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: Glutathione S-transferase pull-down assays, coimmunoprecipitation experiments, and chromatin immunoprecipitation 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 quantitative real-time PCR. The effect on cell cycle was assessed by fluorescence-activated cell sorting analysis on cells overexpressing green fluorescent protein-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 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 show 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 shows that low RIP140 mRNA expression was associated with high E2F1 target gene levels and basal-like tumors.

Conclusion: This study shows 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.

Cell cycle control is a fundamental process that governs cell proliferation and is frequently altered during tumorigenesis. E2Fs and their heterodimer partners (DP) 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, E2F2, and E2F3), whereas the other members were mostly known as transcription repressors, although this classification now seemed too simplistic (reviewed in ref. 2 and references therein). 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; ref. 3). pRB attenuates E2F action by recruiting transcriptional corepressors such as histone deacetylases 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 (i.e., 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 1,158 amino acids, initially identified as a transcription cofactor of estrogen receptors and shown to regulate energy homeostasis in metabolic tissues (see ref. 8 for a review). RIP140 is an atypical coregulator because, despite its repressor activity, RIP140 has deciphered the molecular mechanisms involved in translational modifications being associated with basal-like tumors, which significantly associated with basal-like tumors, which exhibit a strong transcriptional repressive activity. We and others have deciphered the molecular mechanisms involved in translational modifications being associated with basal-like tumors. These findings indicate that this transcription coregulator may play an important role in mammary carcinogenesis and represent a novel prognostic marker or therapeutic target for breast cancer.

**Translational Relevance**

Nuclear receptor transcriptional coregulators are implicated in a large variety of human pathologies. They can be involved in cancer development or with cancer progression or with recurrence following tamoxifen monotherapy. Receptor-interacting protein of 140 kDa (RIP140) is one of these nuclear receptor transcriptional coregulators, and we report here the first study investigating its role in the E2F signaling pathway. We show that RIP140 interacts with E2F1, represses its transcriptional activity, and affects cell cycle progression. In support of these observations, we found that RIP140 expression was inversely correlated with a signature of E2F1 target genes and discriminated breast cancer subtypes, low levels of expression being associated with basal-like tumors. These findings indicate that this transcription coregulator may play an important role in mammary carcinogenesis and represent a novel prognostic marker or therapeutic target for breast cancer.

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. Claude Sardet (Institut de Génétique Moléculaire de Montpellier, Montpellier, France), the (E2F)3-TK-Luc and cyclin E-Luc reporter plasmids by Dr. L. Fajas (Institut de Référence en Cancérologie de Montpellier, Montpellier, France), and the pGL2-ARF-Luc construct (−735 to +75) by Dr. S-Y. Shieh (Institute of Biochemistry and Molecular Biology, Taipei, Taiwan; ref. 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 QuikChange XL from Stratagene. The pRL-CMVbis plasmid (Ozyme) 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). Total RNA (2 μg) was subjected to a reverse transcription step using the SuperScript II reverse transcriptase (Invitrogen). Real-time quantitative PCR was done using a SYBR Green approach (LightCycler, Roche Diagnostics). Primer sequences are available on request. For each sample, results were corrected for R9 mRNA levels (reference gene) and normalized to a calibrator sample.

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

MCF-7 cells were plated in 96-well plates (2.10^4 per well) 24 hours before DNA transfection with JetPEI (0.25 μg 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-green fluorescent protein (GFP) expression plasmid, and the two populations (RIP-GFP⁺ and RIP-GFP⁻) were separated. Cell cycle was analyzed with a FACSVantage flow cytometer (Becton Dickinson) after propidium iodide labeling. The CellQuest and ModFit softwares were used to analyze data.

**In vitro interaction assay and coimmunoprecipitation**

In vitro translation and glutathione S-transferase (GST) pull-down assays were done as previously described (9). For coimmunoprecipitations, expression plasmids for E2F1 or c-myc–tagged RIP140 were transfected in HeLa cells using JetPEI (Ozyme). After cell lysis in 50 mmol/L Tris-HCl (pH 8), 0.5% NP40 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 hours and five 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 chromatin immunoprecipitation analysis)**

