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# ***The transcriptional coregulator RIP140 represses E2F1 activity and discriminates breast cancer subtypes***

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## **Abstract**

### **Purpose**

**Receptor interacting protein of 140 kDa (RIP140) is a transcriptional cofactor for nuclear receptors involved in reproduction and energy homeostasis. Our aim was to investigate its role in the regulation of E2F1 activity and target genes, both in breast cancer cell lines and in tumor biopsies.**

### **Experimental Design**

**GST pull-down assays, coimmunoprecipitation experiments and ChIP analysis were used to evidence interaction between RIP140 and E2F1. The effects of RIP140 expression on E2F1 activity were determined using transient transfection and quantification of E2F-target mRNAs by RT-QPCR. The effect on cell cycle was assessed by FACS analysis on cells overexpressing GFP-tagged RIP140. A tumor microarray data set was used to investigate the expression of RIP140 and E2F1-target genes in 170 breast cancer patients.**

### **Results**

**We first evidenced the complex interaction between the RIP140 and E2F1 and showed that RIP140 represses E2F1 transactivation on various transiently transfected E2F-target promoters and inhibits the expression of several E2F1-target genes (such as CCNE1 and CCNB2). In agreement with a role for RIP140 in the control of E2F activity, we demonstrate that increasing RIP140 levels results in a reduction in the proportion of cells in S phase in various human cell lines. Finally, analysis of human breast cancers demonstrates that low RIP140 mRNA expression was associated with high E2F1-target gene levels and basal-like tumors.**

### **Conclusion**

**This study demonstrates that RIP140 is a regulator of the E2F pathway which discriminates luminal- and basal-like tumors, emphasizing the importance of these regulations for a clinical cancer phenotype.**

**MESH Keywords** Breast Neoplasms ; genetics ; pathology ; Cell Cycle ; Cell Line, Tumor ; E2F1 Transcription Factor ; antagonists & inhibitors ; Female ; Gene Expression Regulation, Neoplastic ; Humans ; Neoplasms, Basal Cell ; genetics ; Nuclear Receptor Co-Repressor 1 ; physiology ; Transcriptional Activation ; Transfection

**Author Keywords** breast cancer ; cell proliferation ; E2F1 ; RIP140 ; transcription

## **INTRODUCTION**

Cell cycle control is a fundamental process that governs cell proliferation and is frequently altered during tumorigenesis. E2Fs and their heterodimer partners, DPs, are central regulators of cell cycle progression and directly regulate the expression of a broad spectrum of genes involved for instance, in cell cycle regulation, DNA replication and repair, apoptosis, differentiation or development [1, 2].

E2F1, which was discovered as a protein promoting the transition to S phase, was the founding member of the E2F family which comprises eight members in mammals. Among this family, some were initially presented as “activator E2Fs” (E2F1, 2, and 3) while the other members were mostly known as transcription repressors although this classification now appeared too simplistic ([2] and reference herein). E2F transcriptional activity was shown to be regulated by a large number of coactivators or corepressors including the so-called pocket proteins which form the retinoblastoma (RB) tumor suppressor family (pRB, together with the related proteins p107 and p130) [3]. RB attenuates E2F action by recruiting transcriptional corepressors such as histone deacetylases (HDACs) to E2F-regulated promoters, thus mediating transcriptional repression of E2F-regulated genes [4, 5]. RB is a critical component of the cell cycle control machinery and, as a consequence, its loss or inactivation is a major mechanism by which cancer cells attain a growth advantage during tumorigenesis [6].

Our laboratory is engaged in the characterization of various transcriptional repressors which regulate another important class of transcription factors, namely nuclear hormone receptors. These receptors such as the estrogen and androgen receptors are also important

regulators of cell proliferation and strongly influence the growth of hormone-dependent cancers [7]. These receptors control gene expression through the recruitment of a large set of coregulatory proteins which regulate, either positively or negatively, chromatin structure and transcription initiation. Our work is mainly focused on RIP140 (Receptor Interacting Protein of 140 kDa also known as NRIP1), a nuclear protein of 1158 amino acids, initially identified as a transcription cofactor of estrogen receptors and shown to regulate energy homeostasis in metabolic tissues (for a review see [8]). RIP140 is an atypical coregulator since, despite its recruitment by agonist-liganded nuclear receptors, it exhibits a strong transcriptional repressive activity. We and others have deciphered the molecular mechanisms involved in this transrepression and identified four repressive domains within the RIP140 molecule [9][10]. We also demonstrated that RIP140 expression was increased upon estrogen or androgen stimulation, in breast [11] and prostate [12] cancer cells, respectively.

The present study identified RIP140 as a novel transcriptional repressor of E2F1 activity which significantly decreased E2F1-target gene expression both on transfected reporter constructs and on endogenous genes. In human breast cancers, a decrease in RIP140 expression is inversely correlated with E2F1-target gene expression and significantly associated with basal-like tumors which exhibit bad prognosis. Altogether this work identifies RIP140 as a new key actor of the E2F pathway and as a potential new prognostic marker in oncology.

## **MATERIALS AND METHODS**

### **Plasmids and reagents**

The E2F1 and DP1 expression vectors were given by Dr C. Sardet (IGMM, Montpellier, France), the E2F(3)TK-Luc and cyclin E-Luc reporter plasmids by Dr L. Fajas (IRCM, Montpellier) and the pGL2-ARF-Luc construct (-735 to +75) by Dr S-Y Shieh (Taipei, Taiwan) [13]. The 17M5βGlob-Luc construct and plasmids allowing RIP140 expression [9, 11, 12] have been described previously. The pEGFP-C2-RIP140 vector was a kind gift of Dr J. Zilliacus [14]. The deletion of the E2F interaction domain in the RIP140 sequence (from residues 119 to 199) was done using the QuickChange XL from Stratagene. The pRL-CMVbis plasmid (Ozyme, Saint Quentin Yvelines, France) was used to normalize transfection efficiency.

