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Multiple domains of the Receptor-Interacting Protein 140 contribute to transcription inhibition

Audrey Castet, Abdelhay Boulahtouf, Gwennaëlle Versini, Sandrine Bonnet, Patrick Augereau, Françoise Vignon, Saadi Khochbin[1], Stéphan Jalaguier and Vincent Cavaillès*

INSERM U540, Endocrinologie Moléculaire et Cellulaire des Cancers and Université de Montpellier I, 60 rue de Navacelles, 34090 Montpellier, France and [1]INSERM U309, Laboratoire de Biologie Moléculaire et Cellulaire de la Différenciation, Domaine de la Merci, 38706, La Tronche, France

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ABSTRACT

In this study, we have investigated the role of C-terminal binding proteins (CtBPs) and histone deacetylases (HDACs) in the repressive activity of the nuclear receptor cofactor Receptor-Interacting Protein 140 (RIP140). We have defined the interaction of both CtBP1 and CtBP2 with RIP140 and delineated two motifs (PIDLS and PINLS) differentially required for *in vitro* interaction. Using different approaches (titration of endogenous CtBPs, mutagenesis and transfection in CtBP knock-out cells), we find that recruitment of CtBPs only partially explains the negative regulation exerted by RIP140. We then demonstrate that RIP140 associates *in vitro* not only with class I HDACs but also with class II enzymes such as HDAC5. This interaction mainly involves the N-terminus of RIP140 (residues 27–199) and two domains of HDAC5. Moreover, the two proteins functionally interfere in transfection experiments, and confocal microscopy indicates that they co-localize in the nucleus. Interestingly, using the specific HDAC inhibitor trichostatin A, we show that HDAC activity is dispensable for active transrepression by RIP140. Finally, we demonstrate that the C-terminal region of RIP140 contains two additional silencing domains and confers strong active transrepression independently of HDAC activity and CtBPs. Altogether, these data indicate that transcriptional inhibition by the cofactor RIP140 involves complex mechanisms relying on multiple domains and partners.

INTRODUCTION

Estrogens are steroid hormones that regulate growth and differentiation of a large number of target tissues such as the mammary gland, the reproductive tract and skeletal and cardiovascular systems. These hormonal effects are mediated through two distinct intracellular receptors, ERα and ERβ, which belong to the superfamily of nuclear receptors. ERs bind as homo- or heterodimers to specific DNA response elements (EREs) located within the regulatory regions of target genes. The ligand-dependent transcriptional activity of ERs is mediated by two distinct activation domains, a constitutive activation function-1 (AF-1) located within the N-terminus of the molecule (A/B domain) and a hormone-dependent AF-2 associated with the ligand-binding domain. Depending on cell and promoter contexts, these two domains function independently or synergistically.

Ligand binding induces a conformational change that promotes the recruitment of a large set of co-activator proteins (1). These transcription mediators act either by stabilizing the formation of a transcription pre-initiation complex or by facilitating chromatin disruption through various enzymatic activities that target histone tails. Several coactivators exhibit histone acetyltransferase activity (CBP/p300, SRC-1, ACTR and p/CAF), whereas others correspond to arginine methyltransferase (CARM1), kinase (TIF1α) or ubiquitin ligase (E6AP). On the other hand, ERα has been shown to interact specifically with co-repressors such as N-CoR in the presence of partial antiestrogens such as 4-hydroxytamoxifen (OHTam). These co-repressors in turn recruit histone deacetylases such as HDAC2 and HDAC4 (2), and we have recently shown that HDAC activity was required for the transrepressive effect of partial antiestrogens (3).

One of the first proteins to be identified as a hormone-recruited cofactor was Receptor-Interacting Protein 140 (RIP140) (4). The human RIP140 gene is located in a gene-poor region of chromosome 21 (5). This gene encodes a protein of 1158 residues which interacts with a large number of nuclear receptors such as ERα, TR, RAR and RXR (6), AR (7), VDR (8) PPARα/LXRα (9), GR (10), SF1 and DAX-1 (11). RIP140 also interacts with other transcription factors such as the aryl hydrocarbon receptor (12), 14-3-3 (13) or c-jun (14).

Despite its recruitment by agonist-liganded receptors, RIP140 exhibits a strong transcriptional repressive activity, which was initially attributed to competition with co-activator binding on nuclear receptors (15). However, RIP140 could also support active repression, and more recent studies have suggested that both class I HDACs and the CtBP1 protein could be recruited and contribute to its inhibitory activity (16,17). HDACs are chromatin-modifying enzymes which

*To whom correspondence should be addressed. Tel: +33 4 67 04 37 68; Fax: +33 4 67 54 05 98; Email: v.cavailles@u540.montp.inserm.fr

participate in the dynamic control of histone acetylation and counterbalance the activity of HATs, thus switching chromatin structure to a condensed state associated with transcription repression. In humans, numerous HDACs have been identified to date and are classified in three classes: class I (HDACs 1–3 and 8), class II (HDACs 4–7 and 9–11) and class III (seven human proteins homologous to yeast SIR2 enzyme) (18). CtBPs (C-terminal binding proteins) are highly conserved transcriptional co-repressors involved in various processes such as development, cell cycle regulation and transformation (19). The two family members (CtBP1 and CtBP2) are largely homologous and play both unique and redundant roles, as shown by gene invalidation (20). In *Drosophila* and vertebrates, CtBPs mediate transcriptional repression by a large number of factors and, according to the promoter, CtBP-mediated repression is dependent or independent of HDAC activity (19).

In this study, we have analysed the mechanisms by which RIP140 inhibits the regulation of gene transcription by estrogens. We have characterized the molecular mechanisms mediating RIP140 inhibitory activity by defining the respective roles and binding domains of the two CtBPs and class II HDACs on the RIP140 molecule. Finally, we show that the C-terminal part of RIP140 also conferred strong active repressive activity. Altogether, our data indicate that inhibition of estrogen signalling by RIP140 involves multiple domains and partners.

MATERIALS AND METHODS

Plasmids

pSV40G5E1BLuc (21) was obtained from Dr Svensson (Uppsala University, Sweden), and pCDNA-HDAC5, L8G5-Luc, LexA-VP16, Gal-HDAC5 and GST–HDAC5 constructs (22,23) were from Dr Khochbin (INSERM U309, La Tronche, France). ERE-βGlob-Luc, ERE-TK-Luc and 17M5βGlob-Luc have been described elsewhere (24). pGEX-2tk-hCTBP2 (25) was obtained from Dr Otte (University of Amsterdam, The Netherlands), pRcCMVT7CtBP1, GST-CtBP1 and pcDNA3-dl1119 (E1A-CID) (26) were from Dr Chinnadurai (St Louis University, MO), pGEX4T1-mHDAC1 (27) from Dr Seiser (VBC, Vienna, Austria), pGST-HDAC2 (28) from Dr Seto (University of South Florida, FL), pSG5-ERα vector (HEGO) from Professor Chambon (IGBMC, Strasbourg, France) and the CFP–HDAC5 from Dr Evans (29) (The Salk Institute for Biological Studies, San Diego, CA). pEFRIP140 was previously described (4). Full-length pCRIP140 was obtained by inserting the SpeI/KpnI cDNA fragment of pEFRIP140 into the XbaI/KpnI sites of pCDNA3.1 (Invitrogen, Cergy-Pontoise, France). Site-directed mutagenesis using the QuickChange kit (Stratagene, Amsterdam, The Netherlands) was performed on pCRIP140 to generate pCRIPmutPIDLS, mutPINLS and mutPI$^{D}/_{N}$LS (containing both sites mutated into PIASS). pCRIP(1–480) was created by inserting a stop codon containing oligonucleotide into the EcoRV restriction site of pCRIP140. pBRIP(682–1158) was previously described (4).

