CRABP2. Ingenuity Pathway Analysis revealed that many genes commonly regulated by CRABP2 and HuR are involved in regulation of oncogenic properties including cell proliferation and survival, migration, invasion, and death with
about 90 clustering as cancer genes (Fig. 2A). Validation of three genes identified by the transcriptome analysis by real time qPCR showed that mRNA for the apoptotic protein CASP7, which was shown previously to be controlled by the CRABP2-HuR complex (27), and the tumor suppressor genes BRCA1 and BRCA2 (34) were markedly down-regulated in cells with decreased expression of either CRABP2 (Fig. 2B) or HuR (Fig. 2C).

CRABP2 Inhibits Mammary Carcinoma Cell Growth by Two Distinct Mechanisms—a nuclear localization-defective CRABP2 mutant was used to assess the relative contributions of the two functions of the protein to its ability to inhibit carcinoma cell growth. This mutant, CRABP2-K20A/R29A/K30A (CRABP2\textsubscript{NLS}), binds RA with native affinity but lacks the nuclear localization signal of the protein and thus does not undergo RA-induced nuclear translocation and does not enhance the transcriptional activity of RAR (7, 27). CRABP2\textsubscript{NLS} nevertheless retains a high affinity for HuR and is indistinguishable from the WT protein in its ability to cooperate with HuR in enhancing mRNA stability (27). M-2\textsuperscript{−/−} mammary carcinoma cells, a line derived from mammary tumors that arose in the murine mammary tumor virus-neu mouse model of breast cancer bred with Crbp2-null mice (24), were used. These cells do not express CRABP2, providing a clean background for examining effects of CRABP2 on cell growth. M-2\textsuperscript{−/−} cells lines that stably overexpress a control vector encoding EGFP, EGFP-tagged CRABP2, or EGFP-CRABP2\textsubscript{NLS} were generated (Fig. 3A). Ectopic expression of CRABP2 suppressed the growth of M-2\textsuperscript{−/−} cells in the absence of RA, and notably CRABP2\textsubscript{NLS} exerted a similar effect (Fig. 3B). Hence, in accordance with its activity in MCF-7 cells (Fig. 1B), CRABP2 can suppress cell growth by an RA- and RAR-independent mechanism. Treatment of these cells with RA markedly facilitated their growth (Fig. 3B). This response reflects that, as these cells lack CRABP2 but express FABP5, RA is directed to PPAR\textbeta/\delta and thus exerts proliferative activities (3, 24). Indeed, expression of CRABP2 converted RA from a proproliferative to a growth-suppressing agent (Fig. 3B). CRABP2\textsubscript{NLS} also inhibited cell growth in the presence of RA, but it did so less efficiently than the WT protein. Interestingly, the rate of proliferation of CRABP2\textsubscript{NLS}-expressing cells in the presence of RA was similar to that of CRABP2-expressing cells devoid of RA.

To further examine the involvement of the two functions of CRABP2 in regulation of carcinoma cell growth, M-2\textsuperscript{−/−} cells that express CRABP2 or CRABP2\textsubscript{NLS} were subcutaneously injected into female NCr\textsuperscript{nu/nu} athymic mice, and tumor growth was monitored. To minimize variability between animals, each mouse was injected with the M-2\textsuperscript{−/−} cells stably expressing a control vector into one flank, and with M-2\textsuperscript{−/−} cells that stably express either CRABP2 or CRABP2\textsubscript{NLS} were injected into the opposite flank. Tumors that arose at sites injected with CRABP2-expressing cells developed at a slower rate than those that arose from control cells (Fig. 3C). Similarly to their behavior in cultured cells, CRABP2\textsubscript{NLS}-expressing cells developed tumors at an intermediate rate, displaying growth that was slower than that displayed by control cells but faster than that observed by cells that express WT-CRABP2 (Fig. 3C). Reflecting activation of RAR, expression of CRABP2 resulted in an increase in mRNA and protein of three established RAR target genes: Rarb, Casp9, and Btg2 (Fig. 3, D and E). In accordance with its inability to cooperate with RAR, CRABP2\textsubscript{NLS} did not affect the levels of these RAR targets (Fig. 3, D and E). However, both CRABP2 and CRABP2\textsubscript{NLS} up-regulated Apaf1, Elavl1, Casp7, Brca1, and Brca2, genes that are controlled by CRABP2 in conjunction with HuR (Fig. 3, F–H and Ref. 28). Taken together, these observations indicate that CRABP2 exerts anticarcinogenic activities through two distinct mechanisms, that one of these mechanisms is mediated through the ability of the protein to enhance RA-induced activation of RAR, and that the other mechanism likely emanates from up-regulation of anti-
proliferative genes brought about through the cooperation with HuR.

Histological analyses revealed that, although the general morphology of all tumors was similar (Fig. 4A), tumors that arose from cells expressing CRABP2 had fewer nuclei that were positively stained for the proliferation marker phosphorylated histone H3, whereas tumors from cells expressing CRABP2/H9004-NLS displayed an intermediate number of positive nuclei (Fig. 4A).