### **Cell culture, RNA extraction and quantitative PCR**

HeLa, MCF-7 and HEK293T human cancer cell lines were cultured as previously described [9]. Total RNA was extracted from cells using the TRIzol reagent (Invitrogen, Cergy Pontoise, France). Total RNA (2 µg) was subjected to a reverse-transcription step using the Superscript II reverse transcriptase (Invitrogen). Real-time quantitative polymerase chain reaction was performed using a SYBR Green approach (LightCycler, Roche Diagnostics, Meylan, France). Primer sequences are available upon request. For each sample, results were corrected for RS9 mRNA levels (reference gene) and were normalized to a calibrator sample.

### **Transient transfection, luciferase assays and cell cycle analysis**

MCF-7 cells were plated in 96-well plates (2.10<sup>4</sup> cells per well) 24h prior to DNA transfection with Jet-PEI (0.25µg of total DNA). Luciferase (firefly and renilla) values from transient transfection were measured [9] and all data were expressed as mean ± SD. For cell cycle analysis, cells were transfected with the RIP-GFP expression plasmid and the two populations (RIP-GFP- and RIP-GFP+) were separated. Cell cycle was analyzed with a FACs-Vatange flow cytometer (Becton-Dickinson, San Jose, CA) after propidium iodide labeling. The CellQuest and ModFit softwares were used to analyze data.

### **In vitro interaction assay and coimmunoprecipitation**

In vitro translation and GST pull-down assays were performed as previously described [9]. For coimmunoprecipitations, expression plasmids for E2F1 or c-myc-tagged RIP140 were transfected in HeLa cells using JetPEI (Ozyme, Saint Quentin Yvelines, France). After cell lysis in 50 mM Tris-HCl, pH8, 0.5% Nonidet P-40 supplemented with protease inhibitors, Transfected RIP140 and E2F1 were immunoprecipitated with the 9E10 monoclonal antibodies against the c-myc epitope or with the anti-E2F1 antibody (C-20) covalently bound to protein G-Sepharose beads. After incubation at 4°C during 2 h and 5 washes, immunoprecipitated proteins were eluted in Laemmli sample buffer, resolved by SDS-PAGE and detected by western blotting using primary antibodies against E2F or c-myc epitope.

### **Interaction of endogenous proteins (coimmunoprecipitation and ChIP analysis)**

For coimmunoprecipitation of endogenous proteins, 700µg of MCF-7 cell nuclear extracts (prepared using the NE-PER kit from Thermo Scientific) were incubated with 2µg of anti-E2F1 monoclonal antibody (KH95 sc-251 Santa Cruz) for 3 hours at room temperature. Beads coupled to Protein G (Ademtech G0433) were added to the immune complex (2 hrs at room temperature) and after 3 washes with lysis buffer, beads were resuspended in 20 µl of lysis buffer and analysed by western blotting using primary antibodies specific for E2F1 (KH95 sc-251 Santa Cruz) and RIP140 2656C6a (sc-81370 Santa Cruz). For ChIP analysis, MCF-7 cells (70% confluent) were synchronized using 4mM hydroxyurea (HU) during 24h and the block was released by changing the medium with 10% FCS supplementation for the indicated time. After PBS washing and cross-linking with 3, 7% formaldehyde during 10 min at 37°C, we

used the ChampionChIP One-Day Kit (SABiosciences) according to the manufacturer's recommendations and using either the antibody KH95 (sc-251 SantaCruz) or 2656C6a (sc-81370 Santa-Cruz) for E2F1 and RIP140 respectively, or no antibody as a control. Q-PCR was then performed using the Power SYBR Green PCR master mix (Applied Biosystems) on an Applied Biosystems 7300 thermal cycler with 2 $\mu$ l of material per point. Primers flanking the E2F site of the cyclin D1 promoter were 5'-GCAGCGGGGCGATTTGCATT-3' and 5'-AGCAAAGATCAAAGCCCGGAGAG-3'. The input DNA fraction corresponded to 1% of the immunoprecipitation.

### Microarray analysis

Microarray data (accession number GSE1992) of the study from Hu et al. [15 ] were obtained from the Gene Expression Omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi> ). Expression data from the 170 sample experiment were downloaded as normalised and log2 transformed Cy5/Cy3 ratios, where tumor sample RNA and human universal reference RNA were labeled with Cy5 and Cy3, respectively. Hierarchical pairwise average-linkage clustering of the 170 tumor specimens was performed on the basis of expression of RIP140 and 6 E2F1-target genes using the Cluster and TreeView software with median-centered gene expression values and Pearson correlation as similarity metrics. Results were analyzed for statistical significance using the 2-tailed Student's t test. For all analyses,  $p < 0.05$  was considered as significant.

## RESULTS

### RIP140 interacts with E2F1

Based on published data reporting that nuclear receptor coregulators were involved in the regulation of E2F1 activity [16–18 ], we hypothesized that RIP140 might also act as a transcriptional modulator of the E2F pathway.

Using in vitro GST pull-down assays, we first investigated whether RIP140 was able to interact with E2F1. We therefore performed pull-down assays with GST-E2F1 and in vitro labeled full-length RIP140. As shown in Figure 1B (left panel), data clearly demonstrated the binding of RIP140 to E2F1. We then tried to delineate the respective binding sites on the two proteins. The use of deletion mutants of E2F1 and RIP140 corresponding to the N-terminal or the C-terminal moiety of the two proteins (fused to GST for E2F1 and in vitro translated for RIP140) suggested the presence of at least two interaction domains on each protein (Figure 1B right panel). Indeed, the two in vitro translated fragments of RIP140 (fragment 1 to 480 and 480 to 1158) were retained by the GST-E2F1 chimaeric proteins encompassing regions from residues 1 to 123 or 123 to 437.

In order to further map the respective binding sites on RIP140, we used deletion mutants of RIP140 fused to GST. Data shown in Figure 1C (left panel) indicated that the N-terminal region of RIP140 encompassing residues 27 to 439 exhibited the strongest binding of in vitro translated full-length E2F1. No binding at all was observed with the central region of RIP140 whereas a faint interaction was detectable upon long exposure with the C-terminal region (residues 683 to 1158). This E2F1 binding region was clearly confirmed when we used the in vitro translated fragment of E2F1 corresponding to residues 1 to 123 (Figure 1C , left panel).