Gal-RIP and the various deletion mutants were obtained by PCR and subcloning into the KpnI site of pSG424 or by inserting a stop codon into the Gal-RIP vector at the BsaBI site for Gal-RIP(27–199). Gal-SHP contains the full-length SHP sequence [pSG5-SHP (30)] inserted into the EcoRI site of the pM vector (Clontech). YFP-RIP140 is encoded by the pcDNAmonocitrin-RIP140 plasmid, obtained by PCR.

GST–RIP plasmids have been previously described (6) or obtained by inserting a stop codon containing oligonucleotides. The GST–RIP(429–582) WT, mutPIDLS, mutPINLS and mutPI$^{D}/_{N}$LS were created by inserting PCR fragments into SmaI sites of the pGEX2TK.

Cell culture and transfection

MCF-7 and HeLa human cancer cells were derived from stocks routinely maintained in the laboratory. Monolayer cell cultures were respectively grown in Ham's F-12/Dulbecco's modified Eagle's medium (1:1) (F12/DMEM) or in DMEM supplemented with 10% fetal calf serum (FCS) (InVitrogen, Cergy-Pontoise, France) and antibiotics. Mouse embryo fibroblasts (MEFs) derived from CtBP1$^{+/-}$CtBP2$^{+/-}$ or from CtBP1$^{-/-}$CtBP2$^{-/-}$ mice (20) were obtained from Dr Hildebrand (Pittsburgh, PA) and cultured in DMEM with 10% FCS. For transient transfection experiments, cells were plated at ~80% confluence (10^6 cells/35 mm diameter well) and transfected in 6-well plates by the calcium phosphate method using CMV-βGal as an internal control. Jurkat T cells were grown in suspension in RPMI supplemented with 10% FCS and transfected using the Lipofectamine 2000 reagent (InVitrogen, Cergy-Pontoise, France) according to the manufacturer's instructions (24-well plates). Cell extract preparation was carried out as recommended by Promega Corporation. Cells were lysed at 4°C for 10 min in 0.4 ml of lysis buffer (25 mM Tris pH 7.8, 2 mM EDTA, 10% glycerol, 1% Triton X-100). Luciferase activity was measured on 100 µl supernatant aliquots during 1 s after injection of 100 µl of luciferase detection solution using a luminometer (Labsystem, Les Ulis, France). When comparing basal levels between different cell lines, transfection data were normalized by the β-galactosidase activities determined as described (31) and expressed as relative luciferase activities. Trichostatin A (TSA) was from Sigma (Saint-Quentin, France).

GST pull-down assay

In vitro translation and GST pull-down assays were performed as previously described (32). Briefly, ^{35}S-labelled proteins were cell-free-synthesized using the TNT lysate system (Promega) and incubated with purified GST fusion proteins overnight at 4°C in NETN buffer containing 0.5% NP-40, 1 mM EDTA, 20 mM Tris pH 8, 100 mM NaCl, 10 mM dithiothreitol and protease inhibitors (Roche Diagnostics, Meylan, France). Protein interactions were analysed by SDS–PAGE followed by quantification using a Phosphorimager (Fujix BAS1000). Gels were stained with Coomassie Brilliant Blue (BioRad) to visualize the GST fusion proteins present in each track.

Confocal fluorescence microscopy

COS-1 cells were plated into 4-well chamber slides (Falcon) and transfected with cyan fluorescent protein (CFP)–HDAC5 (0.125 µg) and yellow fluorescent protein (YFP)–RIP140 (0.5 µg) using Lipofectamine 2000 reagent. After 48 h, cells were washed in phosphate-buffered saline, fixed in 4% paraformaldehyde and mounted with Dako fluorescent mounting

medium. Fluorescence signals were detected with a Leica sp2 confocal microscope and analysed with the corresponding software.

Western blot experiments

Whole-cell extracts were prepared in RIPA buffer [150 mM NaCl, 50 mM Tris pH 7.5, 0.5% NP-40, 0.5% deoxycholate, 0.1% SDS and proteases inhibitors (Roche Diagnostics, Meylan, France)]. Proteins were quantified using the Bradford assay (Bio-Rad Laboratories, Marnes, France) and 60 µg were resolved using SDS–PAGE. The blots were saturated in TBST buffer [50 mM Tris, 150 mM NaCl, 0.1% Tween 20 (v/v), 5% dehydrated milk (w/v)] and incubated with the Sc11390 antibody which detects both CtBP1 and CtBP2 (Tebu, Le Perray, France) and with anti-rabbit second antibody (Sigma, Saint-Quentin, France). Detection was done using the Chemiluminescence Reagent Plus kit (PerkinElmer Life Science, Courtaboeuf, France).

RESULTS

Transcriptional repression by RIP140

RIP140 belongs to an atypical class of nuclear receptor transcription co-regulators which are recruited by agonist-liganded nuclear receptors but negatively regulate their activity. As previously reported by others (33), we found that RIP140 actively inhibits transcription when fused to the heterologous DNA-binding domain (DBD) of the yeast GAL4 transcription factor (Gal-RIP plasmid). In various cell types, transfection of increasing concentrations of Gal-RIP strongly decreased the expression of the luciferase reporter gene driven by different promoters. Moreover, the intrinsic inhibitory effect of RIP140 was also efficient when we used an amplified system based on the activation of the reporter gene by the chimaeric LexA-VP16 fusion protein. As shown in Figure 1, Gal-RIP transfection led to a 10-fold decrease in transcription which was comparable with the effect of Gal-SHP, another ER co-repressor (34), whereas the Gal4 DBD alone was without any effect. This intrinsic ability of RIP140 to silence transcription in transient transfection assays suggested that it may be mediated through interaction with others repressors.

