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New ligands of the Cellular Retinoic Acid-Binding Protein 2 (CRABP2) suggest a role for this protein in chromatin remodeling

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ABSTRACT

Retinoic acid (RA) regulates the transcription of a series of genes involved in cell proliferation, differentiation and apoptosis by binding to the RA Receptor (RAR) and Retinoid X Receptor (RXR) heterodimers. The cellular retinoic acid-binding protein 2 (CRABP2) is involved in the transport of RA from the cytosol to specific RA receptors in the nucleus, acting as a coactivator of nuclear retinoid receptors. In order to have a better understanding of the role of CRABP2 in RA signaling, we used the yeast two-hybrid system as a tool for the identification of physical protein-protein interactions. Twenty-three putative CRABP2-interacting proteins were identified by screening in the presence of RA, five of which are related to transcription regulation or, more specifically, to the process of chromatin remodeling: t-complex 1 (TCP1); H3 histone, family 3A (H3F3A); H3 histone, family 3B (H3F3B); β-tubulin (TUBB) and SR-related CTD-associated factor 1 (SCAF1). These results suggest a more direct role for CRABP2 in chromatin remodeling and may be a starting point for the elucidation of the fine-tuning control of transcription by RA receptors.

Keywords: CRABP2. Saccharomyces cerevisiae and two-hybrid system.

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INTRODUCTION

The cellular retinoic acid binding protein 2 (CRABP2) is involved in the metabolism, storage and transport of retinoic acid (RA) (Dong et al., 1999; Noy, 2010). Together with retinol and retinal, RA is one of 3 related forms of vitamin A, called retinoids, which are responsible for the regulation of multiple biological processes, such as embryogenesis, apoptosis, cell proliferation and differentiation (Altucci and Gronemeyer, 2001; De Luca, 1991). Moreover, RA is used to prevent and treat several types of human cancer. Studies have demonstrated the efficacy of treating head and neck (Büntzel and Küttner, 1998), lung (Arrieta et al., 2011), skin (Niles, 2002) and breast cancers (Bryan et al., 2011) and promyelocytic leukemia (Huang et al., 2012) with RA.

RA inhibits growth by inducing cell differentiation (Rochette-Egly and Chambon, 2001), cell-cycle arrest (Donato et al., 2007) and/or apoptosis (Donato and Noy, 2005). This regulation is mediated by the nuclear receptor family, Retinoic Acid Receptor (RAR) and Retinoid X Receptor (RXR). The RAR and RXR receptors form heterodimers and bind to specific responsive elements in target gene promoters (Retinoic Acid Responsive Elements - RARE), acting as ligand-activated transcription factors (Mangelsdorf et al., 1995).

CRABP2 protein is predominantly cytoplasmic but in the presence of RA is transported to the nucleus, where it can associate with RAR (Budhu and Noy, 2002). The binding of RA to CRABP2 causes conformational changes leading to the emergence of a nuclear localization signal (NLS) recognized by importin α, the protein responsible for CRABP2 transport into the nucleus (Sessler and Noy, 2005).

The biological action of RA can be also mediated by another family of nuclear receptors, the Peroxisomal Proliferation Activated-Receptor β/δ (PPARβ/δ) (Schug et al., 2007). Like RARs, PPARs form heterodimers with RXR and bind to the PPAR Response Elements (PPRE) in regulatory regions of specific target genes (Mangelsdorf

and Evans, 1995). The activation of PPAR β/δ by RA induces the expression of pro-survival genes, resulting in stimulation of cell growth and inhibition of apoptosis (Schug et al., 2008).

Moreover, RA partition between RAR and PPARβ/δ depends on the cytoplasmic levels of two RA transport proteins: CRABP2 and Fatty Acid-Binding Protein 5 (FABP5) (Schug et al., 2007). Either CRABP2 or FABP5 can bind to RA in the cytoplasm and translocate to the nucleus, delivering RA selectively to either RAR or PPARβ/δ, respectively (Budhu and Noy, 2002; Chapellier et al., 2002).

Since the response to RA, mediated by CRABP2/RAR, is responsible for the antiproliferative effects induced by RA treatment in different types of cancer, it is very important to identify new ligands for CRABP2, to reach a better understanding of the RA signaling components necessary for an efficient RA therapy.