Moreover, using a series of deletion mutants in the N- and C-terminal moieties of RIP140, we showed that the minimal N-terminal E2F1 interaction domain (EID1) encompassed a region of 80 amino acids spanning from residues 119 to 199 whereas EID2 was mapped to a region spanning from residues 916 to 1158 (Figure 1A and Figure 1C , right panel). Finally, an E2F1 mutant lacking the transactivation domain which encompasses residues 380–437 was significantly less efficient (more than 7-fold decrease) than wild-type E2F1. This region corresponds to the pRB interaction domain which is strongly impaired by the Y411H mutation [19 ]. Very interestingly, this Y411H E2F1 mutant was also less efficient to interact with RIP140 (Figure 1D ), suggesting that the C-terminal RIP140 binding site on E2F1 might overlap that of pRB.

To demonstrate that this interaction between RIP140 and E2F1 also occurred in intact cells, we then set up co-immunoprecipitation experiments. As illustrated in Figure 2A (top panel), RIP140 was found associated with immunoprecipitated E2F1 and the reverse experiment confirmed the interaction between the two proteins (bottom panel). All controls performed with the sole expression of one partner, or with the use of beads alone confirmed the specificity of the interactions. In order to emphasize these results, we then analyzed the association between endogenous proteins in MCF7 breast cancer cells. Coimmunoprecipitation experiments demonstrated that E2F1 was able to specifically pull-down endogenous RIP140 (Figure 2B ). Finally, chromatin immunoprecipitation (ChIP) analysis was performed in MCF-7 cells on E2F binding sites in the cyclin D1 promoter which is transcriptionally repressed by E2F1 [20 ]. Data indicated a concomitant increase in E2F1 and RIP140 recruitment upon G1/S transition (Figure 2C ). Altogether, these results illustrated the interaction between endogenous RIP140 and E2F1, and binding of RIP140 on an E2F1 target promoter, thus strengthening the data presented in Figure 1 .

### RIP140 inhibits E2F1 transactivation

We next tested the ability of RIP140 to control E2F1 transactivation in transient transfection experiments using expression vectors for both E2F1 and DP1, together with different luciferase reporter vectors known to be regulated by E2Fs. We first used an artificial reporter

plasmid containing three copies of the E2F binding site upstream the thymidine kinase promoter (E2F<sub>3</sub>-TK-Luc). As shown in Figure 3A (left panel), when transfected in MCF-7 breast cancer cells, we observed a significant inhibition of E2F1 activity upon overexpression of RIP140. Similar inhibition of E2F1 transactivation by RIP140 (ranging from 4- to 8-fold) was obtained on two other reporter constructs corresponding to the natural cyclin E or ARF promoters (Figure 3A, middle and right panels, respectively) and, as shown in Figure 3B, the cyclin E promoter was dose-dependently repressed by RIP140. Moreover, the same repressive effect of RIP140 on E2F1 was observed upon transient transfection of other human cancer cell lines such as HeLa or HEK293T (data not shown) and was not restricted to E2F1. Indeed, we found that the two other activator E2Fs i.e. E2F2 and E2F3 not only interacted *in vitro* with RIP140 but were similarly inhibited upon RIP140 overexpression (see supplementary Figure S2).

In order to further characterize the mechanism of this inhibitory effect, we used plasmids allowing the expression of Gal4-E2F1 chimeric proteins fusing the DNA-binding domain of the Gal4 yeast transcription factor to the COOH-terminal moiety of E2F1 (amino acids 123 to 437 or 380 to 437 which both contain the transactivation domain). In these conditions, we obtained a similar dose-dependent transcriptional repression upon RIP140 overexpression (Figure 3C), suggesting that this effect was not due to a regulation of the binding of E2F1 to DNA or to its heterodimerization partners (DP proteins). Finally, we compared, the effect of RIP140 to that of the pocket protein pRB, and found that, in our experimental conditions, E2F1 activity on the cyclin E promoter was repressed to comparable levels although the residual transactivation by E2F1 (compared to basal level) was higher with RIP140 than with pRB (Figure 3D left panel). On the Gal-E2F1(380–437) chimaeric protein, the effect of RIP140 was slightly less efficient than that of pRB probably reflecting the loss of the N-terminal binding site for RIP140 (see above). Altogether, these results demonstrated that RIP140 was able to interact with E2F1 and to repress its transcriptional activity.

### **RIP140 decreases cell cycle progression and regulates E2F-target gene expression**

Having demonstrated that RIP140 was a novel repressor of E2F1 activity, we sought to directly address whether this regulation might impact cell proliferation. We first examined the effect of RIP140 overexpression on cell cycle distribution in various human cancer cell lines. HeLa cells were transfected with a GFP-RIP140 expression vector and two populations overexpressing (GFP-RIP+) or not (GFP-RIP-) RIP140 were separated and analyzed for cell cycle distribution by FACS.

As illustrated in Figure 4A (left panel), 48 hr after transfection, GFP-RIP+ cells (which overexpress RIP140) showed a strong and significant decrease in the S-phase cell population (from 22.1% to 10.1%) with a concomitant increase in the number of G<sub>1</sub>-phase cells (from 65.7% to 86.3%). Interestingly, a similar decrease in the fraction of S-phase cells was observed in two other human cell lines i.e. MCF-7 breast cancer cells and HEK293T transformed embryonic kidney cells (Figure 4A right panel), thus suggesting that this effect of RIP140 could represent a general feature. Controls corresponding to cells transfected with GFP alone and sorted as performed for GFP-RIP+ cells (data not shown) indicated that the effect was indeed due to the overexpression of RIP140.

Since the data shown in Figure 1 suggested that the NH<sub>2</sub>-terminal EID1 (residues 119 to 199) was the strongest binding site for *in vitro* translated full-length E2F1, we generated the corresponding RIP140 mutant ( $\Delta$ EID1 deleted from amino acids 119 to 199) as a fusion with GFP. In transient transfection experiments, we found that this  $\Delta$ EID1 mutant was significantly less efficient ( $p < 0.01$ ) to repress E2F1/DP1 transactivation on the cyclin E promoter (Figure 4B). This effect was specific of E2F transactivation since wild-type RIP140 and the  $\Delta$ EID1 deletion mutant inhibited estrogen receptor transcriptional activity to the same extent (data not shown). We also analyzed the effect of the  $\Delta$ EID1 mutant on cell cycle (Figure 4C). We found that its overexpression in MCF7 cells led to an increase in the proportion of cells in G<sub>1</sub> (as compared to GFP alone) and this increase was significantly lower ( $p < 0.01$ ) than that obtained with the wildtype GFP-RIP140 vector. These results thus indicated that deletion of the main E2F1 interacting domain significantly impaired RIP140 ability to repress E2F transactivation and to block cell cycle progression.

