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HACE1: A Novel Repressor of RAR Transcriptional Activity

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Abstract

The diverse biological actions of retinoic acid (RA) are mediated by RA receptors (RARs) and retinoid X receptors (RXRs). While the coregulatory proteins that interact with the ligand-dependent AF-2 in the E region are well studied, the ligand-independent N-terminal AF-1 domain-interacting partners and their influence(s) on the function of RARs are poorly understood. HECT domain and Ankyrin repeat Containing E3 ubiquitin-protein ligase (HACE1) was isolated as a RARβ_3 AB region interacting protein. HACE1 interacts with RARβ_3 both in *in vitro* GST pull-down and in cell-based coprecipitation assays. The interaction sites map to the N terminus of RARβ_3 and the C terminus of HACE1. HACE1 functionally represses the transcriptional activity of RARα_1, RARβ isoforms 1, 2 and 3, but not RARγ_1 in luciferase reporter assays. In addition, HACE1 represses the endogenous RAR-regulated genes CRABP II, RIG1 and RARβ_2, but not RAI3 in CAOV3 cells. Mutation of the putative catalytic cysteine (C876 of LF HACE1), which is indispensable for its E3 ubiquitin ligase activity, does not alter the repressive effect of HACE1 on the transcriptional activity of RARβ_3. On the other hand, HACE1 inhibits the RA dependent degradation of RARβ_3. It is possible that the repression of RAR-regulated transcription by HACE1 is due to its ability to inhibit the RA-dependent degradation of RARs.

Keywords

retinoic acid; RAR; HACE1; transcription; AF-1

Introduction

The biological function of retinoic acid (RA) is mediated by specific nuclear receptors termed retinoic acid receptors (RARs) and retinoid X receptors (RXRs). Like all members of the steroid/thyroid hormone nuclear receptor superfamily, RARs and RXRs share a common architecture consisting of five or six structurally and functionally distinct regions, termed A to F. Briefly, the N-terminal AB region is the least conserved and contains a ligand-independent transcriptional activation function (AF-1). The C region is the most conserved and contains the DNA binding domain (DBD) that is responsible for binding to the retinoic acid response element (RARE) located in the promoter regions of RAR-regulated genes. Region D represents the hinge that connects regions C and E. Region E is the second most conserved and contains the ligand binding pocket, a dimerization surface, a ligand-dependent transactivation function (AF-2) and binding surfaces for coregulatory proteins.

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The C-terminal region F is absent in RXR and its function in RAR is unknown. [For review, see Chambon, 1996]

The transcriptional activity of RAR is dependent on both the AB region (AF-1) and the E region (AF-2), however the role of only the E region is well understood. In the absence RA, AF-2 recruits corepressors such as NCoR/SMRT that help to maintain the chromatin in a compacted state due to their histone deacetylase activity. Upon RA binding to the ligand binding pocket, a major conformational change occurs in the ligand binding domain causing the release of corepressors and recruitment of coactivators such as SRC, CBP and SWI/SNF. Coactivators function to decompact chromatin by histone modification or shifting of nucleosomes. In addition, some coactivators can interact with certain general transcriptional factors [For review, see Bastien and Rochette-Egly, 2004]. Once repressive chromatin has been decompacted, it has been proposed that coactivators dissociate and general transcription factors including PolII associate with the promoter regions of target genes and initiate their transcription [Dilworth and Chambon, 2001].

In contrast to AF-2, the amino terminal AB regions of RARs are very variable in both size and amino acid sequence along with lacking predictable secondary structures. The mechanism underlying the regulation of transcription by AF-1 is poorly understood. It has been hypothesized that the unfolded AB regions of nuclear receptors can adopt the correct functional secondary structures in the presence of specific interacting proteins by the induced fit model [For review, see Warnmark et al., 2003; Kumar and Thompson, 2003]. Limited studies with the AB regions of RARs indicate that their function can be modulated by modifications such as phosphorylation and by binding interacting proteins. Phosphorylation of serine residues buried in the proline rich region is important for the transcriptional activity of RARγ_2 and RARα_1 [Rochette-Egly et al., 1997; Bastien et al., 2000; f et al., 2002a; Gianni et al., 2002b]. In addition, two AB region interacting proteins, Acinus S' and Vinexin β, repress the transcriptional activity of RARs [Vucetic et al., 2008; Bour et al., 2005].

HECT domain and Ankyrin repeat Containing E3 ubiquitin-protein ligase (HACE1) was initially identified as a novel E3 ubiquitin ligase whose expression is greatly reduced in sporadic Wilms' tumors [Angelesio et al., 2004]. Cys 876 is reported to function as the catalytic cysteine residue and UbcH7 as the partner E2 enzyme in *in vitro* ubiquitination assays [Angelesio et al., 2004]. More recently, HACE1 was reported to be a tumor suppressor [Zhang et al., 2007]. Genetic inactivation of HACE1 in mice resulted in the development of spontaneous, late-onset tumors. Knockdown of HACE1 expression by short hairpin RNAs in HEK293 cells resulted in increased colony formation in soft agar and a marked increase in tumorigenicity *in vivo*.

Based on the hypothesis that AF-1 interacting proteins can modulate the function of RARs, we isolated HACE1 as an AB region-interacting protein in a yeast two-hybrid screen. In the current study, we have demonstrated an interaction between HACE1 and RARβ_3 by both *in vitro* and cell based assays. HACE1 represses RAR-dependent transcription of a RARE-driven reporter gene and several endogenous RAR-regulated genes. Finally, HACE1 inhibits the RA-regulated degradation of RARβ_3. It is possible that the repression of RAR-regulated transcription by HACE1 is due to its ability to inhibit the RA-dependent degradation of RARs.

Materials and Methods

Reagents

RA powder was a generous gift from Hoffmann-LaRoche. Ciglitazone was purchased from Cayman Chemicals, G418 from Sigma, trichostatin A (TSA) from Cayman Chemicals, and cycloheximide from Alexis Biochemicals.

Plasmid constructs

Human HACE1 constructs used are Long Form (LF) HACE1 in pCMX-XL4 (purchased from Origene), LF HACE1 in Invitrogen destination vectors including pcDNA3.1/nV5-DEST (5′ V5), pDEST27 (5′ GST mammalian expression) and pDEST15 (5′ GST bacterial expression), Short Form (SF) HACE1 (obtained as a generous gift from Kazusa Research Institute, Japan) in pcDNAhisC and pGEX-KG. RARβ_3 constructs include RARβ_3 in pOPRSVICAT, pET29a, pDEST27 and pcDNA3.1/nV5-DEST. All RAR constructs used in GST pull down assays were in pET29a. All RARβ constructs used in transactivation assay were in pOPRSVICAT, RARα_1 and RARγ_1 were in pSG5. Estrogen receptor (ER)α, thyroid receptor (TR)α and peroxisome proliferative activated receptor (PPAR)γ in pCMX were a generous gift from Dr. Ronald Evans, Salk Institute. Mutation of Cys 876 to Ala in LF-HACE1 and Cys 529 to Ala in SF-HACE1 was performed using the Quik-Change Kit from Stratagene.

Cell culture

Cos1, NIH3T3 and CAOV3 cells were cultured in DMEM medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 μg/ml penicillin, and 100 units/ml streptomycin. The cells were maintained in an incubator at high humidity, 5% CO2, and 37°C. Stable transfection of CAOV3 cells was performed using the calcium phosphate method as previously described [Ravikumar et al., 2007] using LF HACE1 in pcDNA 3.1/nV5-DEST. Colonies were selected by G418. Individual colonies were isolated and screened by western blot for V5-HACE1 expression using V5 antibody. For electroporation of CAOV3 cells, 6×10^6 cells were resuspended in electroporation buffer EmbryoMax (Chemicon) containing 10 μg V5-LF HACE1 expression plasmid DNA and transferred into an electroporation cuvette (4 mm). The cells were electroporated using GenePulse/Xcell (Biorad) with the following parameters: 1000μF, 230v. Cells were placed in fresh medium and incubated at 37°C with 5% CO2 and humidity overnight. The culture medium was replaced the next day and the cells were treated with ethanol or 1 μM RA, and RNA were extracted after 16 hr. The efficiency of electroporation was determined by immunohistochemistry using V5 antibody.