RESULTS AND DISCUSSION

In order to understand further the involvement of CRABP2 in the regulation of RA transcriptional activities, we performed a yeast two-hybrid screen, using a human fetal brain cDNA library and human CRABP2 as bait. Since CRABP2 undergoes structural modifications when bound to RA (Sessler and Noy, 2005), we performed a two-hybrid screen in the presence of 1 μM RA, using the Saccharomyces cerevisiae L40 strain, which harbors the two-hybrid reporter genes HIS3 and lacZ. The clones in the cDNA library that encode proteins capable of interacting with the protein of interest are identified by the ability to activate transcription of both reporters. The plasmids used in this study are listed in Table S1.

Table S1. Plasmids used in this study.

Plasmids	Description	Source
pVZ993 (pBTMKan)	yeast two-hybrid bait plasmid encoding the LexA DNA binding protein (TRP1, 2μ, kanamycinR)	Nilson Zanchin
pVZ1018 (pBTMKan-CRABP2)	CRABP2 cloned into pBTMKan	This study
pVZ278	TIF51A cloned into pBTM116	Thompson et al., 2003
pVZ549	DYS1 cloned into pBTM116	Thompson et al., 2003
pACT (pSV149)	Yeast two-hybrid prey expression vector encoding the Gal4 activation domain (AD) (LEU2, 2μ, ampicillinR)	Clontech
pACT2	yeast two-hybrid prey plasmid encoding the Gal4 activation domain (AD) (LEU2, 2μ, ampicillinR)	Clontech
pVZ940	LIA1 cloned into pACT	Thompson et al., 2003
pVZ558	DYS1 cloned into pACT	Thompson et al., 2003
pSV424 (pBTM116-PUB1)	PUB1 cloned into pBTM116	Apponi et al., 2007
pRS314 (pSV58)	Yeast centromeric cloning and expression vector (CEN, TRP1, ampicillinR)	Thompson et al., 2003

Initially, the CRABP2 coding sequence was amplified from the human fetal brain cDNA library (Clontech) by employing the oligonucleotide primers SVO 536 (5' CCG GAA TTC ATG CCC AAC TTC TCT G 3') and SVO 537 (5' CCG GAA TTC TAT CCT AGA AGG AGG G 3'), and cloned into the pBTMKan vector to obtain the bait plasmid encoding a fusion of the LexA DNA binding domain (LexA) with the entire coding sequence of CRABP2 (pBTMKan-CRABP2). Next, the L40 yeast was transformed with the pBTMKan-CRABP2 plasmid and the expression of the bait protein (LexA-CRABP2) was analyzed by immunoblotting using an anti-LexA antibody (Millipore). The L40 strain without plasmid and transformed with the empty pBTMKan plasmid were used as negative controls. The expression of the endogenous Pub1 protein was used as a load control. The LexA-CRABP2 fusion protein was expressed with the expected molecular weight of 37 kDa (Figure 1A), confirming the production of the bait protein LexA-CRABP2 by the selected S. cerevisiae transformants.

In order to check that the bait protein LexA-CRABP2 is unable to activate the reporter genes by itself (auto-activation), the L40 strain, already containing the bait plasmid, was transformed with the empty pACT vector (encoding the Gal4 activation domain - AD). The ability of three different transformants to grow on selective medium lacking histidine (HIS3 reporter) and the activity of the β-galactosidase enzyme (lacZ reporter) were tested. Interactions between proteins already characterized in our laboratory (Thompson et al., 2003) were used as controls for this system. The interaction between Dys1 and eIF5A proteins was used as a strong positive interaction control (++) and the interaction between Lia1 and eIF5A proteins as a weak positive interaction control (+), while the L40 strain transformed with pBTM-eIF5A plasmid and empty pACT vector was used as the negative control. All three transformants were unable to grow on medium lacking histidine and there was no development of blue color in the β-galactosidase assay (Figure 1B). Thus, the transformants expressing the LexA-CRABP2 fusion protein did not cause auto-activation of either reporter gene and could be used for the two-hybrid screen using pACT2 fused to the human fetal brain cDNA library encoding putative prey proteins.