In an attempt to explain the growth effect associated with RIP140 expression, we then analyzed by quantitative RT PCR, the steady-state levels of various mRNAs known to be transcribed from E2F-target genes [4]. As shown in Figure 4D, we first noticed that in cells overexpressing the GFP-RIP140 expression vector, the mRNA levels of CCNE, CCNB2, CDC2 and CDC6 were strongly decreased as compared to cells which did not overexpressed GFP-RIP140. The negative regulation was however not general for all E2F-target genes since for instance, the DHFR mRNA levels were not significantly regulated (Figure 4C). Altogether, these results indicated that RIP140 controls cell cycle progression and regulates endogenous E2F-target gene expression.

### **Inverse correlation between RIP140 and E2F1-target genes in human breast cancers**

To determine the biological relevance of E2F control by RIP140 and to validate the regulation by RIP140 of some of the E2F1-target genes in human cancers, we analyzed the expression of RIP140 and E2F1-target genes on a tumor microarray data set representing 170 breast cancer patients [15]. We first selected among 16 known E2F1-target genes shown to be related to tumor proliferation, those presenting the most pronounced differential expression in tumors with low levels of RIP140 vs tumors with high levels of RIP140 (the two groups including tumors with log<sub>2</sub> RIP140 expression values lower and upper than 0, respectively). As shown in Figure 5A, six genes (CCNE1, MYBL2, BIRC5, E2F1, CCNB2 and CDC6) which presented the most important and significant differences in expression ratio

between tumors with low and high RIP140 mRNA levels were selected for clustering analysis. It should be noted that three of these genes i.e. CCNE1, CDC6 and CCNB2 were found regulated by RIP140 in MCF-7 cells (Figure 4C ).

The gene expression signature comprising RIP140 and the 6 selected E2F1-target genes was used to cluster the 170-point data set and is displayed as a condition tree in Figure 5B . This map clearly showed two regions of gene coregulation, low levels of RIP140 mRNA being associated with high levels of the E2F1-target genes whereas in tumors expressing high levels of RIP140 mRNA, the E2F1-target genes were underexpressed. As shown in Figure 5C , the mean expression levels of the six E2F1-target genes were significantly lower ( $p=1.5E-20$ ) in the group of tumors with high levels of RIP140. By contrast, although RIP140 is a known estrogen-target gene, the median expression of ESR1 was not significantly higher in tumors expressing high vs low levels of RIP140 mRNA (respectively 0.41 and 0.29,  $p>0.05$ ). Together, these data showing that RIP140-deficiency is inversely correlated with a signature of E2F1-target genes in human breast cancer, thus strongly strengthen our *in vitro* results.

### Low RIP140 mRNA levels are associated with “basal-like” human breast cancers

Human breast tumors are diverse in their natural history and their responsiveness to treatments. Variations in transcriptional programs account for much of the biological and clinical heterogeneity of breast cancers. Using hierarchical clustering of gene expression patterns, human breast tumors have been classified into five distinct subtypes arising from at least two distinct cell types (basal and luminal epithelial cells) and associated with significant differences in clinical outcome [21 ]. Using the tumor microarray data set from Hu et al. [15 ], we investigated how RIP140 could discriminate between these molecular subtypes and whether the regulation of E2F1-target genes by RIP140 could be observed in these distinct subclasses. We anticipated that it could be the case since a proliferation gene cluster including E2F1-target genes has been shown to be differentially expressed through the breast tumor subtypes [15 ] and reported as a hallmark of a series of prognosis molecular signatures [22 , 23 ]; [24 ]. The 170 tumors were ranked according to RIP140 gene expression and divided into two equal groups of 85 tumors with low and high RIP140 expression levels, respectively. As expected, the E2F1-target metagene expression almost mirrored the RIP140 mRNA levels in these two tumor groups (Figure 6A ). Interestingly, as shown in Figure 6B , the group of tumors with the lowest levels of RIP140 gene expression (left panel) included 87.5% of the tumors identified as basal-like tumors (black box) and only 17.9% of those diagnosed as luminal-like tumors (white box). By contrast, the group with high levels of RIP140 mRNA (right panel) contained 82.1% of the luminal-subtype tumors and only 12.5% of the basal-subtype tumors. It should be noted that 75.2% of the tumors included in this high RIP140 expression group belonged to the luminal subtype while only 5.9% were classified as basal-like tumors. In agreement with the fact that the low and high RIP140 expression groups exhibited opposite contents of luminal- and basal-like tumors, we found that basal-like tumors express low RIP140 and high E2F1-target metagene levels, respectively. By contrast, those identified as luminal-like tumors expressed high RIP140 and low E2F1-target metagene levels, respectively (Figure 6C ). Indeed, the RIP140 gene expression was 3.1-fold higher in luminal- than in basal-like tumors while the E2F1-target metagene level was 2.1-fold higher in basal- than in luminal-like tumors. Using the same data, we also analyzed the partition of luminal-like and basal-like tumors according to the expression of the RB gene or those of the E2F co-activators NCOA3, NCOA6, CBP and PCAF. Interestingly, variations of RIP140 expression were the most powerful to discriminate between luminal- and basal-like tumors in this cohort (Figure S1). Altogether, these data suggested that, in breast cancers, low RIP140 mRNA expression was associated with high E2F1-target gene levels and basal-like tumors.