GST pull-down assays

Glutathione S-transferase (GST) pull-down assays were performed essentially as previously described [Vucetic et al., 2008]. GST–LF HACE1 (amino acids 1 to 909), GST–SF HACE1 (amino acids 1 to 562), GST–AR (amino acids 1 to 358 of LF), GST–SFNT (amino acids 1 to 356 of SF) and GST–SFCT (amino acids 356 to 562) were expressed in BL21-DE3 *E. coli* cells and purified using glutathione-agarose beads (Sigma). Full-length mouse RARα_1, RARβ_1, RARβ_2, RARβ_3, RARβ_4, RARγ_1, RXRα, RARβ deletion mutants including RARβ C-F (amino acids 115-482 of RARβ_3) RARβ D-F (amino acids 181-482 of RARβ_3); RARβ_1 AB (amino acids 1 to 101); RARβ_2 AB (amino acids 1 to 94); RARβ_3 AB (amino acids 1 to 118); RARβ_4 A-D (amino acids 1 to 141); RARβ_3 A-C (amino acids 1-168); RARβ_3 A-D (amino acids 1 to 224), ERα, TRα, and PPARγ were *in vitro* transcribed and translated using TnT kit (Promega) and [^{35}S]-methionine (1,175 Ci/mmol; Perkin-Elmer Life and Analytical Sciences) following the manufacturer's protocol.

Transactivation assays

Transactivation assays were performed as previously described [Vucetic et al., 2008]. Cos1 cells were transfected using GenJet (Genscript) with 0.1 μg RARE-Luc reporter DNA (Panomics), 0. 01 μg pRL DNA (Promega); 0.3 μg RAR expression construct DNA; and 3 μg pCMX-XL4-LF HACE1 DNA or pcDNA3.1/His-SF HACE1 DNA or empty vector DNA. For the SP1 and PPARγ transactivation assays, the transfections were performed with 0.1μg SP1-Luc reporter DNA (Panomics) or PPARγ-Luc reporter DNA (Panomics), 0.01 μg pRL DNA, 0.3 μg RARβ$_3$ expression construct DNA (for SP1 assay) or pCMX-PPARγ DNA (for PPARγ assay), and 3 μg pCMX-XL4-LF HACE1 DNA or empty vector DNA. Twenty-four hr after transfection, cells were treated with 1 μM RA, 1 μM ciglitazone, 100 nM TSA, or ethanol carrier for 24 hr and then harvested. For each experiment, the firefly luciferase activity was normalized to renilla luciferase activity. The change in normalized firefly luciferase activity was calculated relative to cells that were transfected with empty vector DNA and treated with ethanol, which was set as 1 arbitrarily. Values are the mean +/- standard deviation of three independent experiments assayed in triplicate. P values were generated using pairwise student T test.

RNA isolation and real-time PCR

For quantitative reverse transcription-PCR (RT-qPCR), CAOV3 cells were treated with ethanol or 1 μM RA for 16 hr. Total RNA was isolated using RNA-Bee RNA isolation reagent (Tel-Test, Inc.) according to the manufacturer's instructions. One microgram of total RNA was used in the reverse transcription reaction with oligo(dT) primers supplied in the Advantage RT-for-PCR kit (Clontech). Subsequently, 10 μl of the RT reaction mixture was used for quantitative real-time PCR using SYBR green PCR chemistry (Applied Biosystems) according to manufacturer's instructions. Specific PCR primers were synthesized (IDT) and optimized for amplification of each gene. Changes in gene expression were calculated using relative quantitation of each target against the endogenous glyceraldehyde-3-phosphate dehydrogenase (GAPDH) standard. The cycling parameters were 95°C for 10 min and then 40 cycles of 95°C for 45 sec, 55°C for 45 sec, and 72°C for 90 sec. Values are the mean +/- standard deviation of triplicate samples. Primers are: RARβ, GTCACCGAGATA AGA ACTGTGTTA and ACTCAGCTGTCATTTCATAGCTCTC; RAI3, TGCTCACAA AGCAACGAAAC and TGGTTCTGCAGCTGAAAATG; RIG1, GAGATTTTCCGCCTTGGCTAT and CCGTTTCACCTCTGCACTGTT; CRABPII, CCCGAATTCATGCCCAACTTCTCTGG and AGTGGATCCTCACTCTCGGACGTAGA; HPRT, TTCTTTGCTGACCTGCTGG and TCCCCTGTTGACTGGTCAT; GAPDH, AGAAGACTGTGGATGGCCCC and AGGTCCACCACCCTGTGGC.

Coprecipitation

Cos1 cells were cotransfected with V5-HACE1 or GST-HACE1 expression plasmid DNA, GST-RARβ$_3$ or V5-RARβ$_3$ plasmid DNA, RXRα-pSG5 DNA and RARE-luc DNA by GenJet transfection method (Genscript). Twenty-four hr after transfection, cells were treated with 1 μM RA for an additional 24 hr. Whole cell protein extracts were prepared and 50% glutathione beads were added to the whole cell lysate and incubated overnight at 4°C. The beads were washed five times with TNE buffer (0.05M Tris-HCl pH8.0, 0.15M NaCl, 1% NP40, 2mM EDTA pH 8.0). Proteins were released from beads and resolved on 9% SDS-PAGE. Western blots were performed using anti-V5 or anti-GST as primary antibodies, donkey anti-mouse IRdye 800CW or donkey anti-rabbit IRDye 680CW as secondary antibodies, and detected using a LI-COR Odyssey instrument.

Protein stability assay

Cos1 cells were cotransfected with V5-LF HACE1 DNA or empty vector DNA, V5-RARβ_3 DNA, RXRα-pSG5 DNA and RARE-luc DNA by GenJet transfection method. Forty-eight hr after transfection, cells were treated with 10 μg/ml cycloheximide and 1μM RA or ethanol. Whole cell extracts were made at 0, 2, 4, 8 and 16 hr after treatment. The cell lysates obtained at various time points were resolved on a 9% SDS-PAGE gel. Western blots were performed using anti-V5 or anti-GAPDH primary antibodies, donkey anti-mouse IRdye 800CW or donkey anti-rabbit IRDye 680CW secondary antibodies, and detected using a LI-COR Odyssey instrument. The levels of V5-RARβ_3 and V5-LF HACE1 were quantitated using Odyssey software. The half-life of the protein (50% of protein at time 0) was calculated using linear equations generated from the quantitated protein density over time. Values are the mean +/- standard deviation of three independent experiments. P values were generated using pairwise student T test.

Results

Identification of HACE1 as a RARβ_3 AB Domain Interacting Protein

In order to identify proteins that interact with and potentially regulate the activity of the N-terminus of RARs, a yeast two-hybrid screen of an 11-day mouse embryo cDNA library was performed using the A region and first seven amino acids of the B region of RARβ_3. Among the positive clones isolated in the yeast two-hybrid screen, we identified a clone that displays 100% sequence identity to nucleotides 2624-3050 of *Mus musculus* HACE1 (NM_172473) cDNA. This clone encodes the 98-carboxyl terminal amino acids of HACE1 protein (amino acids 811-909) including the putative catalytic cysteine residue (C876) in the HECT domain [Anglesio et al., 2004] (Figure 1A).

Both HACE1 protein and cDNA sequences are highly conserved between *Mus musculus* (NM_172473) and *Homo sapiens* (NM_020771). Their amino acid and nucleotide sequences display 97.3% (884 /909 amino acids) and 91.2% (2490 /2729 nucleotides) sequence identity, respectively. The carboxyl terminal region of human and mouse HACE1 protein corresponding to the region deduced from the yeast two-hybrid clone shows 100% amino acid sequence identity. GenBank contains sequences for two isoforms of *Homo sapiens* HACE1. Human Long Form (LF) HACE1 cDNA (NM_020771) is 4614 base pairs and encodes a 909 amino acid protein and human Short Form (SF) HACE1 cDNA is 5321 base pairs and encodes a 562 amino acid protein. Both transcripts are generated from chromosome 6 at 6q21 presumably by differential promoter usage. The two isoforms of HACE1 share both the HECT domain in the carboxyl terminal end (amino acids 555-909 of LF and amino acids 208-562 of SF) and the functionally unknown region (amino acids 12-207 of SF and amino acids 360-554 of LF). However, these two isoforms differ at their amino terminal ends. Amino acids 1-359 of LF HACE1 contain a region with six ankyrin repeats (amino acids 66-217) while amino acids 1-11 of SF HACE1 have no known functional domain (Figure 1A).