A total of approximately 1.8x105 transformants were screened in this system in the presence of RA, out of which 23 clones confirmed the linkage of both His+ and β-gal+ markers and the presence of the LEU2 library plasmid. To exclude clones encoding proteins that could bind to LexA alone or directly activate transcription, we performed two additional tests. First, the isolated library plasmids were introduced individually in an L40 strain containing the pBTM-PUB1 plasmid (LexA-Pub1) (Apponi et al., 2007) instead of pBTM-CRABP2 (LexA-CRABP2). We also introduced the prey plasmids in an L40 strain containing the pRS314 plasmid, which does not encode

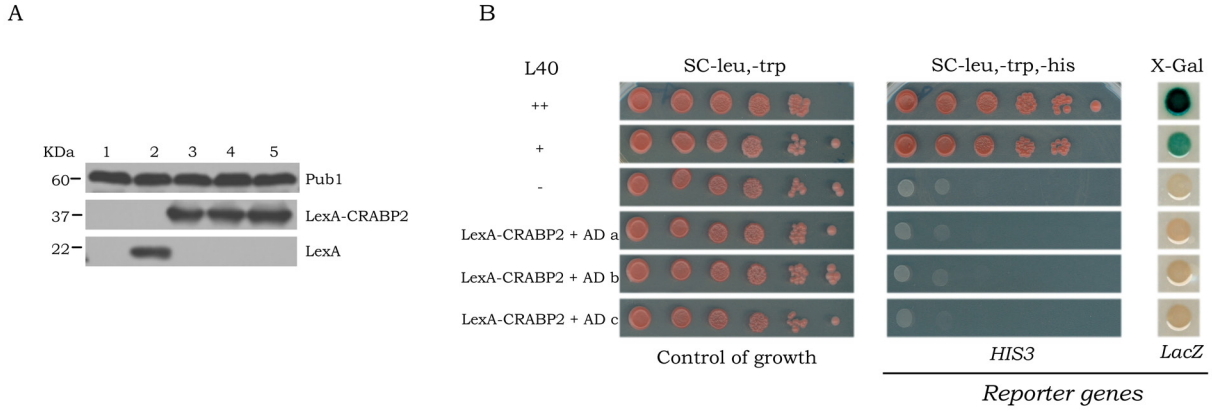

Figure 1. Confirmation of expression and absence of autoactivation by the bait protein LexA-CRABP2 used in the yeast two-hybrid screen. A: Lysates of the L40 strain without plasmid (lane 1), transformed with the empty pBTMKan plasmid (lane 2), and three different clones transformed with the pBTMKan-CRABP2 plasmid encoding the bait protein LexA-CRABP2 (lanes 3, 4 and 5) were subjected to SDS-PAGE and western blot using a polyclonal anti-LexA (1:2000) or anti-Pub1 (1:10,000). B: Yeast L40 strain transformants containing both the pBTMKan-CRABP2 plasmid and the pACT vector (transformants a, b and c) were plated on media SC-leu,-trp and SC-leu,-trp,-his and tested for β-galactosidase activity on nitrocellulose membrane. L40 transformants containing

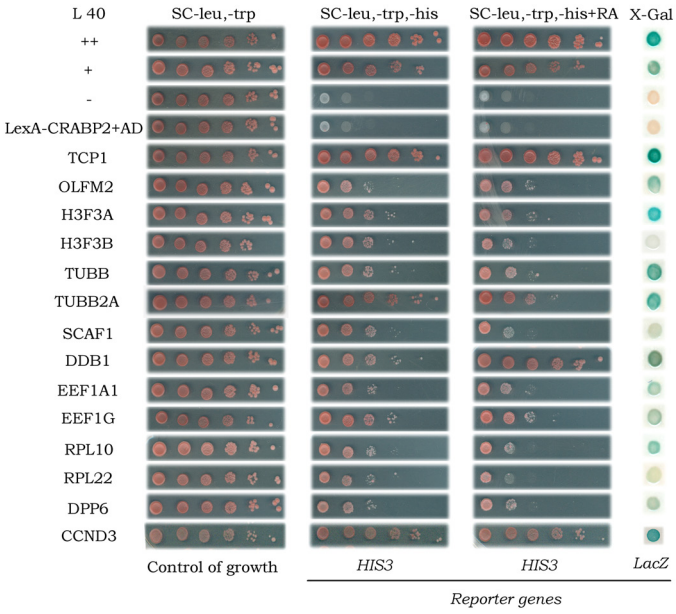

Figure 2. CRABP2 interacting proteins revealed by two-hybrid screen in the presence of RA. Serial dilution (1:10) of cultures of L40 strain containing the plasmid encoding the bait protein pBTMKan-CRABP2 and the plasmid pACT encoding the prey protein (isolated from the cDNA library) were grown in SC-leu,-trp, SC-leu,-trp,-his and SC-leu,-trp,-his+RA and tested for β-galactosidase activity. The L40 strains containing pBTM-eIF5A+pACT-Dys1 (++), pBTM-eIF5A+pACT-Lia1 (+) or pBTM-eIF5A+pACT (-) were used as strong positive, weak positive or negative controls, respectively.

for LexA or the bait protein LexA-CRABP2. Only one of the candidate clones did not pass these tests and was excluded from our analysis (Figure S1).