## DISCUSSION

RIP140 was initially identified as a transcriptional repressor of ligand-activated nuclear hormone receptor, involved in the control of ovarian functions and metabolic pathways (for a review, see [25 ]). In the present study, we identified RIP140 as a novel transcriptional repressor of E2F1 and as a new important regulator of cell proliferation. Based on both *in vitro* protein-protein interaction assays, coimmunoprecipitation and ChIP experiments, our data clearly demonstrate that RIP140 is an E2F1 partner. Transcriptional repression of E2F1 activity by RIP140 was observed in transient transfections on various E2F-target promoters and strong inverse correlations between RIP140 and E2F-target genes were noted upon overexpression of RIP140 in breast cancer cells. More interestingly, the same observation ( i.e. low levels of RIP140 associated with high expression of E2F1 targets) was made using data obtained on a set of 170 human breast cancer samples.

Other nuclear receptor cofactors were previously reported as E2F regulators. However, most of these studies dealt with transcriptional activators such as CBP [26 ], PCAF [16 ] or more recently ASC-2/NCOA6 [17 ] or AIB1/NCOA3 [18 ]. RIP140, acting as a negative regulator of E2F1, thus appears to act similarly to the well-known pocket proteins [3 ] although no structural similarities could be detected between RIP140 and pRB, p107 or p130. It has been shown that pocket proteins exhibit some specificity towards E2F family members. Indeed, pRB targets preferentially the activator E2Fs (E2F1, 2 and 3), whereas p107 and p130 are involved in the regulation of E2F4 and E2F5 [1 ]. We are currently trying to determine whether RIP140 exhibits a similar specificity amongst E2F transcription factors and our preliminary results indicate that RIP140 is not specific for E2F1, E2F2 and E2F3 and also interacts with repressor E2Fs (Figure S2).

Very interestingly, our data also identified RIP140 as a new regulator of cell proliferation with a significant impact on cell cycle progression since transient overexpression of RIP140 significantly decreased the number of cells in S phase. The function of RIP140 in cell proliferation was supported by the observation that DNA synthesis (assessed by incorporation of 5-ethynyl-2'-deoxyuridine) was significantly increased in tissues from RIP140 knock-out mice [27] as compared to wild-type littermates (M. Lapierre, unpublished data). Moreover, in addition to their roles on cell cycle and cell proliferation, E2F1 and/or RB are also key players in the control of major biological processes such as apoptosis or differentiation and deregulation of this pathway is of major relevance in tumorigenesis [1, 3]. Experiments are therefore currently in progress in our laboratory to evaluate the relevance of RIP140 on the control of these parameters and more globally to determine whether RIP140 loss has a direct impact on cancer formation. Based on the data published on pocket proteins, this might not be obvious since, although RB<sup>-/+</sup> mice are prone to develop pituitary and thyroid tumors [28], no increase in tumor formation upon p107 or p130 gene invalidation was observed, except in an RB<sup>-/+</sup> background [3][29]. In addition, several studies have reported that deregulation of the E2F signaling pathway could be linked to antiestrogen resistance in breast cancers [30]. It will be therefore very important to investigate whether RIP140, through its effects on E2F signaling, could be involved in resistance of mammary tumors to antiestrogen therapy.

Concerning breast cancer, hierarchical clustering of microarray data led to a classification into at least four groups: luminal-like (including luminal A and B), basal-like, ERBB2+ and normal-like showing distinct clinical outcomes [21]. Such unsupervised analyses [15][31] as well as supervised analyses performed to define prognosis classifiers [22][23][24], identify proliferation genes as interesting markers for predicting relapse in breast cancer. Interestingly, when we analyzed published transcriptomic data obtained on human breast tumors [15], we clearly demonstrated that RIP140 mRNA levels discriminate between the different cancer subtypes classified as basal- or luminal-like based on the basis of molecular profiling. Our results indicate that low RIP140 mRNA levels correlate with basal-like tumors whereas those expressing high levels of RIP140 mRNA are mostly luminal-like (see Figure 6 and S1).

These data therefore suggest that RIP140 may help to improve molecular signatures used to classify breast cancers. However, further work is needed to assess the association of RIP140 expression with clinical outcome and to determine the relative contribution of this gene as compared to the different markers previously identified.

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## Footnotes:

**CONFLICT OF INTEREST** The authors declare no conflict of interest.

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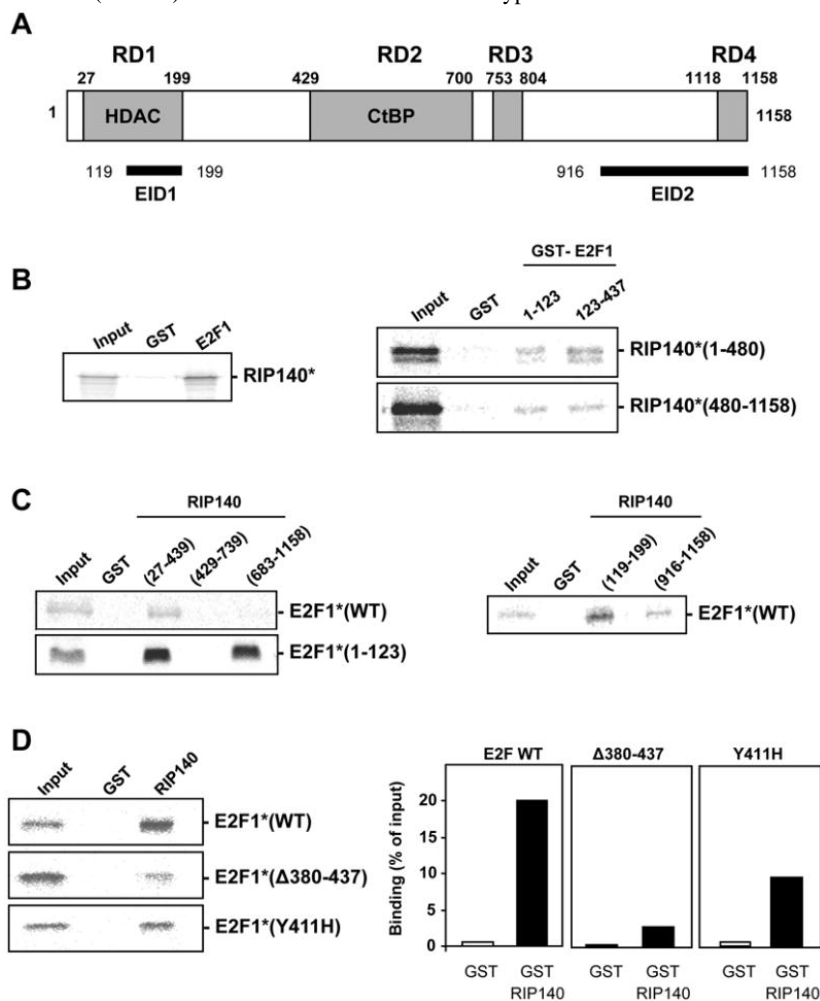
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**Figure 1**