Interaction between HACE1 and RARs

To confirm the physical interaction between HACE1 and RARβ_3, we performed an *in vivo* coprecipitation assay in Cos1 cells. Figure 1B demonstrates that V5-RARβ_3 associates in vivo with GST-LF HACE1 but not with GST alone. Similarly, V5-LF HACE1 associates in vivo with GST-RARβ_3 but not with GST alone.

To further study the physical interaction between HACE1 and other nuclear receptors, we performed *in vitro* GST pull-down assays. As seen in Figure 1C, RARβ isoforms 1, 2 and 3 specifically bound both full length GST-SF HACE1 and GST-LF HACE1 but not GST

alone, while RARβ$_4$ did not specifically interact with either GST-SF HACE1 or GST-LF HACE1. As expected for a negative control, luciferase did not interact with full length GST-SF HACE1, GST-LF HACE1 or GST alone. In addition, GST-SF HACE1 interacts with RARα$_1$, RARγ$_1$, ERα and TRα but not with RXRα$_1$ and PPARγ (Figure 1D). Finally, the interaction between RARβ$_3$ and SF HACE1 is not affected by the RAR ligand, RA (data not shown).

Mapping of the Interaction Site between HACE1 and RARβ$_3$

We further mapped the interaction site on both HACE1 and RARβ$_3$ by performing GST pull-down assays using GST-tagged full length and portions of HACE1 (Figure 1A) and [^{35}S]-methionine labeled full length and portions of RARβ (Figure 2A). As seen in Figures 1C & 1E, AR (amino acids 1-358 of LF HACE1) and SFNT (amino acids 1-356 of SF HACE1) do not appear to interact with any of the isoforms of RARβ, while SFCT (amino acids 356-562 of SF HACE1) interacts with RARβ isoforms 1, 2 and 3 with similar intensity. Taken together these data suggest that only the C terminus of HACE1 (amino acids 356-562 of SF HACE1, amino acids 703-909 of LF HACE1) interacts with RARβ. This is consistent with the fact that the RARβ$_3$ interacting clone identified in the yeast two-hybrid screen encodes the carboxyl terminal region of LF HACE1 (amino acids 811-909).

Figure 2 demonstrates that the A-D regions of RARβ$_3$ (panel B) as well as A-C regions of RARβ$_3$ (panel C) interact with SF HACE1 while neither C-F regions nor D-F regions of RARβ interact with SF HACE1 (panel E). However, the AB regions of RARβ$_1$, RARβ$_2$ and RARβ$_3$ showed weak or no interaction with SF HACE1 (panel D). Taken together, the N terminus (A-C regions) of RARβ$_3$ contains the interacting site however the C region in the C-F regions of RARβ does not interact with SF HACE. This is consistent with the fact that the A/partial B regions were originally used as the bait in the yeast two-hybrid screen. Taking into consideration the fact that the AB regions are a small peptide whose conformation may not be optimal for interaction with HACE1 when fused to the C terminal end of GST, the interaction site most likely resides in the AB regions and the additional amino acids in the C region (GST-RARβ$_3$ A-C) or GAL4 DBD (yeast two-hybrid bait) may allow the AB region to fold properly.

Effect of HACE1 on RAR/RARE Dependent Transcription

To test the functional role of the HACE1-RAR interaction, we first assessed the effect of HACE1 on the transcriptional activity of RAR using a luciferase reporter which is controlled by a minimal TA promoter and DR5 type RARE in Cos1 cells. LF HACE1 caused a repression in the transcriptional activity of both endogenous RARs and exogenous RARβ$_3$ when the cells were treated with RA (Figure 3A & 3B). This repression of RARβ$_3$ transcriptional activity was dependent on the amount of HACE1 expression plasmid DNA transfected into the cells, with the highest amount of LF HACE1 DNA resulting in a ~80% repression of RARβ$_3$ transcriptional activity over mock control (Figure 3A).

In addition, we chose three luciferase reporter controls (SP-1 luc, PPRE-luc and empty luc) to examine the specificity of the effect of LF HACE1 on RAR-dependent transcription. As seen in Figure 3B, LF HACE1 has no effect on SP1-dependent, PPARγ-dependent and empty luciferase reporter (data not shown) transcriptional activity in Cos1 cells. In addition to Cos1 cells, transactivation assays were also performed in NIH3T3 cells (Figure 3B). We also observed that LF HACE1 repressed the transcriptional activity of both endogenous RAR and exogenous RARβ$_3$ with ~50-60% repression over mock control. On the other hand, LF HACE1 had little effect on the transcriptional activity of PPARγ and SP1. Taken together these results demonstrate that LF HACE1 acts as a specific repressor of RAR/RARE dependent transcription.

Finally, we also examined the effect of LF HACE1 and SF HACE1 on the transcriptional activity of other RAR subtypes including RARα_1, RARβ isoforms 1-3 and RARγ_1 using the transcriptional transactivation assay (Figure 3C). Both LF HACE1 and SF HACE1 repressed the transcriptional activity of RARα_1 and RARβ isoforms 1-3 significantly; however they had no effect on RARγ_1 transcriptional activity.

Analysis of RAR-Regulated Gene Expression in HACE1-Expressing CAOV3 Cells

We stably overexpressed V5-LF HACE1 in CAOV3 cells (see Supplemental Figure 1) and examined the effect of LF HACE1 expression on the mRNA levels of endogenous RAR-regulated genes including cellular retinoic acid binding protein (CRABP) II, retinoid inducible gene (RIG) 1, RARβ_2 and retinoic acid induced (RAI) 3. All four genes contain a functional DR5 RARE in their promoter region [Astrom et al., 1994; Tao et al., 2004; Jiang et al., 2005; Zelent et al., 1991]. As seen in Figure 4, left panel, overexpression of LF HACE1 repressed the RA-dependent induction of CRABP II, RIG1 and RARβ_2 mRNA levels in all five independently isolated HACE1 overexpressing CAOV3 clones (H1, H4, H9, H11 and H14) but not that of RAI3. No change was observed in the mRNA level of the control housekeeping gene hypoxanthine phosphoribosyltransferase (HPRT).

In addition, the effect of LF HACE1 on RAR regulated gene expression was measured in CAOV3 cells transiently expressing V5-LF HACE1. Electroporation efficiency was measured by immunohistochemistry using V5 antibody. Approximately 90% of the cells were positive for V5-LF HACE1 expression. Similar to that observed in CAOV3 cells stably expressing HACE1, transient expression of HACE1 repressed the RA-dependent induction of CRABP II, RIG1 and RARβ2 in HACE1 overexpressing CAOV3 cells after 16 hr RA treatment, but not that of RAI3 (Figure 4, right panel). No change was observed in the mRNA level of the control housekeeping gene HPRT.

How HACE1 Functions as a RAR Transcriptional Repressor

Since HACE1 was reported to be an E3 ubiquitin ligase, we asked whether its function as an E3 ubiquitin ligase plays a role in mediating repression of RAR transcriptional activity. We mutated the catalytic cysteine residue to alanine in SF HACE1 (Cys529) and LF HACE1 (Cys876) (Anglesio et al., 2004), and examined the ability of Cys529Ala SF HACE 1 to bind RARβ_3 and Cys876Ala LF HACE1 to repress RARβ_3–dependent transcription. As seen in Figure 5A and 5B, both wild type HACE1 and the putative catalytic cysteine mutants of HACE1 interacted similarly with RARβ_3 and displayed a similar level of repression of RARβ transcriptional activity. This suggests that the effect of HACE1 on RARβ transcriptional activity is not mediated by the catalytic cysteine residue associated with its E3 ubiquitin ligase activity.

We next asked whether HACE1 represses the transcriptional activity of RARs by deacetylation of histone proteins. Since TSA is a commonly used histone deacetylase inhibitor, we asked whether TSA treatment could relieve HACE1 repressive effect on the transcriptional activity of RARβ_3. As seen in Figure 5C, treatment with TSA increases RARβ_3 transcriptional activity in the presence and absence of LF HACE1, however LF HACE1 represses the activity of RARβ_3 activity with same level of repression (80%) both in the presence and in the absence of TSA. This suggests that the repressive effect of HACE1 on the transcriptional activity of RARβ_3 was not due to histone deacetylation.