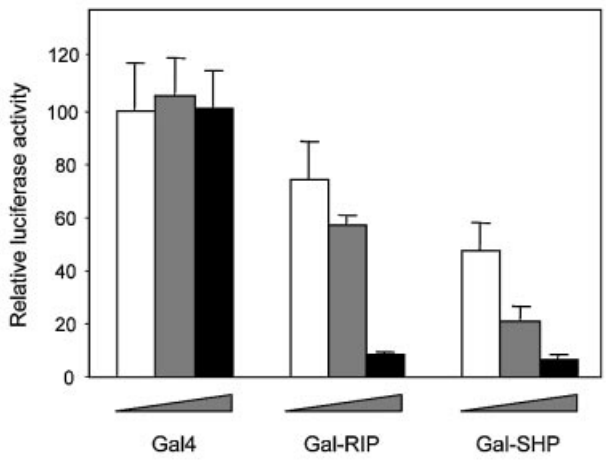

Figure 1. Intrinsic repression activity of RIP140. HeLa cells were transiently transfected with the L8G5-Luc reporter construct (0.4 µg) together with the expression vector for LexA-VP16 (0.2 µg) and increasing concentrations of Gal4, Gal-RIP or Gal-SHP (0.25, 0.5 and 1 µg). Luciferase activity was quantified as indicated in Materials and Methods. Results are expressed relative to control and are the mean (±SD) of three independent experiments.

Interaction of RIP140 with CtBPs

CtBP1 was previously shown to interact with RIP140 both *in vitro* and *in vivo* through a PIDLSCK sequence (17). The data presented in Figure 2A confirmed that CtBP1 expressed as a GST fusion protein was able to bind *in vitro* translated full-length RIP140. Interestingly, we observed a comparable interaction of RIP140 with CtBP2, another member of the family highly homologous to CtBP1.

To further characterize the domains of RIP140 required for binding to CtBPs, we used deletion mutants of RIP140 fused to GST (Fig. 2B). The results indicated that the central region of the molecule encompassing residues 429–739 was the main interacting domain for CtBP1, although a faint binding was detected with the N-terminus of RIP140. The central region contains the known PIDLS motif (residues 440–444) and a second potential CtBP-binding motif (sequence PINLS located between residues 565 and 569) which was not previously identified. Interestingly, a search of sequence databases indicated that the two motifs are conserved in mouse and zebrafish cDNAs, suggesting that they both could play functional roles (A.Castet, unpublished observation).

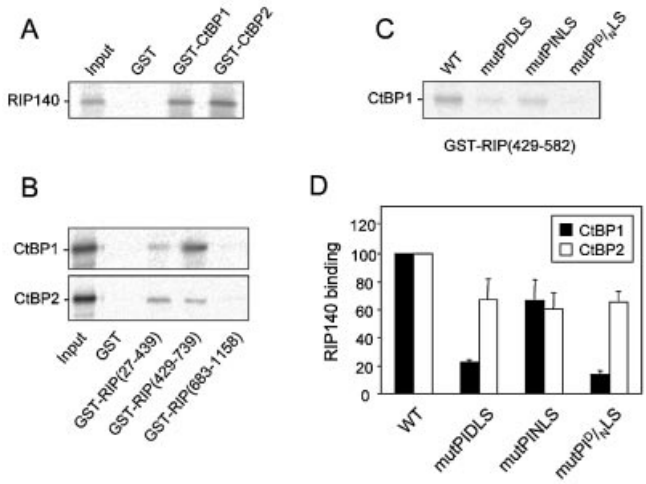

Figure 2. *In vitro* interaction of RIP140 with CtBPs. (**A**) GST pull-down assays were carried out as described in Materials and Methods using bacterially expressed GST–CtBP1 or GST–CtBP2 proteins to retain ^{35}S-labelled RIP140. (**B**) GST–RIP140 proteins containing different regions of the molecule, i.e. GST–RIP(27–439), GST–RIP(429–739) or GST–RIP(683–1158), were tested for their interaction with ^{35}S-labelled CtBP1 and CtBP2. (**C**) GST pull-down assays were performed to precipitate ^{35}S-labelled CtBP1 with the GST–RIP(429–582) protein containing the wild-type or mutated sequences for the PIDLS (mutPIDLS), PINLS (mutPINLS) or both (mutPI^{D}/$_{N}$LS) sites. (**D**) GST–CtBP1 or GST–CtBP2 proteins were used to pull-down radiolabelled RIP140 either wild type or mutated for the PIDLS (mutPIDLS), PINLS (mutPINLS) or both sites (mutPI^{D}/$_{N}$LS). Results are expressed as a percentage of the binding of the wild-type RIP140.

Site-directed mutagenesis was performed to inactivate the two sites independently or simultaneously (mutPIDLS,

mutPINLS and mutPI^{D}/$_{N}$LS, respectively), either in the context of the GST–RIP(429–582) chimaeric protein (Fig. 2C) or in the full-length RIP140 molecule (Fig. 2D). *In vitro* interaction assays performed in both ways essentially gave similar results and demonstrated that both sites were involved in the interaction between RIP140 and CtBP1, with the PIDLS motif being clearly more potent than the PINLS sequence.

Interestingly, differences appeared in the *in vitro* binding of RIP140 to CtBP1 and CtBP2 since the central region of RIP140 was less efficient at recruiting CtBP2 than CtBP1, the N-terminus and central domains of RIP140 being equally able to retain labelled CtBP2 (Fig. 2B). In support of this observation, the residual binding of full-length labelled RIP140 mutated on either PIDLS site alone or both PIDLS and PINLS sites was more important on GST–CtBP2 than on GST–CtBP1 (Fig. 2D).

Together, these results indicate that RIP140 (i) interacts *in vitro* with both CtBP1 and CtBP2; and (ii) contains several binding sites for CtBPs (including two PIxLS-binding motifs) which are differentially involved in these interactions.

CtBPs and RIP140 repression

By western blot analysis, we showed that CtBPs were expressed at high levels in various human cancer cell lines (Fig. 3A). A single band migrating with an apparent mol. wt of 48 kDa and corresponding to CtBP1 and CtBP2 was detected with a comparable intensity in ER-positive (MCF-7 and T-47D) or ER-negative (MDA-MB231, MDA-MB435 and MDA-MB436) human breast cancer cell lines. Similar results were obtained in other cells of human or simian origin (HeLa, COS-1 or CV1 cells) whereas, in two of three human endometrial cancer cell lines, an additional faster migrating band was detected [corresponding probably to the previously described N-terminally truncated version of CtBP1 (35)]. The high expression of endogenous CtBPs could explain why overexpression of CtBP1 or CtBP2 in transient transfection did not significantly increase RIP140 repressive activity [(17) and data not shown].

To determine the relative role of CtBPs in RIP140-mediated transcriptional repression, we therefore introduced the double mutation of the PIDLS and PINLS sites in the RIP140 protein and compared its inhibitory activity with that of the wild-type protein. As shown in Figure 3B, the mutant RIP140 protein efficiently repressed the reporter gene transcription (42% of repression as compared with 75% for the wild-type protein), suggesting that, in these conditions, transcriptional inhibition by RIP140 was in part CtBP independent. We performed the same type of experiment with the Gal-RIP chimaeric proteins and obtained exactly the same results (53% repression with the PI^{D}/$_{N}$LS mutant instead of 75% for the wild-type protein) (Fig. 3C).