The selected clones were then sequenced and subjected to DNA sequence alignment. The 22 clones obtained in this screen revealed 14 ligands of CRABP2: β-tubulin (TUBB), t-complex 1 (TCP1), tubulin beta 2A chain (TUBB2A), eukaryotic translation elongation factor 1 gamma (EEF1G), olfactomedin 2 (OLFM2), damage-specific DNA binding protein 1 (DDB1), eukaryotic translation elongation factor 1 alpha 1 (EEF1A1), H3 histone, family 3A (H3F3A), H3 histone, family 3B (H3F3B), SR-like CTD-associated factor 1 (SCAF1), ribosomal protein L10 (RPL10), ribosomal protein L22 (RPL22), dipeptidyl-peptidase 6 (DPP6) and cyclin D3 (CCND3) (Table 1).

Table 1. New ligands of CRABP2 revealed by yeast two-hybrid screen.

Number of clones	Gene name	Description
1	CCND3	cyclin D3
2	TCP1	t-complex 1
2	TUBB2	beta 2A tubulin
6	EEF1G	eukaryotic translation elongation factor 1 gamma
1	OLFM2	olfactomedin 2
1	DDB1	damage-specific DNA binding protein
2	TUBB	beta tubulin
1	H3F3B	H3 histone, family 3B
1	EEF1A1	eukaryotic translation elongation factor 1alpha 1
1	H3F3A	H3 histone, family 3A
1	SCAF1	SR-like CTD-associated factor 1
1	RPL10	ribosomal protein L10
1	RPL22	ribosomal protein L22
1	DPP6	dipeptidyl-peptidase 6

The plasmid linkage test for one representative clone of each of the 14 ligands is shown in Figure 2. All clones were positive in the β-galactosidase assay. Although most clones showed a decrease in growth in the presence of RA, this is unlikely to be due to toxicity, since the positive and negative controls did not show any alteration in growth and one of the clones (DDB1) showed increased growth in the presence of RA. Thus, these results are more likely to reflect some specificity of the ligand towards the bound or unbound forms of CRABP2.

The protein cyclin D3 (CCND3) is among the ligands identified in this screen and this protein has already been described as a ligand of CRABP2 (Despouy et al., 2003), validating the CRABP2 screen used in this study. Moreover, two-hybrid experiments performed in our laboratory have also shown interactions of CRABP2 with Ubc9 and SUMO (Brantis, C.E., unpublished data). Both Ubc9 and SUMO have been described previously as physical partners of CRABP2 (Majumdar et al., 2011; Tatham et al., 2011), confirming that the bait protein LexA-CRABP2 used in this screen is functional and able to reveal new CRABP2 ligands.

Interestingly, five of the ligands revealed in this CRABP2 screen are proteins involved in transcriptional regulation, more specifically in the process of chromatin remodeling: TUBB, H3F3A, H3F3B, TCP1 and SCAF1.

The TUBB gene encodes β-tubulin, which, besides being a component of microtubules, has been observed to interact with nuclear receptor coactivator 6 (NCOA6) (Goo et al., 2003). These proteins are part of two protein complexes, ASCOM and ALR/MLL2, which possess H3K4 methyltransferase activity and are involved in transcriptional activation of RAR receptor (Goo et al.,