RARs are degraded by the proteasome system in response to retinoids and RARγ_2 degradation is indispensable for the induction of RARγ_2 transcriptional activity [For review, see Bour et al., 2007]. We therefore asked whether LF HACE1 affects RARβ_3 transcriptional activity by modulating RARβ_3 protein stability. As expected, in the absence

of LF HACE1, RARβ$_3$ in the ethanol treated cells has a half life of 7.0+/-1.1 hr while in the RA treated cells the half life is reduced to 4.5+/-0.6 hr (Figure 6). Interestingly, in the presence of LF HACE1, RARβ$_3$ displays a similar half life in both the ethanol and RA treated cells, 7.0+/-1.1 hr and 6.9+/-1.0 hr, respectively (Figure 6). Note that the half-life of RARβ$_3$ in the presence of LF HACE1 with or without RA treatment is the same as that of the ethanol treated cells in the absence of LF HACE1 Finally there is no difference in the half-life of LF HACE1 in the presence or absence of RA. These studies suggest that HACE1 is repressing the transcription of RARβ$_3$ by inhibiting the RA-dependent degradation of RARβ$_3$.

Discussion

In this report, we have demonstrated an interaction between LF HACE1 and RARβ$_3$ by both *in vitro* GST pull-down and *in vivo* cell based copurification assays. The sites of interaction between HACE1 and RARβ$_3$ map to the common C-terminus of the two HACE1 isoforms (amino acids 356-562 of SF HACE1, amino acids 703-909 of LF HACE1) and the N-terminus (ABC regions) of RARβ$_3$. In addition, HACE1 interacts with RARβ$_1$, RARβ$_2$, RARβ$_3$, RARα$_1$, RARγ$_1$, ERα and TRα, but not with RARβ$_4$, RXRα and PPARγ in *in vitro* GST pull-down assays. Functionally both HACE1 isoforms repress the transcriptional activity of RARα$_1$, RARβ isoforms 1, 2 and 3, but not RARγ$_1$ in transactivation assays. LF HACE1 also represses the expression of the endogenous RAR-regulated genes RIG1, CRABP II and RARβ$_2$, but not RAI3 in CAOV3 cells. This repression of RAR-dependent gene expression does not appear to be mediated by the E3 ubiquitin ligase activity of HACE1 nor by deacetylation of histone proteins. On the other hand, HACE1 inhibited the RA-dependent degradation of RARβ$_3$.

It was surprising that the AB regions of RARβ isoforms 1, 2, 3 have very weak binding to HACE1 in *in vitro* GST pull-down assays. On the other hand, the ABC regions of RARβ$_3$ strongly bound HACE1. Failure of RARβ$_4$ to bind HACE1 suggests that amino acids in the A region are necessary for HACE1 binding since the A region of this receptor is truncated, consisting of only 4 amino acids [Nagpal et al., 1992]. Furthermore, since the C-F regions of RARβ did not bind to HACE1, it is likely that the C region without the A and B regions is not sufficient to bind HACE1. The addition of the DBD (C region) of RARβ$_3$ in the GST pull-down assays or the GAL4 DBD in the yeast two-hybrid screen may enable the AB regions to properly fold to allow binding of HACE1. This is supported by studies utilizing nuclear magnetic resonance, circular dichroism spectroscopy and limited proteolysis of several steroid nuclear receptor N terminal domains /AF-1s (GR, ERα, ERβ, PR) which indicate that this region exists in an unfolded state]Dahlman-Wright et al., 1995; Warnmark et al., 2000; Birnbaumer et al., 1983; Bain et al., 2000]. Interestingly, when the AB regions and DBD of either PR or AR were expressed, they tended to be more structured than the AF-1 domain alone [Lavery and McEwan, 2005; Bain et al., 2000; Kumar et al., 1999]. Taken together, it is likely that the AB regions of RARβ contains the binding site/surface for HACE1 however additional amino acid residues in the C region enable proper structural folding of the AB region in order to bind HACE1. Such a model would be consistent with our finding that HACE1 binds to TR and ER as well as all three RAR subtypes. Since the AB regions of TR and ER shares little primary amino acid sequence identity with the AB regions of the RARs, it is possible that the conformation of the combined ABC regions of TR and ER share common feature(s) with that of RARs that permit binding to HACE1. The amino terminal regions of several nuclear receptors, including ER and AR, undergo a transition to a proper folded state upon interaction with coregulatory proteins [Warnmark et al., 2001; Reid et al., 2002; Warnmark et al., 2003]. Such features would not be present in the conformation of the ABC regions of RXR and PPARγ.

HACE1 functionally represses the transcriptional activity of RARα_1, RARβ isoforms 1, 2 and 3, but not RARγ_1 in transactivation assays using a luciferase reporter gene under the control of a DR5 RARE. It is unclear why HACE1 fails to repress the RARγ_1 regulated transcription of the luciferase reporter gene despite strong *in vitro* binding of HACE1 and RARγ_1. It is possible that RARγ_1 interacts in Cos1 cells with specific cofactor(s) that blocks its binding to HACE1. In addition, HACE1 represses the expression of the endogenous RAR-regulated genes RIG1, CRABP II and RARβ_2, but not RAI3. Each of these RAR-regulated genes contains a DR5 RARE in their promoter region [Jiang et al., 2005; Tao et al., 2004; Zelent et al., 1991; Astrom et al., 1994]. RARα and/or RARβ have been shown to be involved in the transcription of CRABP II, RIG1 and RARβ_2. CRABPII has been shown to be regulated by RARα [Astrom et al., 1994], RIG1 DR5 interacts with RARα/RXR in *in vitro* EMSA assays [Jiang et al., 2005], and RARβ_2 expression can be regulated by all of the three RAR isotypes (Taneja et al., 1996). However, it is unknown which subtype of RAR is involved in regulation of RAI3 transcription [Tao et al., 2004]. These data demonstrate that HACE1 displays a type of specificity in the modulation of RAR-regulated genes that might be related to a RAR subtype functional selectivity and/or promoter context.

Although HACE1 was reported to be an E3 ubiquitin ligase [Anglesio et al., 2004], mutation of the catalytic Cys 876 (LF HACE1)/Cys 529 (SF HACE1) reported to be responsible for its ubiquitin ligase activity to an Ala does not affect HACE1-dependent repression of RARβ_3 transcriptional activity or HACE1/RARβ_3 binding, respectively. The E3 ubiquitin ligase activity of HACE1 is dependent on the conserved HECT domain that is the signature domain for this class of HECT containing E3 ligases. Structurally HECT domains form two lobes. The N-lobe interacts with E2 ubiquitin transferase and the C-lobe is responsible for transferring ubiquitin to its substrate [Huang et al., 1999; Wang et al., 1999; Verdecia et al., 2003]. Other well studied HECT domain containing E3 ubiquitin ligases interact with their substrates through their N terminal domains rather than their HECT domains (for example, RLD domain of HERC5, WW domain of NEDD family and LXXLL motif of E6-AP) [Huang et al., 1999; Wang et al., 1999]. Interaction of HACE1 with RARβ_3 via its C terminal HECT domain is not consistent with HACE1 ubiquitinating RARβ_3 and could possibly block its E3 ubiquitin ligase activity. Taken together, our data do not support the notion that HACE1 affects the transcriptional activity of RARs through its E3 ubiquitin ligase activity.

It has been shown that proteins with E3 ubiquitin ligase activity have other functions in addition to ubiquitination of target proteins. HERC1 has been shown to bind and act as guanine nucleotide exchange factor for ARF1. However, ARF1 does not appear to be degraded by HERC1 [Rosa et al., 1996]. In spite of the importance of Cbl E3 ubiquitin ligase activity in the degradation of a number of proteins such as EGFR and Vav, Cbl also functions as an adaptor molecule by forming complexes with numerous proteins. In addition, Cbl is also involved in activation of MAP kinases [For review, see Swaminathan and Tsygankov, 2006].

Both RAR and RXR are degraded by the 26S proteasome system in response to retinoids [Boudjelal et al., 2000; Kopf et al., 2000; Osburn et al., 2001; Tanaka et al., 2001; Gianni et al., 2002; Gianni et al., 2003; Bour and Rochette-Egly, 2007]. Similarly, our data demonstrate that RA potentiates the degradation of RARβ_3 (half life of 4.5+/-0.6 hrs for RA treated sample versus 7.0+/-1.1 hrs for ethanol treated sample). Interestingly, HACE1 inhibits this RA-dependent increase in RARβ_3 degradation.