We then used another approach based on the functional knock-out of CtBPs. Over-expression of the CtBP-interacting domain of the adenovirus E1A protein (E1A-CID) (21) again only partially relieved RIP140 transrepression activity (50% repression instead of 71%), either in the context of a fusion with the Gal4 DBD (Fig. 3D) or when evaluated on ERα activity (data not shown). Finally, we investigated the repressive activity of RIP140 on ERα activity in MEFs derived from an animal knock-out for both the CtBP1 and

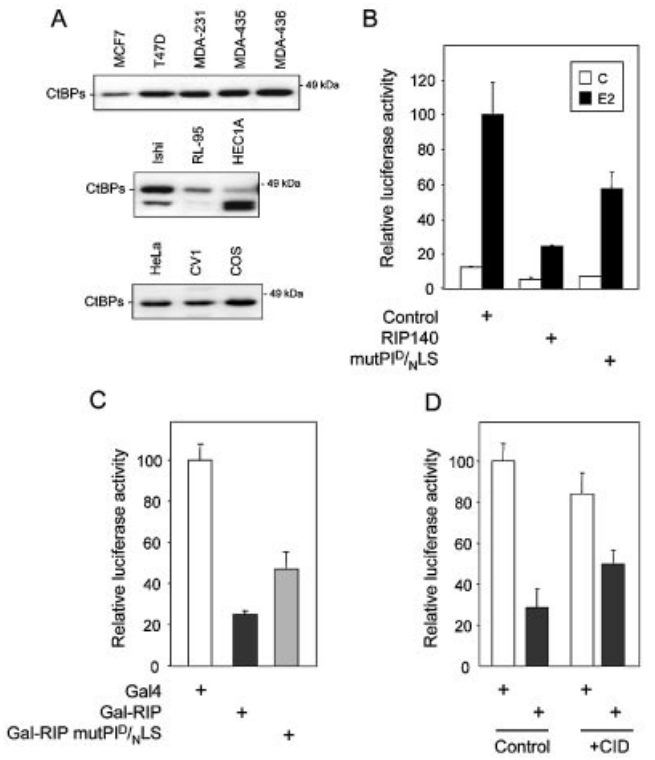

Figure 3. Role of CtBPs in transcriptional repression by RIP140. (**A**) A western blot experiment using an antibody specific for CtBP1 and CtBP2 was performed as described in Materials and Methods. Cell lines from various tissues were analysed. MCF-7 and T47-D are derived from ERα-positive breast carcinoma, whereas MDA-MB231, MDA-MB435 or MDA-MB 436 are from ERα-negative breast carcinoma. Ishikawa (Ishi), RL-95 and HEC1A are from endometrium, HeLa cells from a cervix carcinoma, and CV-1 and COS-1 from African green monkey kidney. (**B**) Transient transfections of HeLa cells were performed using Lipofectamine 2000 reagent as described in Materials and Methods. The ERE-βGlob-Luc reporter (125 ng) and ERα (50 ng) expression plasmid were transfected together with either the vectors encoding RIP140 wild type, mutated for the PIDLS and PINLS sites (mutPI^{D}/$_{N}$LS), or the empty vector only (750 ng). Relative luciferase activity was measured 24 h after the treatment of cells with 17β-estradiol (E2, 10^{-8} M) or vehicle alone and is expressed as a percentage of control in the presence of E2. (**C**) HeLa cells were transiently transfected with the 17M5βGlob-Luc reporter construct (1 μg) together with 1.5 μg of Gal-RIP wild type (black box) or mutated for the two motifs PIDLS and PINLS (Gal-RIPmutPI^{D}/$_{N}$LS, grey box) versus empty vector alone (white box). Luciferase activity was measured 48 h after transfection and is expressed as a percentage of control with Gal4 alone. Results are the mean (±SD) of three values obtained in three independent experiments. (**D**) HeLa cells were transiently transfected with the pSV40G5E1BLuc reporter (1 μg) together with 1 μg of Gal-RIP plasmid (black boxes) or empty vector (white boxes) in the presence or absence of the pcDNA3-dl1119 plasmid (2.5 μg) allowing the expression of the E1A exon 2 (E1A-CID). Results are expressed as relative luciferase activity (% of control) and are the mean (±SD) of six values from two independent experiments.

CtBP2 genes (20). As shown in Figure 4A, western blot analysis confirmed that the expression of CtBPs was lost in CtBP$^{-/-}$ MEFs when compared with CtBP$^{+/-}$ cells. However, when we analysed the effect of RIP140 expression on ERα activity, we found that transrepression was still effective in MEFs which do not express CtBPs (41% repression versus 51% in CtBP$^{+/-}$).

We also analysed the inhibitory potential of the Gal-RIP protein in CtBP$^{-/-}$ or CtBP$^{+/-}$ cells. As shown in Figure 4B, a