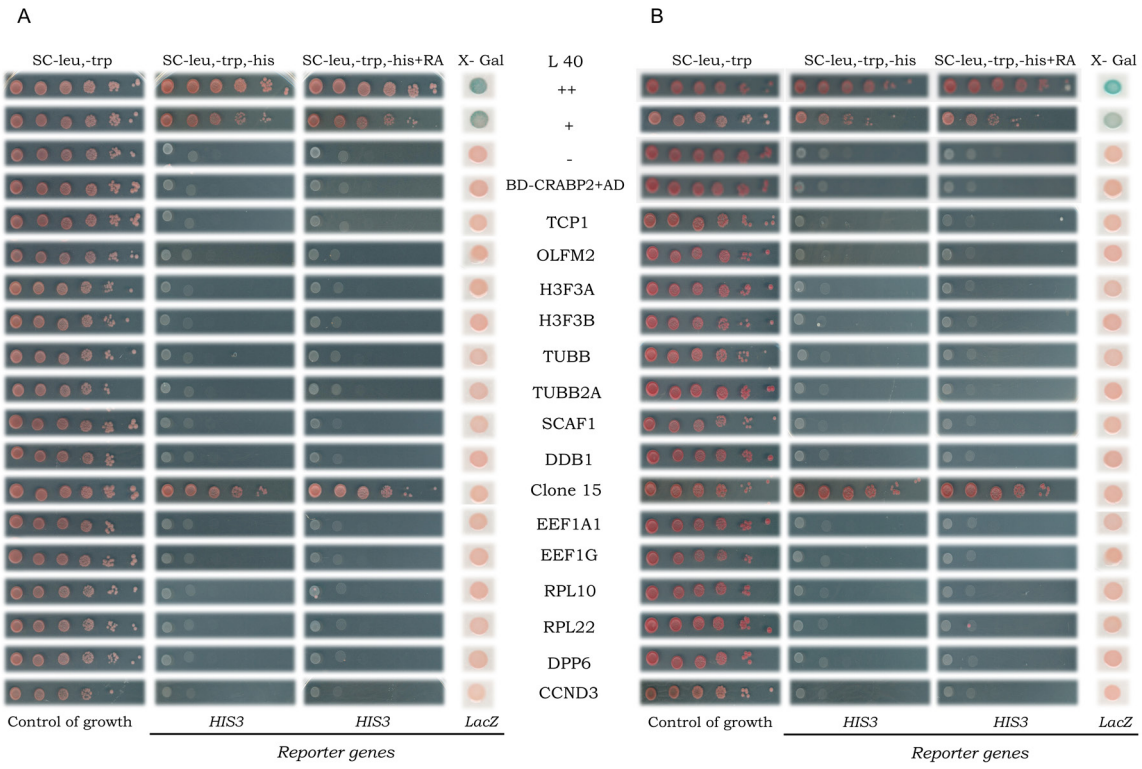

Figure S1 - Test of specificity of the new ligands revealed in the yeast two-hybrid screen.

2003; Issaeva et al., 2007). It has been demonstrated that nuclear tubulin interacts specifically with histone H3, suggesting that the recruitment of the ASCOM complex to target DNA response elements could be facilitated by the tubulin-histone H3 interaction (Akoumianaki et al., 2009). Accordingly, histone H3 (H3F3A and H3F3B) also emerged as ligands of CRABP2 in this study. Thus, the interaction of CRABP2 with TUBB and histone H3, together with the specific interaction of nuclear tubulins with histone H3 (Akoumianaki et al., 2009) and the participation of tubulin in the transcriptional coactivator complex of RAR (Goo et al., 2003), suggest an involvement of a CRABP2-tubulin-histone H3 complex in the transcription regulation of RA-target genes.

TCP1 is a molecular chaperone and is part of a eukaryotic chaperonin complex, known as TRiC/CCT (TCP1-ring complex or chaperonin-containing TCP1 complex) (Lewis et al., 1992). Initially, it was proposed that this complex is involved only in the folding of tubulin and actin into their functional structure (Sternlicht et al., 1993), but many other proteins have now been identified as substrates for this complex, such as the histone deacetylase 3 enzyme (HDAC3) (Guenther et al., 2002). Besides that, HDAC3 is activated by forming a stable complex with the RAR corepressor protein, SMRT/N-CoR (Wen et al., 2000; Guenther et al., 2001). The inactive HDAC3 is associated primarily with the TRiC/CCT complex, which allows the interaction with SMRT/N-CoR and activation of HDAC3 (Guenther et al., 2002). Thus, the interaction between CRABP2 and TCP1, and the participation of the TRiC/CCT complex in the activation of HDAC3, further suggest an involvement of CRABP2 in the control of transcriptional repression of RA-target genes.

Another CRABP2 ligand revealed in this screen was SCAF1, a serine arginine-rich pre-mRNA splicing factor SR-A1 (Scorilas et al., 2001). The SCAFs (SR-like CTD-associated factors) belong to the SR-related protein family, which are characterized as containing a conserved domain that interacts with the C-terminal domain (CTD) of RNA polymerase II (Corden and Patturajan, 1997). It has been shown that the C-terminal CTD-interacting domain of SCAF1 can bind either to partially or highly phosphorylated RNA pol II; for this reason, it has been proposed that this protein may be a link between transcription and pre-mRNA processing (Yuryev et al., 1996).