It is possible that the repression of RARβ_3 transcriptional activity by HACE1 is due to its ability to inhibit the RA-dependent degradation of RARβ_3. Prior reports have demonstrated that the proteolytic function of the proteasome system on RARγ_2 upon RA treatment is

intimately linked with the transcriptional activity of RARγ_2 [Gianni et al., 2003; Gianni et al., 2002a & b]. When RARγ_2 degradation is blocked, the RA-dependent transcriptional activity of RARγ_2 is dramatically impaired. In addition, E6-AP, a HECT domain containing E3 ubiquitin ligase for ER, has dual roles including degradation of ER and transactivation of ER activity [For review, see Ramamoorthy and Nawaz, 2008]. When the ubiquitin ligase activity of E6AP is abolished, the transcriptional activity of ER is repressed. In addition, inhibition of proteosome degradation significantly diminished the ligand-induced transcriptional activity of many of the nuclear receptors including AR, ER, PR, RARα, TR, PPAR and RXR [For review, see Alarid, 2006]. Moreover, it has been postulated that the proteosome system may orchestrate the dynamics of ER mediated transcription by modulating the degradation of the ER and cofactor complexes on chromatin [Shang et al., 2000; Metevier et al., 2003; Reid et al., 2003].

We still do not understand how HACE1 inhibits the RA dependent degradation of RARβ_3. One possible mechanism is that the interaction of HACE1 and RARβ_3 may interfere with the function of its real E_3 ubiquitin ligase preventing ubiquitination of RARβ_3. One example of this phenomenon is the interaction of calmodulin with ER enhances the stability of ER through interfering with the interaction of E6AP and ER [Li et al., 2006]. Alternatively, the interaction of HACE1 with RAR may interfere with a signal for RAR degradation, such as phosphorylation. Phosphorylation of serine residues in the proline rich region in B domain was suggested to be required for both RARγ_2 degradation and RARγ_2 transactivation [Gianni et al., 2003; Gianni et al., 2002a&b]. If the interaction of HACE1 with RAR blocks the binding and/or action of kinases on critical amino acid residues then the degradation of the receptor may be prevented and transcription will be repressed. Interestingly, vinexin β interacts with unphosphorylated RARγ and represses its transcriptional activity [Bour et al., 2005].

In conclusion, our data demonstrate that the N-terminal AB region of RARs can bind HACE1. In addition, HACE1 represses the transcriptional activity of RARs and inhibits the RA-dependent degradation of RARs. Finally, these data suggest that HACE1 has additional function(s) beyond its role as an E3 ubiquitin ligase.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

Alarid ET. Lives and times of nuclear receptors. Mol Endocrinol. 2006; 20:1972–1981. [PubMed: 16423879]

Anglesio MS, Evdokimova V, Melnyk N, Zhang L, Fernandez CV, Grundy PE, Leach S, Marra MA, Brooks-Wilson AR, Penninger J, Sorensen PH. Differential expression of a novel ankyrin containing E3 ubiquitin-protein ligase, Hace1, in sporadic Wilms' tumor versus normal kidney. Hum Mol Genet. 2004; 13:2061–2074. [PubMed: 15254018]

Astrom A, Pettersson U, Chambon P, Voorhees JJ. Retinoic acid induction of human cellular retinoic acid-binding protein-II gene transcription is mediated by retinoic acid receptor-retinoid X receptor

heterodimers bound to one far upstream retinoic acid-responsive element with 5-base pair spacing. J Biol Chem. 1994; 269:22334–22339. [PubMed: 8071361]

Bain DL, Franden MA, McManaman JL, Takimoto GS, Horwitz KB. The N-terminal region of the human progesterone A-receptor. Structural analysis and the influence of the DNA binding domain. J Biol Chem. 2000; 275:7313–7320. [PubMed: 10702302]

Bastien J, Adam-Stitah S, Riedl T, Egly JM, Chambon P, Rochette-Egly C. TFIIH interacts with the retinoic acid receptor gamma and phosphorylates its AF-1-activating domain through cdk7. J Biol Chem. 2000; 275:21896–21904. [PubMed: 10748061]

Bastien J, Rochette-Egly C. Nuclear retinoid receptors and the transcription of retinoid-target genes. Gene. 2004; 328:1–16. [PubMed: 15019979]

Birnbaumer M, Schrader WT, O'Malley BW. Assessment of structural similarities in chick oviduct progesterone receptor subunits by partial proteolysis of photoaffinity-labeled proteins. J Biol Chem. 1983; 258:7331–7337. [PubMed: 6345521]

Boudjelal M, Wang Z, Voorhees JJ, Fisher GJ. Ubiquitin/proteasome pathway regulates levels of retinoic acid receptor gamma and retinoid X receptor alpha in human keratinocytes. Cancer Res. 2000; 60:2247–2252. [PubMed: 10786691]

Bour G, Lalevee S, Rochette-Egly C. Protein kinases and the proteasome join in the combinatorial control of transcription by nuclear retinoic acid receptors. Trends Cell Biol. 2007; 17:302–309. [PubMed: 17467991]

Bour G, Plassat JL, Bauer A, Lalevee S, Rochette-Egly C. Vinexin beta interacts with the non-phosphorylated AF-1 domain of retinoid receptor gamma (RARgamma) and represses RARgamma-mediated transcription. J Biol Chem. 2005; 280:17027–17037. [PubMed: 15734736]

Chambon P. A decade of molecular biology of retinoic acid receptors. FASEB J. 1996; 10:940–954. [PubMed: 8801176]

Dahlman-Wright K, Baumann H, McEwan IJ, Almlof T, Wright AP, Gustafsson JA, Hard T. Structural characterization of a minimal functional transactivation domain from the human glucocorticoid receptor. Proc Natl Acad Sci USA. 1995; 92:1699–1703. [PubMed: 7878043]

Dilworth FJ, Chambon P. Nuclear receptors coordinate the activities of chromatin remodeling complexes and coactivators to facilitate initiation of transcription. Oncogene. 2001; 20:3047–3054. [PubMed: 11420720]

Gianni M, Bauer A, Garattini E, Chambon P, Rochette-Egly C. Phosphorylation by p38MAPK and recruitment of SUG-1 are required for RA-induced RAR gamma degradation and transactivation. EMBO J. 2002a; 21:3760–3769. [PubMed: 12110588]

Gianni M, Kopf E, Bastien J, Oulad-Abdelghani M, Garattini E, Chambon P, Rochette-Egly C. Down-regulation of the phosphatidylinositol 3-kinase/Akt pathway is involved in retinoic acid-induced phosphorylation, degradation, and transcriptional activity of retinoic acid receptor gamma 2. J Biol Chem. 2002b; 277:24859–24862. [PubMed: 12032135]

Gianni M, Tarrade A, Nigro EA, Garattini E, Rochette-Egly C. The AF-1 and AF-2 domains of RAR gamma 2 and RXR alpha cooperate for triggering the transactivation and the degradation of RAR gamma 2/RXR alpha heterodimers. J Biol Chem. 2003; 278:34458–34466. [PubMed: 12824162]

Huang L, Kinnucan E, Wang G, Beaudenon S, Howley PM, Huibregtse JM, Pavletich NP. Structure of an E6AP-UbcH7 complex: insights into ubiquitination by the E2-E3 enzyme cascade. Science. 1999; 286:1321–1326. [PubMed: 10558980]

Jiang SY, Wu MS, Chen LM, Hung MW, Lin HE, Chang GG, Chang TC. Identification and characterization of the retinoic acid response elements in the human RIG1 gene promoter. Biochem Biophys Res Commun. 2005; 331:630–639. [PubMed: 15850806]

Kopf E, Plassat JL, Vivat V, de The H, Chambon P, Rochette-Egly C. Dimerization with retinoid X receptors and phosphorylation modulate the retinoic acid-induced degradation of retinoic acid receptors alpha and gamma through the ubiquitin-proteasome pathway. J Biol Chem. 2000; 275:33280–33288. [PubMed: 10869350]

Kumar R, Thompson EB. Transactivation functions of the N-terminal domains of nuclear hormone receptors: protein folding and coactivator interactions. Mol Endocrinol. 2003; 17:1–10. [PubMed: 12511601]

Lavery DN, McEwan IJ. Structure and function of steroid receptor AF1 transactivation domains: induction of active conformations. Biochem J. 2005; 391:449–464. [PubMed: 16238547]

Li L, Li Z, Howley PM, Sacks DB. E6AP and calmodulin reciprocally regulate estrogen receptor stability. J Biol Chem. 2006; 281:1978–1985. [PubMed: 16314411]

Metivier R, Penot G, Hubner MR, Reid G, Brand H, Kos M, Gannon F. Estrogen receptor-alpha directs ordered, cyclical, and combinatorial recruitment of cofactors on a natural target promoter. Cell. 2003; 115:751–763. [PubMed: 14675539]

Nagpal S, Zelent A, Chambon P. RAR-beta 4, a retinoic acid receptor isoform is generated from RAR-beta 2 by alternative splicing and usage of a CUG initiator codon. Proc Natl Acad Sci USA. 1992; 89:2718–2722. [PubMed: 1313565]

Osburn DL, Shao G, Seidel HM, Schulman IG. Ligand-dependent degradation of retinoid X receptors does not require transcriptional activity or coactivator interactions. Mol Cell Biol. 2001; 21:4909–4918. [PubMed: 11438648]

Ramamoorthy S, Nawaz Z. E6-associated protein (E6-AP) is a dual function coactivator of steroid hormone receptors. Nucl Recept Signal. 2008; 6:1–9.