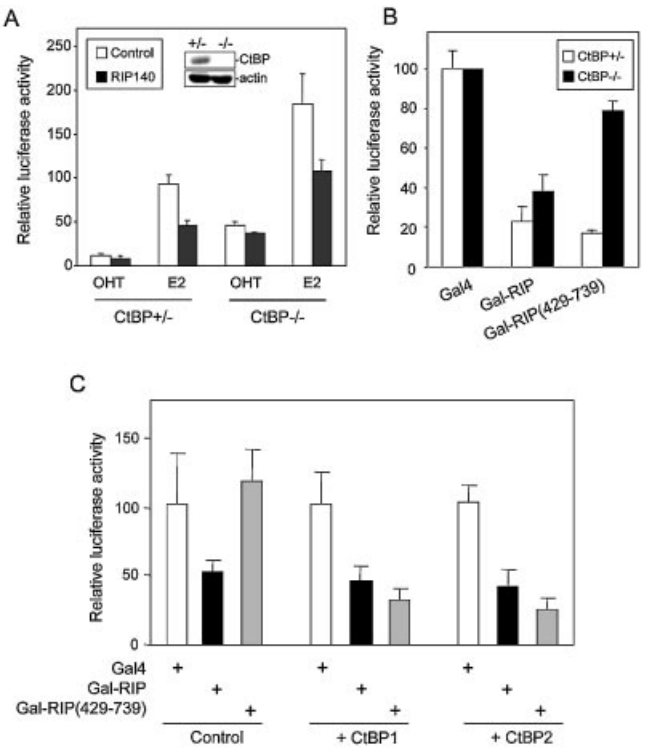

Figure 4. Role of CtBPs in transcriptional repression by RIP140. (**A**) MEFs (either CtBP$^{+/-}$ or CtBP$^{-/-}$) were transiently transfected with the ERE-βGlob-Luc reporter (0.5 μg) together with the ERα (0.2 μg) and RIP140 (1.8 μg) expression plasmid (black boxes) or empty vector (white boxes) in the presence of 17β-estradiol (E2) or 4-hydroxytamoxifen (OHTam, 10^{-8} M). Results are expressed as raw luciferase activity and are the mean (±SD) of six values from two independent experiments. The level of expression of CtBPs assessed by western blot analysis in the two types of MEFs is also shown. (**B**) Empty vector (1 μg), Gal-RIP full-length construct and its central part comprised between residues 429 and 739 were transiently transfected in CtBP$^{+/-}$ (white boxes) or CtBP$^{-/-}$ (black boxes) MEFs together with the L8G5-Luc reporter and the LexA-VP16 activator plasmids. Cells were transfected using Lipofectamine 2000 reagent. Results are expressed as relative luciferase activity (percentage of control Gal4) and are the mean of three values. (**C**) Transient transfections were realized in CtBP$^{-/-}$ MEFs using Lipofectamine 2000 reagent. Gal4, Gal-RIP or Gal-RIP(429–739) expression plasmids (1 μg) have been transfected together with the L8G5-Luc reporter and the LexA-VP16 activator plasmids in the presence or absence of CtBP1 or CtBP2. Luciferase activity which represents the mean (±SD) of three values was measured 48 h after the transfection and is expressed as a percentage of each control without RIP140.

slight but not significant reduction in the ability to repress transcription was noticed in CtBP-deficient cells (61% versus 73% in CtBP$^{+/-}$ cells). We then analysed the activity of the isolated central domain of RIP140 encompassing the two CtBP-binding sites. This region clearly repressed luciferase activity in CtBP-expressing fibroblasts (79% inhibition) but was only poorly active in CtBP$^{-/-}$ cells (21% repression). As expected, re-expression of either CtBP1 or CtBP2 in knock-out cells restored a full repression of the central domain which became as active as the full-length molecule (Fig. 4C).

Altogether, these results supported a strong *in vitro* interaction of CtBPs with the central domain of RIP140 exhibiting a convincing CtBP-dependent transrepression when tested as an isolated module. However, in our cell system, when we considered the full-length RIP140 molecule, CtBPs were clearly not the main mediators of RIP140 repressive activity, suggesting that other effectors might exist.

RIP140 interaction with class II HDACs

Amongst the mechanisms that might explain the inhibitory effect of RIP140 on nuclear receptor activity, the recruitment of class I HDACs (HDAC1 and 3) has been evoked in the case of RAR/RXR heterodimers (16). As shown in Figure 5A, we confirmed that class I HDACs could be recruited *in vitro* by RIP140. In GST pull-down experiments, we observed a specific interaction of RIP140 not only with HDAC1 [as previously described (16)] but also with HDAC2.

We then tested whether RIP140 could interact with class II HDACs, and our results showed that both HDAC5 (Fig. 5B–D) and HDAC6 (data not shown) could bind to RIP140. In order to define more precisely the interaction domains on RIP140 and HDAC5, we performed interaction assays with deletion mutants of the two partners. When tested as GST fusion proteins, we found that the major interacting domain of HDAC5 on RIP140 was located in the N-terminus of the molecule (Fig. 5B). However, both the central and the C-terminal regions of RIP140 also retained *in vitro* translated HDAC5, though the strength of these interactions appeared less intense. By deletion analysis in the N-terminus region of RIP140, we found that the main interaction domain for HDACs (both class I and II) corresponds to a region encompassing residues 27–199 and requires a sequence between residues 115 and 199 (Fig. 5C and data not shown for class I enzymes). On the other hand, both fragments of HDAC5 corresponding to residues 123–673 and 674–1113 (encompassing the catalytic site) interacted with *in vitro* translated RIP140, whereas no retention was observed with the N-terminus of HDAC5 (residues 1–122) (Fig. 5D). Again, we observed a preferential interaction of the two HDAC domains with the N-terminus region of RIP140, and the binding of the sequence corresponding to the last 576 residues of RIP140 (amino acids 682–1158) was almost undetectable.

To further support the *in vitro* interaction between RIP140 and HDAC5, we used confocal microscopy to define whether the two proteins could co-localize in intact cells. We transiently co-transfected expression vectors for YFP–RIP140 together with CFP–HDAC5 in COS-1 cells. As expected from previous reports (13,29), both proteins were detected in the nuclear foci, and confocal analysis revealed that the two signals merge nicely (Fig. 6A). This therefore suggested that over-expressed RIP140 and HDAC5 co-localized in subnuclear compartments.

Finally, to evidence a functional link between the two factors, we investigated if over-expression of one of the two molecules could modulate the transcriptional activity of the other. In our transient transfection experiments, HDAC5 produced almost no effects on RIP140 transcriptional repression (data not shown). In contrast, as shown in Figure 6B, we noticed that RIP140 over-expression significantly amplified the transcriptional repression of Gal-HDAC5 proteins. Interestingly, this effect was obtained only with constructs corresponding to the sequences that we found to interact *in vitro* with RIP140 (domains 123–673 and 180–1113). No decrease in luciferase activity was noticed when RIP140 was co-transfected with the Gal4 DBD alone or with the

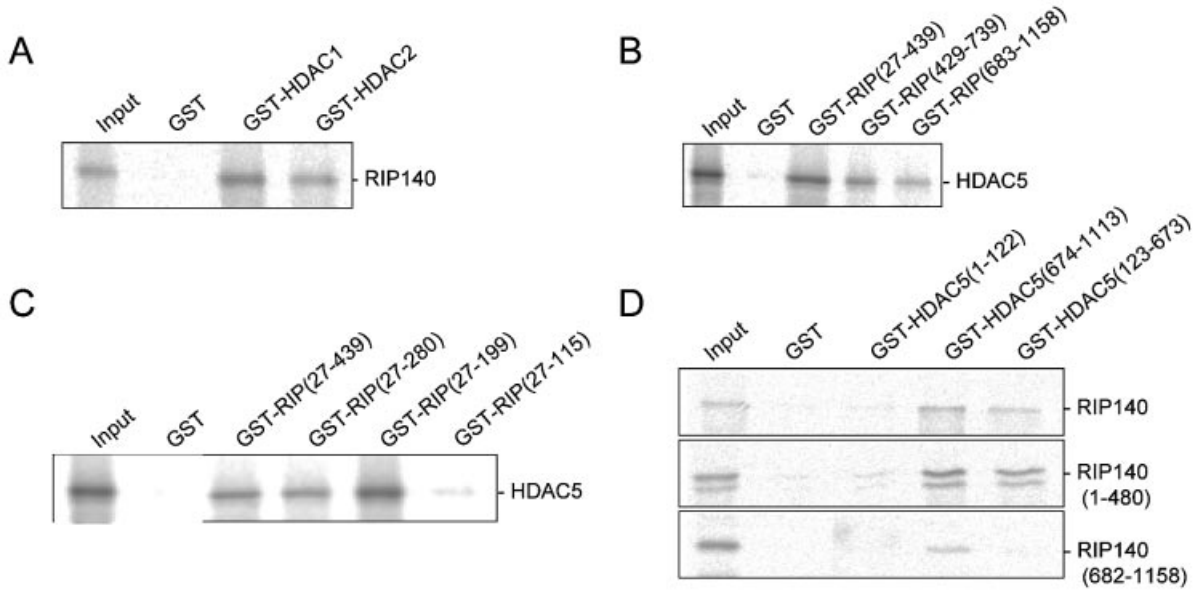

Figure 5. RIP140 recruits both class I and II HDACs. (**A**) The GST–HDAC1 and GST–HDAC2 fusion proteins were tested for their ability to interact with radiolabelled RIP140 in GST pull-down experiments. (**B** and **C**) Various bacterially expressed GST–RIP140 mutants were analysed for their interaction with [^{35}S]HDAC5. (**D**) Interaction between different GST–HDAC5 mutants and ^{35}S-labelled RIP140, either full-length or C- or N-terminus deleted. Inputs represent 10% of the material used in the assays.