Tumors that arose from cells that express either CRABP2 or CRABP2/H9004-NLS similarly displayed a marked increase in cleavage of the apoptotic protein poly(ADP-ribose) polymerase (Fig. 4B). The data thus suggest that suppression of cell growth by CRABP2 is mediated both by RAR and by HuR, whereas proapoptotic activities of the protein are exerted primarily through its cooperation with HuR.

**HuR Is Required for CRABP2-mediated Activation of RAR**—Depletion of retinoids and the use of CRABP2/H9004-NLS allowed for dissection between the two functions of CRABP2 by negating its cooperation with RAR. To further examine the relative contributions of these activities, M-2−/− cell lines that express different levels of HuR in the absence or presence of ectopically expressed CRABP2 were generated (Fig. 5A). Cells were cultured in the presence of 200 nM RA, and cell growth was monitored (Fig. 5B). Ectopic expression of CRABP2 markedly suppressed proliferation. In agreement with previous reports that HuR displays antiproliferative activities (27, 35–37), decreasing the expression level of this protein enhanced cell growth. Surprisingly, however, despite the presence of RA, decreasing the expression of HuR negated the ability of CRABP2 to inhibit cell growth. Cells with reduced expression of HuR and counterparts that express CRABP2 were then injected into NCrnu/nu athymic mice, and tumor growth was monitored (Fig. 5C). Similarly to the behavior of cultured cells, ectopic expression of CRABP2 in M-2−/− cells failed to suppress tumor development from cells with a reduced level of HuR (Fig. 5C). These observations surprisingly suggest that HuR not only directly cooperates with CRABP2 in mediating growth inhibition but that its presence is also necessary for enabling CRABP2 to inhibit proliferation in
conjunction with RAR. In support of this conclusion, ectopic expression of CRABP2 had no effect on the RAR target genes Casp9 and Btg2 in tumors that arose from cells with reduced expression of HuR (Fig. 5D). Transcriptional activation assays were carried out to directly examine whether HuR affects the transcriptional activity of the CRABP2/RAR pathway. Cells that stably express different levels of CRABP2 and HuR (Fig. 5A) were transfected with a luciferase reporter driven by an RAR response element and treated with RA, and luciferase activity was measured (Fig. 5E). In control cells, RA activated the reporter, and CRABP2 enhanced the response. However, although expression of HuR was reduced in these cells by only 40–50% (Fig. 5A), the decrease inhibited RA-induced reporter activation both in the absence and presence of CRABP2.

**DISCUSSION**

CRABP2 suppresses the growth of various carcinomas, and it has been established that this activity is exerted at least in part by CRABP2-mediated direct delivery of RA to RAR, leading to induction of antiproliferative RAR target genes (11–13, 23, 24). The observations described here show that CRABP2 also exerts anticarcinogenic activities through its ability to cooperate with HuR. Transcriptome analyses revealed that, in the absence of RA, a large cohort of transcripts is regulated in common by CRABP2 and HuR (Fig. 1, E–G) and that many of these are involved in regulation of oncogenic properties (Fig. 2A). Notably, the analyses failed to identify some proapoptotic transcripts known to be regulated by the cooperation of CRABP2
and HuR such as APAF1 and CASP7 (Ref. 27 and Fig. 2B), reflecting the sensitivity limit of the method. The complete spectrum of transcripts co-regulated by CRABP2 and HuR and their involvement in cancer cell biology remain to be elucidated.

CRABP2 cooperates with HuR in the absence of RA as well as in the absence of the nuclear localization signal of the protein that is essential for enabling it to deliver RA to RAR (27). In contrast, the RAR-mediated activities of CRABP2 strictly depend on the presence of RA and on an intact ability to undergo RA-induced nuclear localization. Consequently, CRABP2 and its nuclear localization-defective mutant similarly inhibited cell growth in the absence of retinoïds (Fig. 3B), whereas in the presence of RA CRABP2 was more effective. In accordance, ectopic expression of CRABP2ΔNLS inhibited tumor growth in a xenograft mouse model, but CRABP2 was more efficient in this capacity (Fig. 3C), reflecting the additional growth-suppressing activity of RAR. Indeed, although both CRABP2 and CRABPΔNLS increased the expression of HuR target genes, only the WT protein activated RAR (Fig. 3, D–H). The data thus indicate that CRABP2 inhibits tumorigenesis both by cooperating with RAR and by enhancing HuR-mediated transcript stabilization. Notably, the data indicate that the contribution of the CRABP2/HuR pathway to the growth-inhibitory activities of CRABP2 is more substantial than that of CRABP2/RAR arm (Fig. 3B, 4B).

Surprisingly, down-regulation of HuR inhibited the transcriptional activity of RAR (Fig. 5E) and abolished the ability of CRABP2 to inhibit carcinoma cell growth (Fig. 5, B and C). The observations that HuR is critical for enabling the nuclear import of CRABP2 (Fig. 6B) and that down-regulation of this protein results in decreased expression of multiple genes involved in nuclear pore formation and in nuclear import and export (Fig. 6C) suggest a mechanism by which HuR is involved in regulating transcriptional activities. Taken together with the observations that HuR is necessary for the transcriptional activity of RAR even in the absence of CRABP2 (Fig. 5E), the data indicate that HuR does not specifically regulate the nuclear import of CRABP2 but is generally involved in regulating nuclear pore formation and nuclear entry and exit. The observations thus point at a novel function for this important protein.

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