Ravikumar S, Perez-Liz G, Del Vale L, Soprano DR, Soprano KJ. Insulin receptor substrate-1 is an important mediator of ovarian cancer cell growth suppression by all-trans retinoic acid. Cancer Res. 2007; 67:9266–9275. [PubMed: 17909034]

Reid G, Hubner MR, Metivier R, Brand H, Denger S, Manu D, Beaudouin J, Ellenberg J, Gannon F. Cyclic, proteasome-mediated turnover of unliganded and liganded ERalpha on responsive promoters is an integral feature of estrogen signaling. Mol Cell. 2003; 11:695–707. [PubMed: 12667452]

Reid J, Kelly SM, Watt K, Price NC, McEwan IJ. Conformational analysis of the androgen receptor amino-terminal domain involved in transactivation. Influence of structure-stabilizing solutes and protein-protein interactions. J Biol Chem. 2002; 277:20079–20086. [PubMed: 11896058]

Rochette-Egly C, Adam S, Rossignol M, Egly JM, Chambon P. Stimulation of RAR alpha activation function AF-1 through binding to the general transcription factor TFIIH and phosphorylation by CDK7. Cell. 1997; 90:97–107. [PubMed: 9230306]

Rosa JL, Casaroli-Marano RP, Buckler AJ, Vilaro S, Barbacid M. p619, a giant protein related to the chromosome condensation regulator RCC1, stimulates guanine nucleotide exchange on ARF1 and Rab proteins. EMBO J. 1996; 15:4262–4273. [PubMed: 8861955]

Shang Y, Hu X, DiRenzo J, Lazar MA, Brown M. Cofactor dynamics and sufficiency in estrogen receptor-regulated transcription. Cell. 2000; 103:843–852. [PubMed: 11136970]

Swaminathan G, Tsygankov AY. The Cbl family proteins: ring leaders in regulation of cell signaling. J Cell Physiol. 2006; 209:21–43. [PubMed: 16741904]

Taneja R, Roy B, Plassat JL, Zusi CF, Ostrowski J, Reczek PR, Chambon P. Cell-type and promoter-context dependent retinoic acid receptor (RAR) redundancies for RARβ_2 and Hoxa-1 activation in F9 and P19 cells can be artefactually generated by gene knockout. Proc Natl Acad Sci USA. 1996; 93:6197–6202. [PubMed: 8650243]

Tanaka T, Rodriguez de la Concepcion ML, De Luca LM. Involvement of all-trans-retinoic acid in the breakdown of retinoic acid receptors alpha and gamma through proteasomes in MCF-7 human breast cancer cells. Biochem Pharmacol. 2001; 61:1347–1355. [PubMed: 11331070]

Tao Q, Cheng Y, Clifford J, Lotan R. Characterization of the murine orphan G-protein-coupled receptor gene Rai3 and its regulation by retinoic acid. Genomics. 2004; 83:270–280. [PubMed: 14706456]

Verdecia MA, Joazeiro CA, Wells NJ, Ferrer JL, Bowman ME, Hunter T, Noel JP. Conformational flexibility underlies ubiquitin ligation mediated by the WWP1 HECT domain E3 ligase. Mol Cell. 2003; 11:249–259. [PubMed: 12535537]

Vucetic Z, Zhang ZP, Zhao J, Wang F, Soprano KJ, Soprano DR. Acinus-S' represses retinoic acid receptor (RAR)-regulated gene expression through interaction with the B domains of RARs. Mol Cell Biol. 2008; 28:2549–2558. [PubMed: 18250153]

Wang G, Yang J, Huibregtse JM. Functional domains of the Rsp5 ubiquitin-protein ligase. Mol Cell Biol. 1999; 19:342–352. [PubMed: 9858558]

Warnmark A, Gustafsson JA, Wright AP. Architectural principles for the structure and function of the glucocorticoid receptor tau 1 core activation domain. J Biol Chem. 2000; 275:15014–15018. [PubMed: 10747977]

Warnmark A, Treuter E, Wright AP, Gustafsson JA. Activation functions 1 and 2 of nuclear receptors: molecular strategies for transcriptional activation. Mol Endocrinol. 2003; 17:1901–1909. [PubMed: 12893880]

Warnmark A, Wikstrom A, Wright AP, Gustafsson JA, Hard T. The N-terminal regions of estrogen receptor alpha and beta are unstructured in vitro and show different TBP binding properties. J Biol Chem. 2001; 276:45939–45944. [PubMed: 11595744]

Zelent A, Mendelsohn C, Kastner P, Krust A, Garnier JM, Ruffenach F, Leroy P, Chambon P. Differentially expressed isoforms of the mouse retinoic acid receptor beta generated by usage of two promoters and alternative splicing. EMBO J. 1991; 10:71–81. [PubMed: 1846599]

Zhang L, Anglesio MS, O'Sullivan M, Zhang F, Yang G, Sarao R, Mai PN, Cronin S, Hara H, Melnyk N, Li L, Wada T, Liu PP, Farrar J, Arceci RJ, Sorensen PH, Penninger JM. The E3 ligase HACE1 is a critical chromosome 6q21 tumor suppressor involved in multiple cancers. Nat Med. 2007; 13:1060–1069. [PubMed: 17694067]

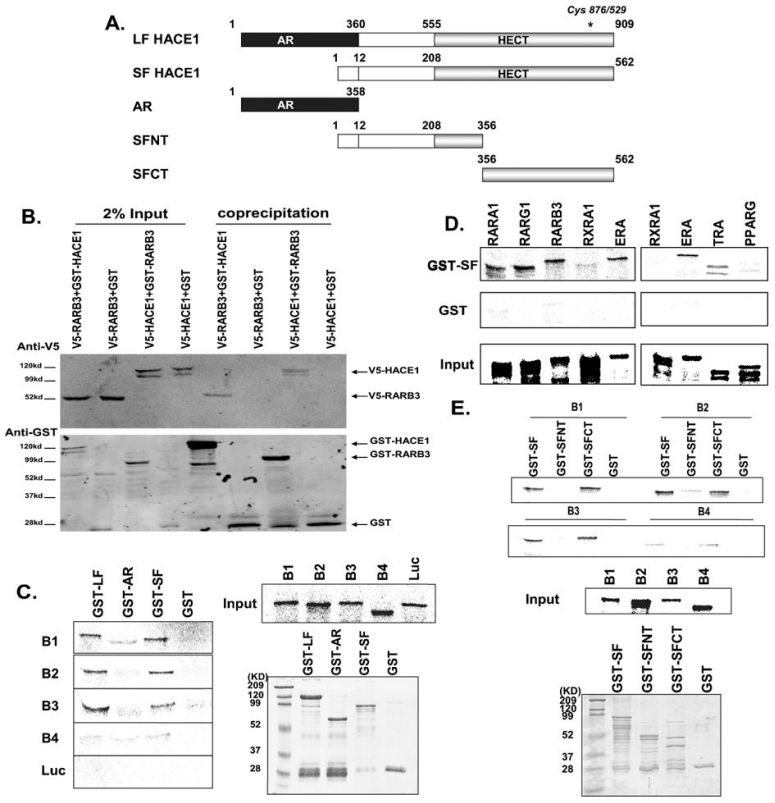

Figure 1. Interaction of SF HACE1, LF HACE1 and HACE1 regions with RARs and other nuclear receptors

A. Schematic representation of full length LF HACE1 (909 amino acids), SF HACE1 (562 amino acids) and regions of HACE1 including AR (amino acids 1-358 of LF HACE1), SFNT (amino acids 1-356 of SF HACE1) and SFCT (amino acids 356-562 of SF HACE1). B. Interaction of LF HACE1 and RARβ_3 *in vivo*. Cos1 cells were cotransfected with V5-LF HACE1 DNA and GST-RARβ_3 DNA or empty GST vector DNA, or V5- RARβ_3 DNA along with GST-LF HACE1 DNA or empty GST DNA as indicated. Protein complexes were purified using glutathione beads and resolved by SDS-PAGE. Western blots were performed using anti-V5 or anti-GST primary antibodies, donkey anti-mouse IRDdye800CW or donkey anti-rabbit IRDye680CW secondary antibodies, and detected using LI-COR Odyssey instrument. C. Interaction of LF HACE1, AR and SF HACE1 with RARβ isoforms. D. Interaction of SF HACE1 with RARα_1, RARγ_1 and several steroid/ thyroid hormone receptors including ER, TR and PPARγ. E. Interaction of SF HACE1, SFNT, SFCT with RARβ isoforms. GST pull-down assays were performed with purified GST fused LF HACE1, SF HACE1 and HACE1 regions or GST alone and the indicated *in vitro* transcribed and translated [^{35}S]-methionine labeled nuclear receptors and luciferase. Input (5% for panels C and E and 10% for panel D) and purified GST fusion proteins (panels C and E) are also shown.