Gal4-HDAC5 mutant corresponding to residues 1–122. Altogether, these data suggested that RIP140 and HDAC5 could functionally interact in the cell nucleus.

HDAC activity is dispensable for RIP140-dependent transcription repression

We then investigated whether the enzymatic activity of HDACs was required for their *in vitro* interaction with RIP140 and for its transrepressive activity. It has been reported in some cases that HDAC could inhibit transcription independently of their deacetylase activity. As shown in Figure 7A, TSA, a potent HDAC inhibitor, did not significantly modify the binding of radiolabelled RIP140 on HDAC1, HDAC2 or HDAC5 (domain 674–1113) expressed as GST fusion proteins.

To investigate whether TSA could relieve RIP140 transcriptional repression, we first used the simplified system with the full-length RIP140 or different regions of the molecule fused to the Gal4 DBD (Fig. 7B). In these conditions, we found, as expected, that transrepression of the HDAC-interacting domain [Gal-RIP(27–199)] was severely impaired by TSA treatment. Interestingly, the same effect was noticed for the CtBP-binding region [Gal-RIP(429–739)], suggesting a CtBP-mediated HDAC recruitment on this isolated domain. However, in the same conditions, the Gal-RIP still efficiently repressed transcription even in the presence of TSA, suggesting that in the context of the full-length protein, HDAC activity was dispensable for active repression.

Similar results were obtained on RIP140-mediated repression of ERα-dependent transactivation. As shown in Figure 7C, treatment with increasing concentrations of TSA did not relieve the repressive effect of RIP140 on estradiol-dependent transactivation of ERα. This was obtained in different cell types (data not shown), on two reporter plasmids with either the β-globin or the thymidine kinase promoters and also evidenced when ERα transactivates through an ERE-independent mechanism (14). Together, these results did not support the idea that HDAC enzymatic activity was required for transcriptional repression of ERs by RIP140.

Role of the C-terminal region of RIP140

To investigate whether other domains of RIP140 could be involved in transcriptional repression, we generated a Gal4 fusion protein containing only the COOH moiety of RIP140 [Gal-RIP(753–1158)]. When tested in transient transfection, this domain strongly repressed transcription and, in dose–response experiments, this region was even more potent in silencing transcription than the full-length protein (Fig. 8A), or than the two other repressive regions corresponding to the N-terminus or the central part of RIP140 (data not shown).

The C-terminal domain was active on several reporter constructs bearing different promoters and was found to confer a strong repression in all the cell lines that we tested (Fig. 8B and data not shown). As expected from the *in vitro* interaction data (Figs 2B, and 5B and D) and from the results obtained in transient transfection with the full-length protein, repression conferred by the C-terminal part of RIP140 was not sensitive to TSA treatment and was very potent in CtBP$^{-/-}$ MEFs (Fig. 8C).

To delineate more precisely the C-terminal repression domain(s), we generated other deletion mutants. As shown in Figure 8D, this allowed us to demonstrate that two regions (corresponding to residues 753–804 and 848–1158) supported efficient transcriptional repression in transfection experiments. Together these results indicated that the intrinsic repressive activity of RIP140 involved multiple domains. The two main domains, which are independent of CtBP and HDAC activities, are located in the C-terminal part of the molecule.

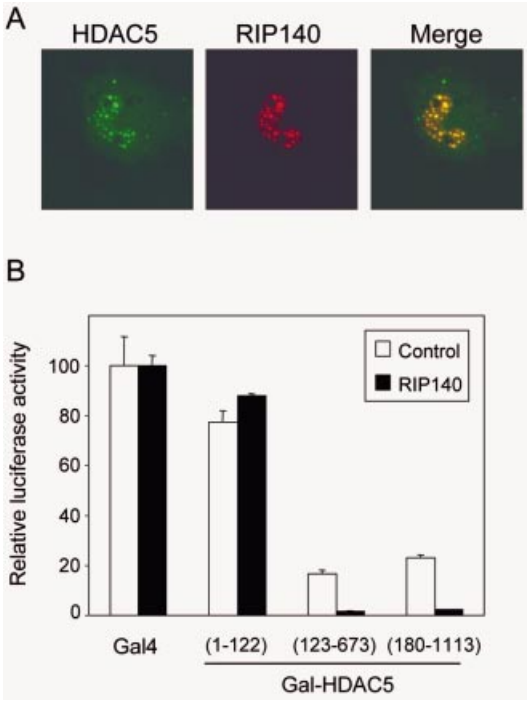

Figure 6. RIP140 and HDAC5 co-localize and functionally interact. (**A**) CFP–HDAC5 and YFP–RIP140 expression plasmids were co-transfected in COS-1 cells using Lipofectamine 2000 reagent. Fluorescence signals were detected independently or simultaneously (merge) with a Leica sp2 confocal microscope. (**B**) HeLa cells were transiently transfected with various expressing plasmids encoding Gal-HDAC5 domains (0.25 μg), together with the L8G5-Luc reporter and the LexA-VP16 activator plasmids, in the presence or absence of the expression vector for RIP140 (1.5 μg).