RIP140 binds to E2F1

(A) **Schematic representation of the RIP140 molecule.** The figure shows the four repressive domains (RD) and the two-E2F1 interaction domains (EID) mapped on RIP140. (B and C) **In vitro interaction between RIP140 and E2F1.** GST pull-down assays were carried out as described in Material and Methods using GST or GST-E2F1 with <sup>35</sup>S-labelled wild-type RIP140 (B) or GST-RIP140 mutant proteins to retain <sup>35</sup>S-labelled wild-type E2F1 (C) Inputs represent 25% of the material used in the assays. (D) GST pull-down assays carried out with the GST-RIP(27-439) construct and <sup>35</sup>S-labelled wild-type or mutants E2F1.

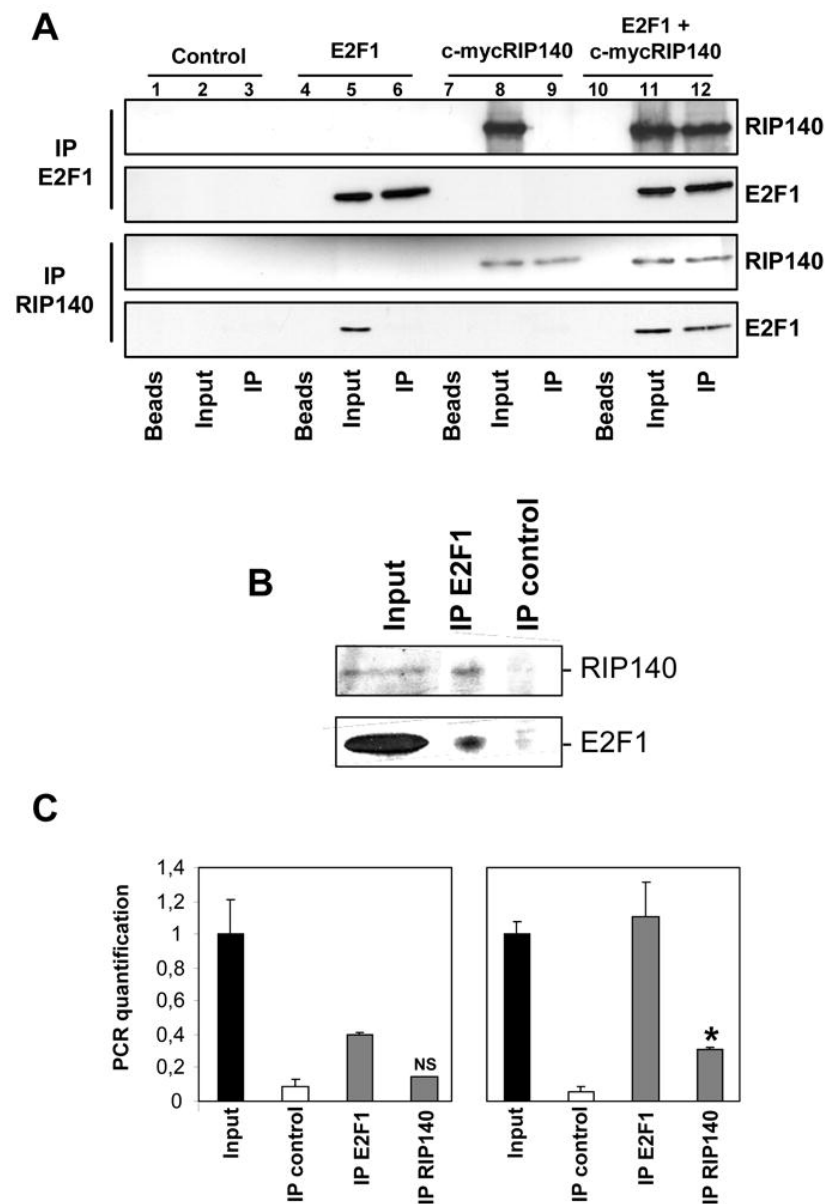




**Figure 2**

Interaction between RIP140 and E2F1 in intact cells

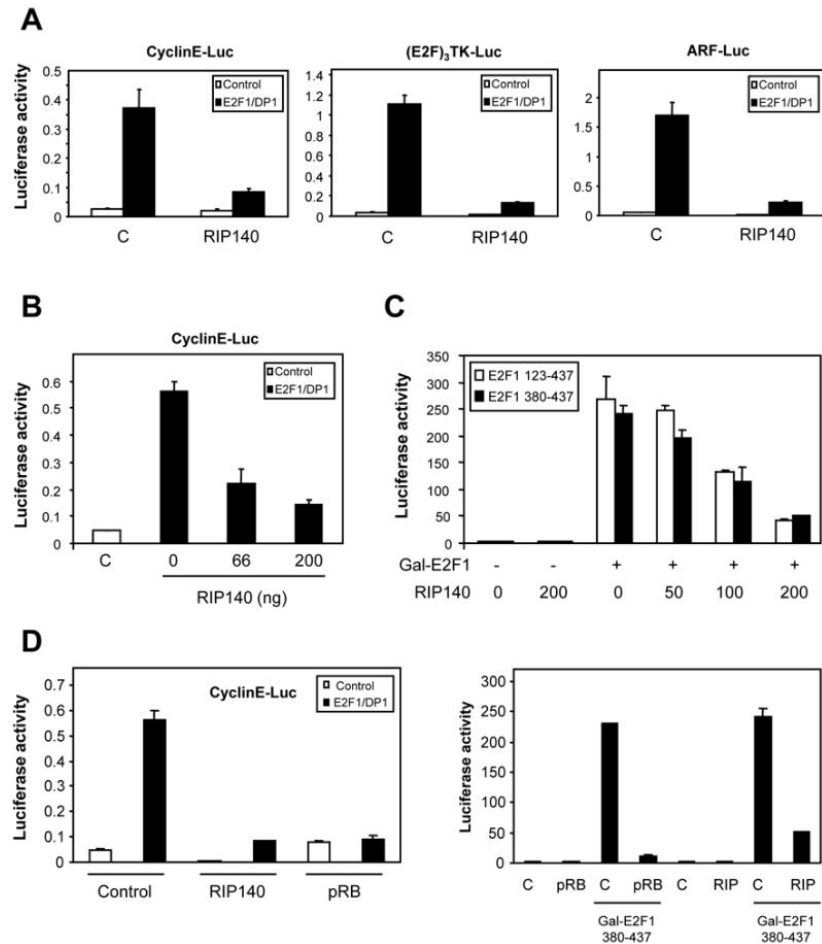
**(A) Co-immunoprecipitation of RIP140 and E2F1.** HeLa cells were either transfected with pCDNA3-E2F1 (lanes 4–6) or pEF-c-mycRIP140 (lanes 7–9) alone or co-transfected with pCDNA3-E2F1 and pEF-c-mycRIP140 (lanes 10–12). Immunoprecipitations were done using the anti-E2F1 polyclonal antibody (IP E2F1) or the anti-c-myc monoclonal antibody (IP RIP140) and Western blots were performed with the same antibodies as indicated. For each immunoprecipitation (IP), inputs and non-specific retention on beads alone are shown. **(B) Coimmunoprecipitation of endogenous proteins.** Nuclear extracts from MCF-7 human breast cancer cells were immunoprecipitated using the anti-E2F1 polyclonal antibody C20 (sc-193 Santa-Cruz) or an irrelevant antibody (sc-510 anti-Gal4 DBD antibody Santa-Cruz). Western blots were performed as indicated with the same E2F1 antibody and with the anti-RIP140 monoclonal antibody (sc-81370 Santa Cruz). Input representing 10% of the amount used for immunoprecipitation is also shown. **(C) Chromatin immunoprecipitation.