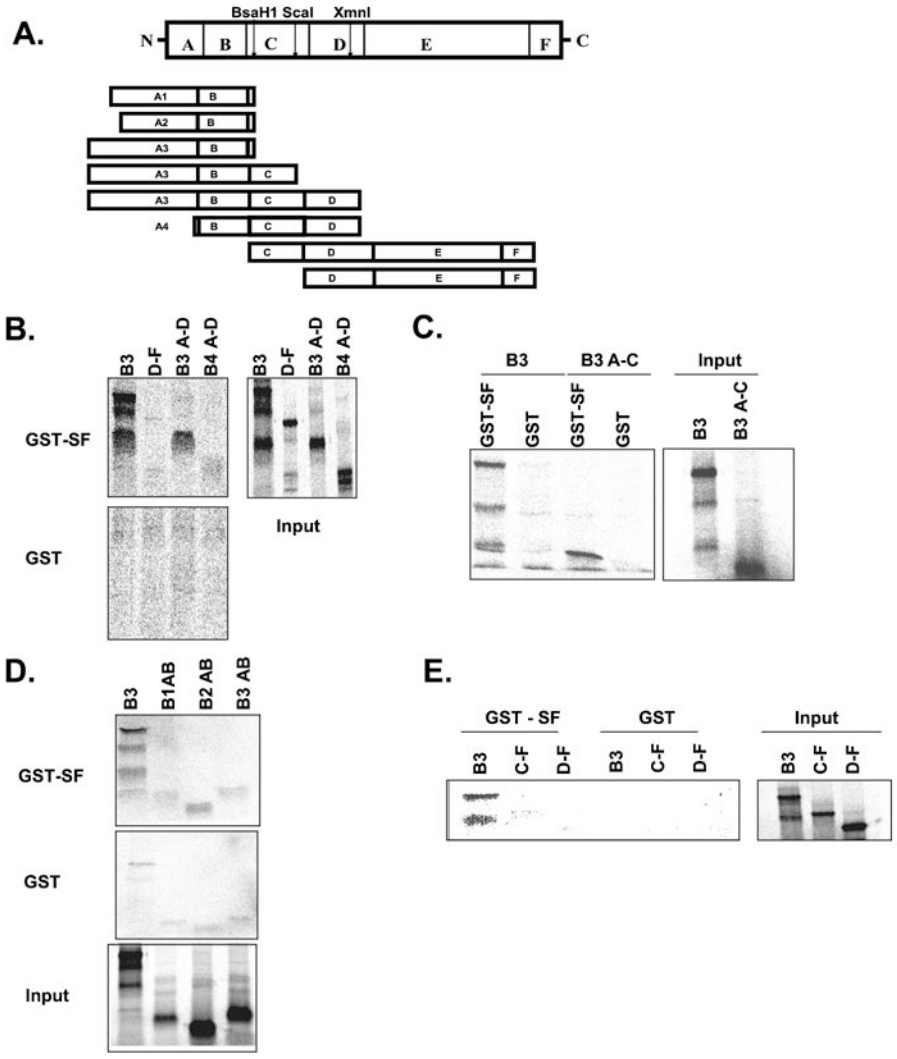

Figure 2. Mapping of the HACE1 interaction site on RARβ

A. Schematic representation of the truncations of RARβ. B. Interaction of D-F domains of RARβ, A-D domains of $RAR\beta_3$, A-D domains of $RAR\beta_4$ and full length $RAR\beta_3$ with SF HACE1. C. Interaction of A-C domains of $RAR\beta_3$ and full length $RAR\beta_3$ with SF HACE1. D. Interaction of AB domains of $RAR\beta_1$, $RAR\beta_2$, $RAR\beta_3$, and full length $RAR\beta_3$ with SF HACE1. E. Interaction of C-F domains of RARβ, D-F domains of RARβ and full length $RAR\beta_3$ with SF HACE1.

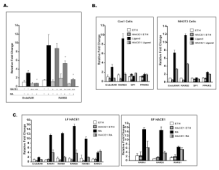

Figure 3. The effect of HACE1 on the transcriptional activity of RARs

A. LF HACE1 causes a dose dependent repression of RARβ$_3$-dependent transcriptional activity. Transactivation assay were performed in Cos1 cells transfected with increasing amount of HACE1 expression plasmid DNA (pCMVXL4-LF HACE1) including 0 μg, 0.3 μg (+), 1 μg (++) and 3 μg (+++). B. The effect of LF HACE1 on RARβ$_3$-, SP1- and PPARγ-dependent transcription in Cos1 and NIH3T3 cell lines. Transactivation assays were performed in Cos1 or NIH3T3 cells transfected with 3 μg of HACE1 expression plasmid DNA (pCMVXL4-LF HACE1). C. The effect of LF HACE1 and SF HACE1 on RAR-dependent transcriptional activity in Cos1 cells. Transactivation assays were performed in Cos1 cells transfected with 3 μg of either LF HACE1 expression plasmid DNA (pCMVXL4-LF HACE1) or SF HACE1 expression plasmid DNA (pcDNA3.1/His-SF HACE1). For panels A-C, the fold change was calculated relative to cells that were transfected with empty vector DNA and treated with ethanol arbitrarily set to 1. Values are the Mean ± SD of 3 independent experiments performed in triplicate. P values in part A were generated by pairwise student T test, *P < 0.01.

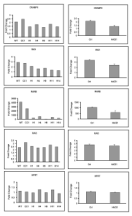

Figure 4. Effect of overexpressing HACE1 on the mRNA levels of RAR responsive genes in stable HACE1 expressing CAOV3 clones (Left Panels) and transiently HACE1 expressing CAOV3 cells (Right Panel)

Wild type CAOV3 cells (WT), empty control clone (CC1) and LF HACE1 overexpressing clones (H1, H4, H9, H11 and H14), (Left Panels), or CAOV3 cells electroporated with either LF HACE1 expression plasmid DNA or control empty plasmid DNA (Right Panels) were treated with 10^{-6} M RA or ethanol for 16 hr. RNA was extracted and reverse transcribed. The expression levels of CRABP II, RIG1, RARβ_2, RAI3 and HPRT were determined by RT-qPCR. The expression level of each gene tested was normalized to the endogenous GAPDH levels and the fold changes upon RA treatment calculated relative to the gene expression level in the respective ethanol treated sample. Left panel, each clone was assayed one time; right panel, values are Mean +/- SD.