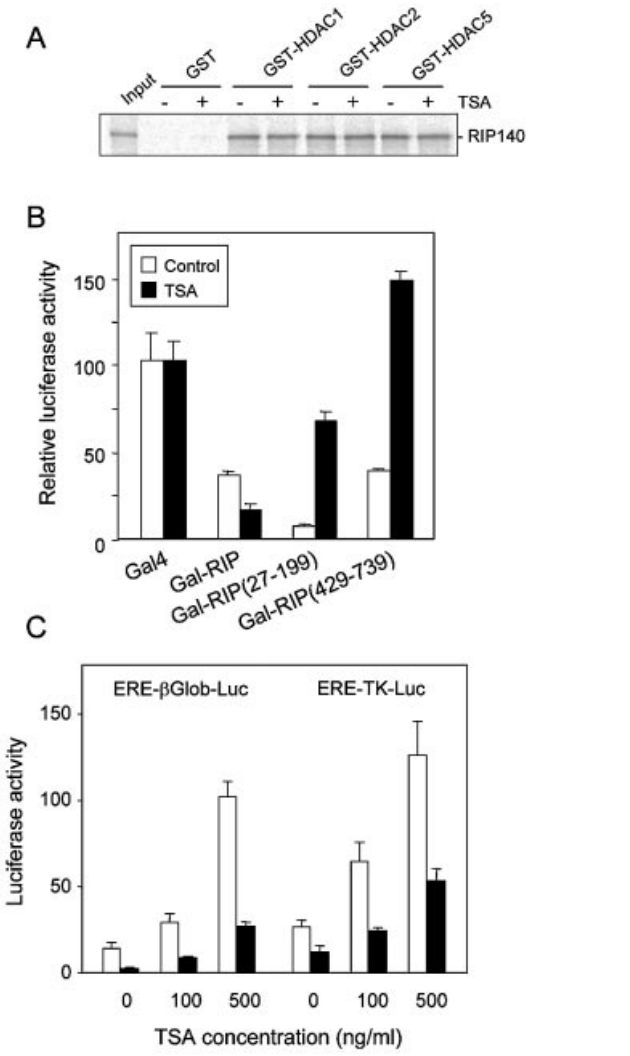

Figure 7. HDAC inhibition does not relieve RIP140 transcription repression. (**A**) GST pull-down assays were performed as described in Materials and Methods by incubating GST–HDAC1, 2 and 5 (encompassing residues 674–1113) or GST alone, together with radiolabelled RIP140 in the presence or absence of TSA (5 mg/ml). (**B**) HeLa cells were transiently transfected with the L8G5-Luc reporter construct together with the expression vector for LexA-VP16, Gal-RIP, Gal-RIP(27–199), Gal-RIP(429–739) or empty vector Gal4 alone (2 μg). Cells were then treated or not by TSA (500 ng/ml) for 24 h and luciferase activity was quantified as indicated in Materials and Methods. Results are expressed as relative luciferase activity and are the mean (±SD) of three values. (**C**) HeLa cells were transfected with the ERE-βGlob-Luc or ERE-TK-Luc reporter constructs (0.5 μg) together with the ERα-encoding plasmid and the expression vector for RIP140 (black boxes) or empty vector alone (white boxes). Luciferase activity was quantified as indicated in Materials and Methods after 24 h of stimulation by estradiol together with increasing concentrations of TSA. Results are expressed as raw luciferase activity and are the mean (±SD) of three values.

DISCUSSION

RIP140 is an atypical transcription regulator, which, despite its hormone-dependent recruitment by nuclear receptors, negatively regulates their transcriptional activation. In this study, we have characterized the interaction of RIP140 with different interacting proteins (CtBP and HDAC) which could act as downstream effectors, and we have defined the role of these molecules in estrogen transcriptional signalling.

Our results demonstrate that RIP140 interacts *in vitro* with both CtBP1 and CtBP2 by means of various domains. The association of CtBP1 with the central region of RIP140 involves two motifs (PIDLS and PINLS). Previous studies have identified CtBP-interacting proteins harbouring two binding sites, such as EBNA3A (36), xTcf-3 (37) and Evi-1 (38). This supports the notion that CtBPs could bind some of their target proteins as homo- or hetero-dimers (39) but such a binding stoichiometry remains to be demonstrated in the case of RIP140. In addition, a direct interaction between CtBPs and HDACs has been demonstrated [for a review see Chinnadurai (19)], and it is therefore conceivable that ternary complexes comprising HDAC/CtBP and RIP140 may exist under some conditions. Interestingly, since reticulocyte lysates contain detectable HDAC activity, we could speculate that the binding of CtBPs that we observed with the N-terminal moiety of RIP140 could be in fact indirect and mediated by HDACs through regions that do not contain a recognizable PxDLS motif [for a review see Turner and Crossley (40)]. Our data suggest that the relative importance of these different modes of recruitment by RIP140 may vary for CtBP1 and CtBP2 (see Fig. 2B and D). Several lines of evidence obtained from the corresponding gene inactivation studies in mice indicate that the two CtBPs might exhibit different properties (20). Moreover, it has been shown that SUMOylation preferentially affects the CtBP1 protein, controlling its cellular localization and its co-repressor function (41).