From the ligands found in this two-hybrid screen, a model may be proposed in which CRABP2 plays a more active role in transcription mediated by RAR, instead of acting merely to increase RA transport and its load to RAR (Figure 3). In the absence of retinoic acid, RAR/RXR heterodimers associate with a corepressor complex that includes the HDAC enzyme, inducing chromatin condensation and repression of transcription. CRABP2 could act in the inactivation of this corepressor complex,

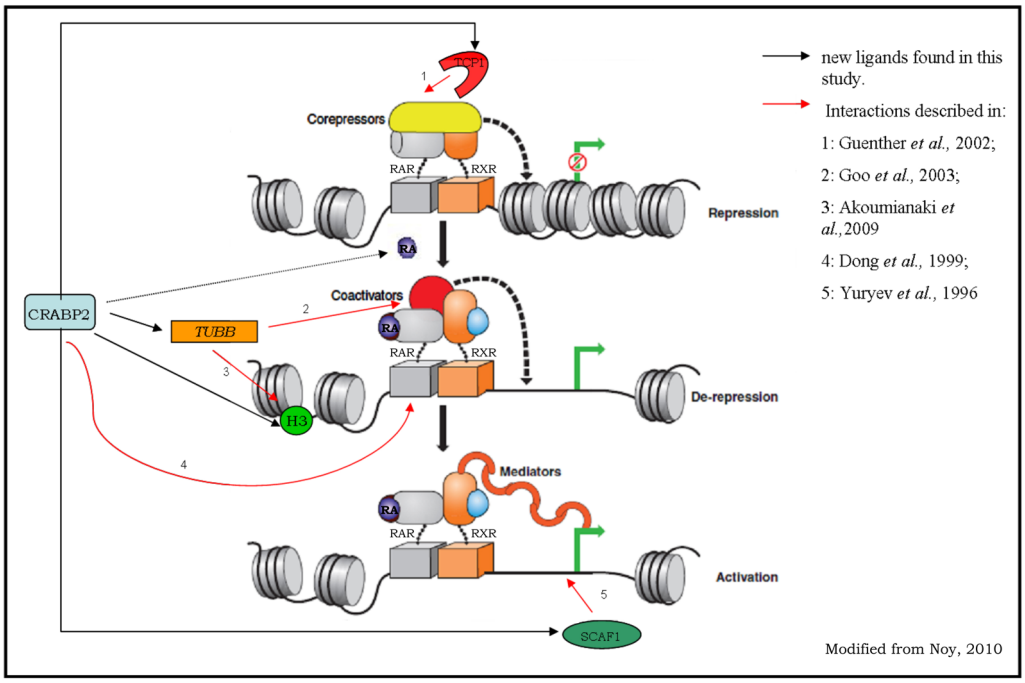

Figure 3. CRABP2 in the context of the new ligands revealed in the two-hybrid screen. Model of action for CRABP2 in chromatin remodeling, including the new ligands found in this study. TCP1 is a chaperone and member of the TCP1 ring complex involved in the activation of the HDAC3 enzyme, which is part of the corepressor complex of nuclear receptor RAR. The TUBB gene encodes β-tubulin, which physically interacts with a coactivator of nuclear receptor RAR and histone 3 in the nucleus. SCAF1 binds to either highly or partially phosphorylated RNA polymerase II.

through its interaction with TCP1, an activator of the HDAC3 enzyme (Guenther et al., 2002). Upon binding to RA, the nuclear receptors recruit a coactivator complex, which contains the histone acetylase transferase activity required for transcriptional activation of retinoid-responsive genes (reviewed by Noy, 2010). CRABP2 may also interact with TUBB and histone H3, facilitating the recruitment of the coactivator complex to target DNA response elements, since TUBB is part of a co-activator complex (Goo et al., 2003) and also interacts with histone H3 (Akoumianaki et al., 2009). At the same time, as SCAF1 interacts with RNA pol II, CRABP2 may also help to mediate the recruitment of RNA pol II through its interaction with SCAF1, leading to induction of transcription by the RAR receptor (Figure 3).

CONCLUSION

In summary, by using the yeast two-hybrid system, we have identified new ligands for CRABP2 that suggest a role for this protein in chromatin remodeling. Thus, CRABP2 may participate more actively in the control of transcription by RA. Further analysis will help to clarify the mechanism by which CRABP2 modulates transcription.

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