** MCF-7 cells were analyzed either when HU-synchronised (left panel) or 2hrs after block release (right panel). The proportions of cells in G0/G1 and S phases were 92.4% vs 2.7% (left panel) and 62.3% vs 31.4% (right panel). ChIP analysis was performed as described in Material and Methods. Graphics represent the results of PCR quantifications using amplimers in the cyclin D1 promoter. Inputs were arbitrary set at 1 and represent 1/1000 of immunoprecipitation values. Statistical analysis was performed using the Mann-Whitney test with reference to the control without antibody (\*  $p < 0.05$  and NS non significant).



**Figure 3**

RIP140 represses its transcriptional activity

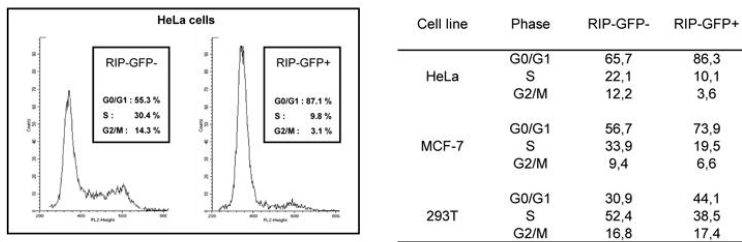
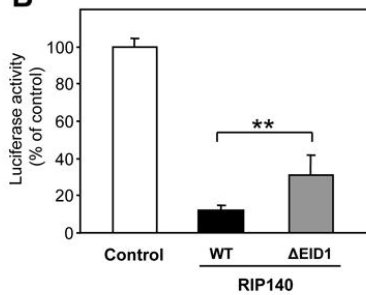
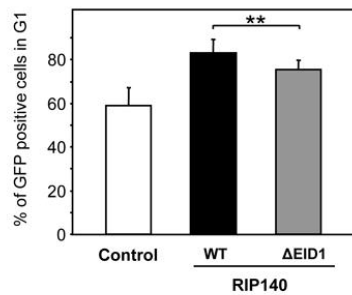
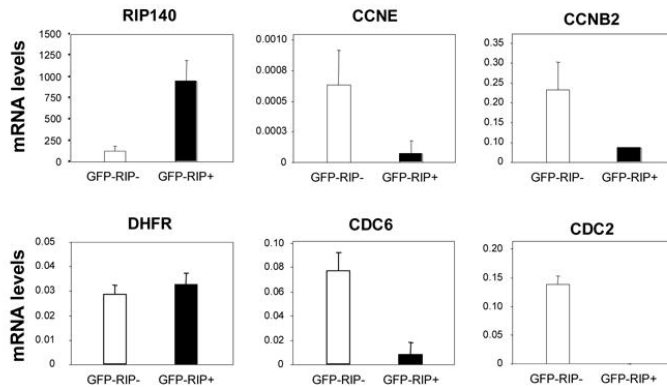
**(A) Effect on different reporter constructs.** MCF-7 human breast cancer cells were transiently transfected as indicated in Materials and Methods, with the E2F<sub>3</sub>-TK-Luc (left panel), cyclinE-Luc (middle panel) or ARF-Luc (right panel) reporter plasmid (25ng) together with expression vectors for E2F1 and DP1 (25ng each) in the presence or absence of RIP140 expression vector (250ng). Luciferase activity represents the mean (±SD) of three values. **(B) Dose-dependent regulation of cyclin E reporter.** MCF-7 cells were transfected as in (A) with increasing concentrations of RIP140 expression vector. **(C) Effect on Gal4-E2F1 fusion proteins.** MCF-7 cells were transiently transfected as indicated in Materials and Methods with the 17M5βGlob-Luc reporter plasmid (25ng) together with 25 ng of expression vectors for Gal4-E2F1(123–437 or Gal4-E2F1(380–437) in the presence or absence of increasing concentrations of RIP140 expression vector as indicated. Luciferase activity represents the mean (±SD) of three values. **(D) Comparison with the repressive activity of pRB.** MCF-7 cells were transiently transfected as in (B) and (C) respectively for the left and right panels with expression vectors for RIP140 or pRB (200ng).