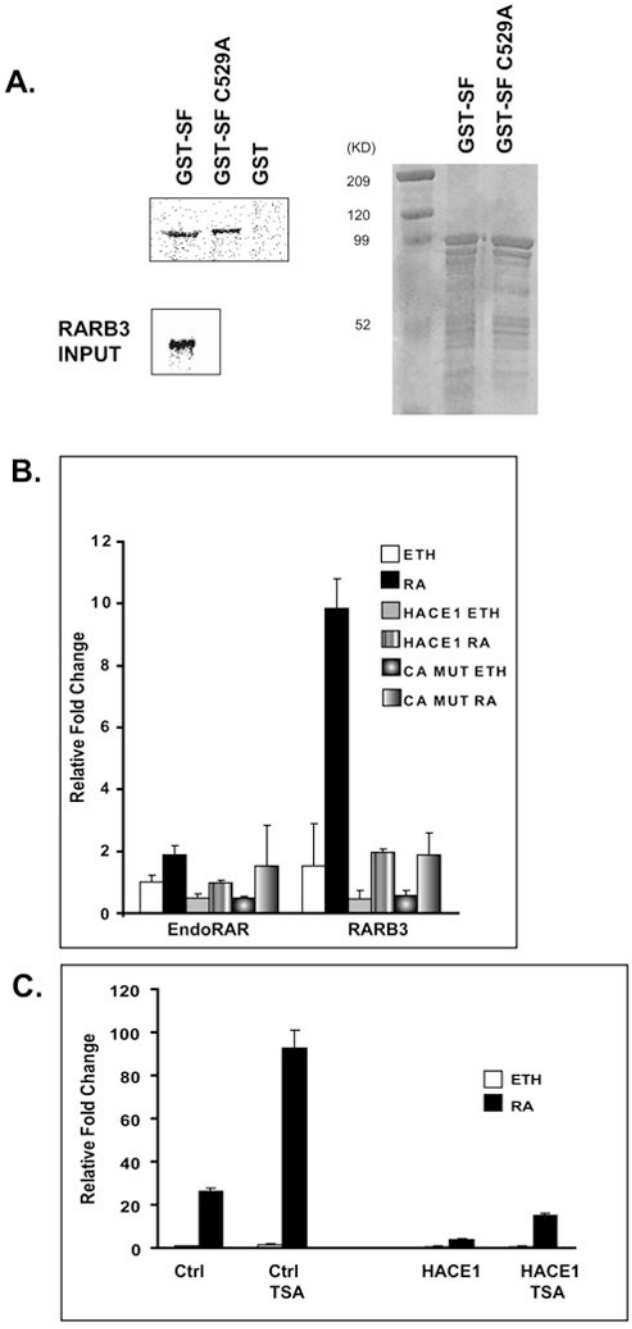

Figure 5. The role of E_3 ubiquitin ligase activity (Panels A and B) and histone deacetylase activity (Panel C) in the repression of RARβ_3 transcriptional activity by HACE1

A. GST-pulldown assays were performed with wild type GST-SF HACE 1 or GST-SF HACE1 C529A and *in vitro* transcribed and translated [^{35}S]-methionine labeled RARβ_3. B. Cos1 cells were co-transfected with DNA constructs including HACE1 expression plasmid (pCMVXL4-LF HACE1), HACE1 CA mutant expression plasmid (pCMVXL4-LF HACE1 C876A) or empty plasmid along with pTL-RARE-luc reporter plasmid, pRL reporter plasmid, and RARβ_3 expression plasmid (pOPRSVICAT- RARβ_3) or empty expression plasmid. C. Cos1 cells were cotransfected with expression plasmid of LF HACE1 (pCMVXL4-LF HACE1) or empty expression plasmid, pTL-RARE-luc reporter plasmid,

pRL reporter plasmid and expression plasmid of RARβ_3. Twenty-four hr after transfection, cells were treated with combinations of ethanol/10^{-6} M RA and 100ng/ml TSA for an additional 24 hr. For panel B, the fold changes were calculated relative to cells that were transfected with empty expression plasmid DNA and treated with ethanol arbitrarily set to 1. For panel C, the fold changes were calculated relative to cells that were transfected with empty expression plasmid DNA, treated with ethanol and no TSA arbitrarily set to 1. For panels B and C, values are the Mean + SD of 3 independent experiments performed in triplicate.

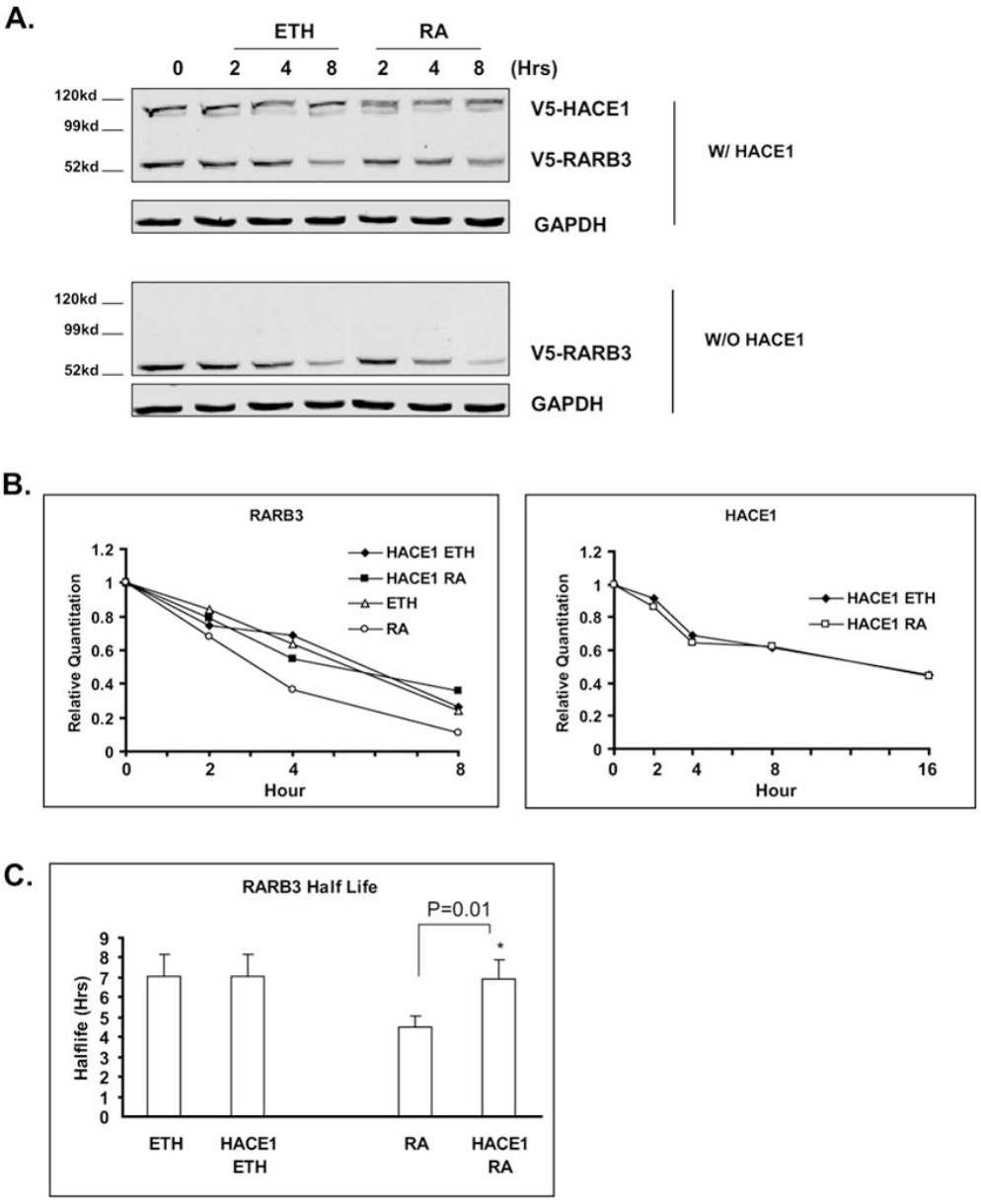

Figure 6. Analysis of RARβ$_3$ half life in the absence and presence of HACE1

Cos1 cells were transfected with V5-LF HACE1 DNA or empty vector DNA, V5-RARβ$_3$ DNA, RXRα DNA and RARE-luc DNA. Cells were treated with 10 μg/ml cycloheximide, and 10^{-6} M RA or ethanol for the indicated times. (A). Whole cell extracts were prepared from cells with indicated times of treatment and western blots were performed using V5 or GAPDH primary antibodies, donkey anti-mouse IRdye 800CW or donkey anti-rabbit IRDye 680CW secondary antibodies, and detected using a LI-COR Odyssey instrument. GAPDH was used as a loading control. A representative figure from one of three independent experiments is shown. (B). Densitometric values of each band from western blots were quantitated using LI-COR software. V5-RARβ$_3$ and V5-HACE1 values were normalized with corresponding GAPDH values. The normalized values of V5-RARβ$_3$ and V5-HACE1 were plotted. A representative plot from one of three independent experiments is shown, time 0 was set to 1 arbitrarily. (C). Half life (protein level = 50% of time 0) of RARβ$_3$ was

calculated based on the linear equations generated from quantitated protein density over time from three independent experiments. Values are the Mean ± SD of 3 independent experiments. P value was generated by pairwise student T test, *P < 0.01.