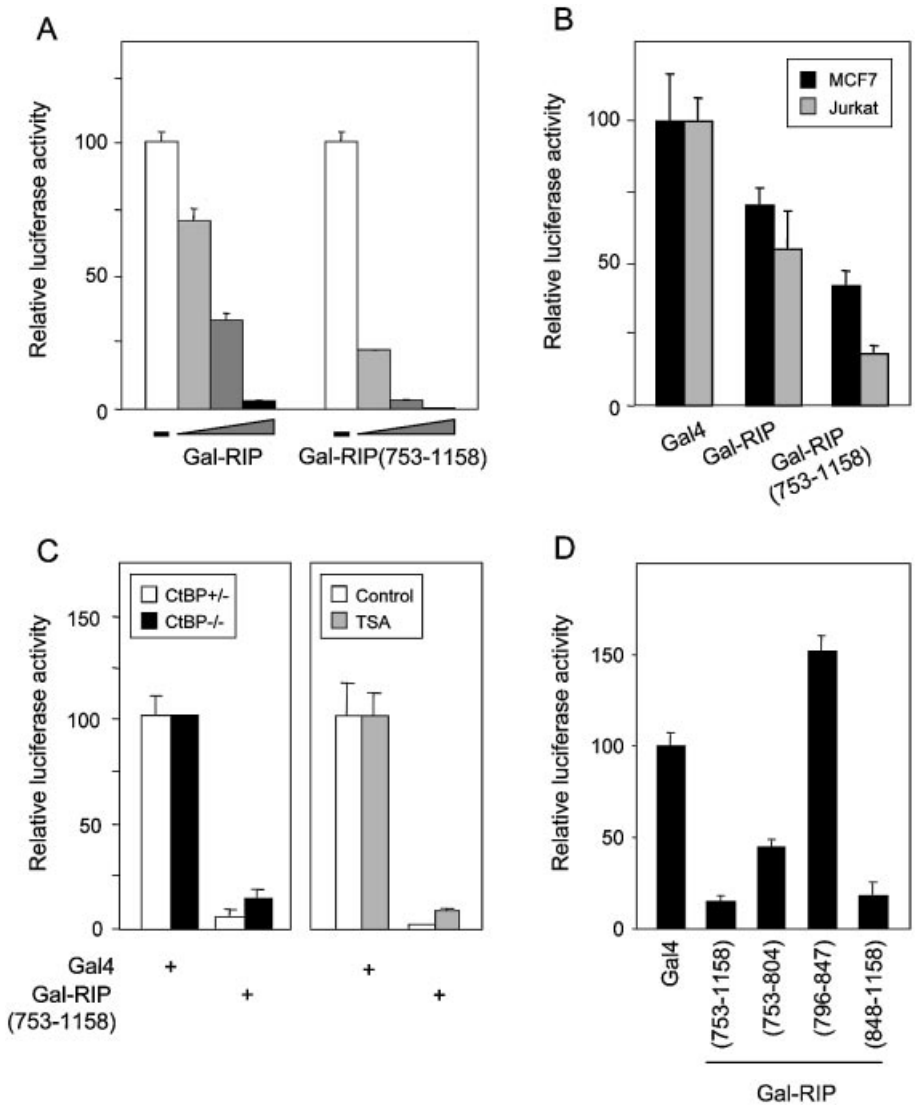

Figure 8. Two independent domains of the C-terminal region of RIP140 mediate transcriptional repression. (**A**) HeLa cells were transfected with the L8G5-Luc reporter and LexA-VP16, together with increasing amounts of Gal-RIP, Gal-RIP(753–1158) or empty Gal4 vectors (0.5, 1 and 2 μg). Luciferase activity was quantified 48 h after transfection, and the results, expressed as a percentage of control, are the mean (±SD) of three values. (**B**) MCF-7 cells (black boxes) and Jurkat T cells (grey boxes) were transfected with the pSV40G5E1BLuc reporter construct (1 μg) together with 1.5 μg of Gal-RIP, Gal-RIP(753–1158) mutant or Gal4 vector alone. Results are expressed as relative luciferase activity (% of control Gal4) and are the mean (±SD) of six values. (**C**) Gal-RIP(753–1158) or empty vector was transfected together with the L8G5 reporter and the LexA-VP16 in either $CtBP^{+/-}$ or $CtBP^{-/-}$ cells, using Lipofectamine 2000 reagent (left panel) or in HeLa cells (right panel). Relative luciferase activity was measured 24 h after TSA treatment (500 ng/ml) in HeLa cells and 48 h after transfection for MEFs. Results are expressed as a percentage of Gal4 activity. (**D**) HeLa cells were transiently transfected with the 17M5βGlob-Luc reporter construct (1 μg) together with the indicated Gal-RIP mutants (3 μg). Results are expressed as relative luciferase activity (% of control Gal4) and are the mean (±SD) of three values.

Despite the clear interaction *in vitro* with RIP140, we found that, in our model, CtBPs could only partly explain its repressive action. This was based on the functional inactivation of CtBPs using the CID of E1A and on the use of MEFs devoid of CtBP1 and CtBP2. In addition, as it has been shown that the co-repressor function of CtBPs could be regulated by the NAD^+/NADH ratio (42), we have tested whether agents that perturb the cellular redox status could modify the intrinsic repressive activity of RIP140. Treatment of cells with 200 μM $CoCl_2$, which has been shown to significantly enhance the repression of ZEB and its interaction with CtBP1 (42), did not modify the repressive activity of Gal-RIP (data not shown). Our results are in accordance with the data published by Vo *et al.* showing that the mutation of the PIDLS motif in the context of the full-length RIP140 only partially relieved the repression of an (ERE)2-pS2-CAT reporter gene (17). More recent data have confirmed that the CtBP-binding motif is not essential for intrinsic repression by RIP140 (43). However, it has been shown recently that Pak1 could phosphorylate CtBP and decrease its transcriptional repressive activity (44). The amount of phosphorylated CtBP, which may vary from one cell to another, could therefore account for variation in the level of CtBP-dependent repression by RIP140.

In the present study, we have also demonstrated that RIP140 associates with class II HDACs and have shown that a sequence comprised between residues 115 and 199 of RIP140

is needed for the interaction. Surprisingly, we found that the repressive activity of full-length RIP140 was not affected by inhibition of HDAC enzymatic activity. This contrasts with two previously published studies, which described a TSA-sensitive repression by RIP140 of RAR/RXR (16) or SF-1 (45). Our results are, however, not inconsistent, since several groups have reported that class IIa HDACs, i.e. HDAC4 (46,47), HDAC5 (22), HDAC7 (29) and HDAC9 (48), have the potential to repress transcription in a deacetylase-independent manner. HDAC10, which is a class IIb enzyme, exhibits the same property (49). Moreover, the highly homologous HDAC6 and HDAC10 both appear more resistant to inhibitors of HDAC activity (49,50). The precise pattern of expression of the different HDACs in estradiol-responsive cells is not known and it would be interesting to define the relative expression of class I and class II enzymes in these cells. The apparent discrepancy of our results with the data showing that the inhibitory effect of RIP140 on RAR/RXR is relieved by TSA (16) could be explained by the nature of the nuclear receptor and/or cell or promoter context. As pointed out by Fernandes *et al.* (51), the inhibition of estrogen- and glucocorticoid-dependent transcription by LCoR appeared sensitive to TSA, whereas progesterone- or vitamin D-dependent transcription was not affected. The demonstration of a regulatory role of HDACs in RIP140-mediated repression, independently of their deacetylase activity, would require their invalidation by either genetic recombination or small interfering RNA approaches. Interestingly, HDACs and CtBPs have been shown to participate in the negative regulation of a large number of transcription factors and, in the case of MEF2, both proteins appear to be recruited by MITR in a multicomponent co-repressor complex (52). Based on our results showing that RIP140 over-expression co-localizes with HDAC5 and amplifies its silencing activity (Fig. 6), it might be important to determine whether RIP140 could participate in the repression of other transcription factors such as MEF2.

Finally, our results indicate that RIP140 is a complex transcription factor, which possesses multiple transcription regulatory modules, including two newly identified domains in the C-terminal region of the protein (Fig. 9). During the revision of this manuscript, Christian *et al.* (53) also reported, in support of our conclusions, that the C-terminal region of RIP140 contained silencing domains. This C-terminal region supports strong repressive activity but does not require HDAC activity or CtBPs. We have investigated whether this region could bind other known transcription repressors such as Suv39H1 and HP1 proteins, but failed to detect a significant interaction, and we are currently trying to identify which partners could mediate this negative control of transcription. Interestingly, the mapping of several distinct domains is reminiscent of the situation observed for ER transcriptional coactivators such as members of the SRC family and CBP/p300, which also contain several separate activation domains (1). In addition, this also suggests that the overall repressive activity of RIP140 may depend on the interplay among these negative regulatory modules, which could be differently active according to the cell type, promoter or nuclear receptor considered.

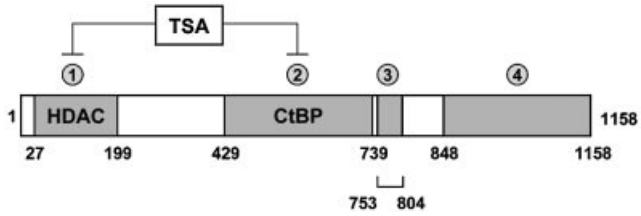

Figure 9. Schematic representation of the different silencing domains identified in RIP140. The position of each domain is indicated with the corresponding amino acid coordinates. The preferential binding of HDAC and CtBP on domains 1 and 2 is also shown, together with their sensitivity to TSA.

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