**Figure 4**

## RIP140 controls cell cycle progression

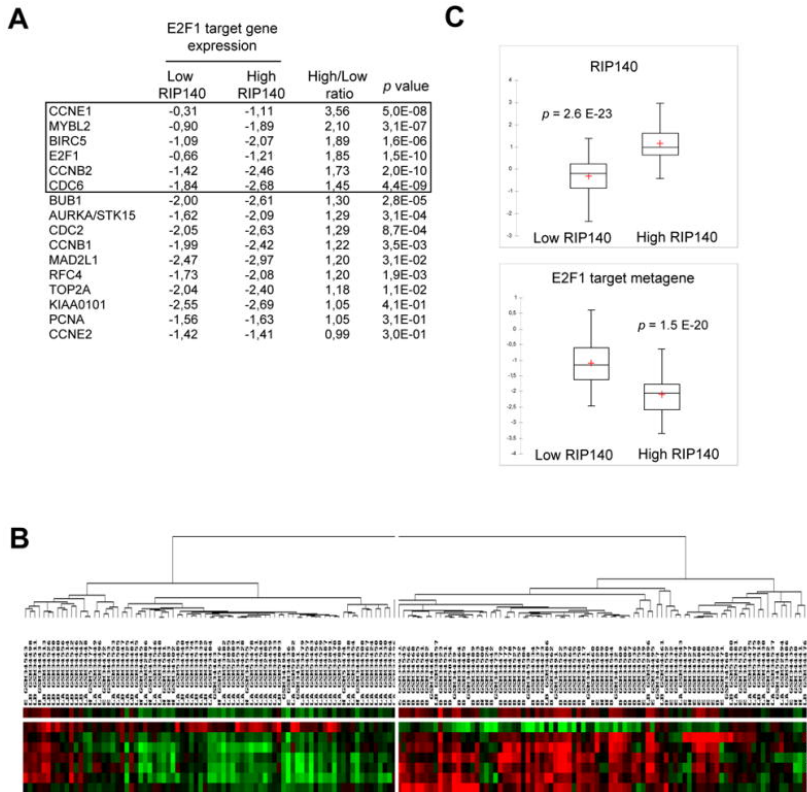
**(A) Effect of RIP140 over-expression on cell cycle** . Cells were transfected by pEGFP-C2-RIP140 plasmid and analyzed by flow cytometry as described in Materials and Methods. Left panel shows an experiment performed in HeLa cells with the profiles of relative iodide fluorescence versus the number of cells for each population (GFP-RIP+ vs GFP-RIP-). In the right panel, the breakdown of the population according to cell cycle phase is indicated for each condition (mean of 3 independent experiments). **(B and C) Deletion of the N-terminal E2F1 interaction domain**. (B). MCF-7 cells were transiently transfected with E2F1 and DP1 together with the cyclinE-Luc and expression vectors for wild-type RIP140 or the  $\Delta$ EID1 mutant. (C) Analysis of the proportion of MCF-7 cells in G1 after transfection of expression vectors for wild-type or  $\Delta$ EID1 mutated RIP140. Statistical analysis were performed using the Mann-Whitney test (\*\*  $p < 0.01$ ). **(D) Analysis of mRNA expression** . Quantitative real-time PCR analysis of gene expression was performed using total RNA extracted from MCF-7 cells as described in Material and Methods . The nature of the corresponding mRNAs is indicated above graphs. Results are represented as raw data after correction with RS9 values.

**A****B****C****D**

**Figure 5**

Inverse correlation between RIP140 and E2F1-target gene expression in breast cancer

**(A) Differential statistical analyses between 2 groups of tumor specimens defined by RIP140 expression values.** The 170 samples from the Hu et al study [15] were separated into two groups according to their RIP140 expression levels. Fifty-five (low RIP140 expression) and 115 (high RIP140 expression) tumors exhibited log<sub>2</sub>-transformed RIP140 expression values lower and upper than 0, respectively. The median expression of each of 16 E2F1-target genes was determined within each of the 2 groups and the ratios of the respective median values were calculated. **(B) Hierarchical clustering of the 170 samples on the basis of expression of RIP140 and E2F1-target genes.** Six E2F1-target genes with expression ratios  $\geq 1.45$  between high and low RIP140 expression tumor groups were selected (boxed in panel A) and used for cluster analysis of the 170 tumor specimens. **(C) Boxplot analyses of the expression values for RIP140 and E2F1-target metagene into the 2 groups defined by RIP140 expression.** The expression value of the E2F1-target metagene was defined in each tumor as the mean expression of the 6 E2F1-target genes selected in (A). RIP140 and metagene expression values were visualised for the low and high RIP140 tumor groups using boxplots.



**Figure 6**

RIP140 expression discriminates luminal and basal breast cancer subtypes

**(A) E2F1-target metagene expression in the low and high RIP140 expression tumor groups.** The 170 breast tumors of the study from Hu et al. [15] were ranked according to RIP140 gene expression and divided into two equal groups (85 tumors) exhibiting low and high RIP140 expression, respectively. Expression values for RIP140 and E2F1-target metagene (defined as the mean expression of the 6 E2F1-target genes selected in Figure 4A) are expressed as means  $\pm$  SE and p-values (Student's t test) are indicated. **(B) Relationship between RIP140 gene expression levels and molecular breast tumor subtypes.** For the two groups of tumors defined in (A) and expressing low and high RIP140 mRNA (left and right panel, respectively), luminal-like (white), basal-like (black), ERBB2+ (left-hatched), normal-like (grey) and unclassified (right-hatched) tumors are indicated. The statistical significance was assessed using a Chi2 analysis ( $p = 3.9E-13$ ). **(C) RIP140 and E2F1-target metagene expression levels in luminal-and basal-like tumors.** The expression values of RIP140 and E2F1-target metagene in the basal- ( $n=40$ ) and luminal-like ( $n=78$ ) tumors of the cohort are expressed as means  $\pm$  SE and p-values are indicated.