For coimmunoprecipitation of endogenous proteins, 700 μg of MCF-7 cell nuclear extracts (prepared using the NE-PER kit from Thermo Scientific) were incubated with 2 μg of anti-E2F1 monoclonal antibody (KH95; Santa Cruz Biotechnology) for 3 hours at room temperature. Beads coupled to protein G (Ademtech) were added to the immune complex (2 h at room temperature), and after three washes with lysis buffer, beads were resuspended in 20 μL of lysis buffer and analyzed by Western blotting using primary antibodies specific for E2F1 (KH95) and RIP140 2656C6a (Santa Cruz Biotechnology). For chromatin immunoprecipitation (ChIP) analysis, MCF-7 cells (70% confluent) were synchronized using 4 mmol/L hydroxyurea during 24 hours, 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 minutes at 37°C, we used the ChampionChIP One-Day kit (SABiosciences) according to the manufacturer's recommendations and using either the antibody KH95 or 2656C6a for E2F1 and RIP140, respectively, or no antibody as a control. Quantitative PCR was then done using the Power SYBR Green PCR master mix (Applied Biosystems) on an Applied Biosystems 7300 thermal cycler with 2 μL of material per point. Primers flanking the E2F site of the cyclin D1 promoter were 5′-GCAGCGGGGCGATT-3′ and 5′-AGCAAAGATCAAAGGCCGAGAG-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 database (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi). Expression data from the 170 sample experiment were downloaded as normalized 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 done on the basis of expression of RIP140 and six 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 two-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 did pull-down assays with GST-E2F1 and in vitro labeled full-length RIP140. As shown in Fig. 1B (left), data clearly showed 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 NH2-terminal or the COOH-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 (Fig. 1B, right). Indeed, the two in vitro translated fragments of RIP140 (fragments 1–480 and 480–1158) were retained by the GST-E2F1 chimeric proteins encompassing regions from residues 1 to 123 or 123 to 437.

To further map the respective binding sites on RIP140, we used deletion mutants of RIP140 fused to GST. Data...
### Table

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### Figures

#### A

A histogram showing the distribution of counts for R<sup>+</sup> and R<sup>-</sup> in HeLa cells.

#### B

A bar graph showing luciferase activity (% of control) for Control, WT, and ΔEID1 in RIP140.

#### C

A bar graph showing the percentage of GFP-positive cells in G<sub>1</sub> for Control, WT, and ΔEID1 in RIP140.

#### D

A panel showing mRNA levels for RIP140, CCNE, CCNB2, DHFR, CDC6, and CDC2 for GFP-RIP<sup>-</sup> and GFP-RIP<sup>+</sup>.
shown in Fig. 1C (left) indicated that the NH₂-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 on long exposure with the COOH-terminal region (residues 683–1158). This E2F1-binding region was clearly confirmed when we used the in vitro translated fragment of E2F1 corresponding to residues 1 to 123 (Fig. 1C, left).

Moreover, using a series of deletion mutants in the NH₂- and COOH-terminal moieties of RIP140, we showed that the minimal NH₂-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 (Fig. 1A and C, right). Finally, an E2F1 mutant lacking the transactivation domain that encompasses residues 380 to 437 was significantly less efficient (>7-fold decrease) than wild-type (WT) 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 (Fig. 1D), suggesting that the COOH-terminal RIP140-binding site on E2F1 might overlap that of pRB.

To show that this interaction between RIP140 and E2F1 also occurred in intact cells, we then set up coimmunoprecipitation experiments. As illustrated in Fig. 2A (top), RIP140 was found associated with immunoprecipitated E2F1 and the reverse experiment confirmed the interaction between the two proteins (bottom). All controls done with the sole expression of one partner or with the use of beads alone confirmed the specificity of the interactions. To emphasize these results, we then analyzed the association between endogenous proteins in MCF-7 breast cancer cells. Coimmunoprecipitation experiments showed that E2F1 was able to specifically pull down endogenous RIP140 (Fig. 2B). Finally, ChIP analysis was done 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 on G1-S transition (Fig. 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 Fig. 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)₃-TK-Luc]. As shown in Fig. 3A (left), when transfected in MCF-7 breast cancer cells, we observed a significant inhibition of E2F1 activity on 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 (Fig. 3A, middle and right, respectively), and as shown in Fig. 3B, the cyclin E promoter was dose dependently repressed by RIP140. Moreover, the same repressive effect of RIP140 on E2F1 was observed on 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 also were similarly inhibited on RIP140 overexpression (see Supplementary Fig. S2).

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 123–437 or 380–437, which both contain the transactivation domain). In these conditions, we obtained a similar dose-dependent transcriptional repression on RIP140 overexpression (Fig. 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 with 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 with basal level) was higher with RIP140 than with pRB (Fig. 3D, left). On the Gal4-E2F1(380–437) chimeric protein, the effect of RIP140 was slightly less efficient than that of pRB, probably reflecting the loss of the NH₂-terminal–binding site for RIP140 (see above). Altogether, these results showed 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 shown that RIP140 was a novel repressor of E2F1 activity, we sought to directly address whether this
regulation might affect 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 (RIP-GFP+) or not (RIP-GFP−) RIP140 were separated and analyzed for cell cycle distribution by fluorescence-activated cell sorting.

As illustrated in Fig. 4A (left), 48 hours after transfection, RIP-GFP+ 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 G1-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; Fig. 4A, right), thus suggesting that this effect of RIP140 could represent a general feature. Controls corresponding to cells transfected with GFP alone and sorted as done for RIP-GFP+ cells (data not shown) indicated that the effect was indeed due to the overexpression of RIP140.

Because the data shown in Fig. 1 suggested that the NH2-terminal EID1 (residues 119–199) was the strongest binding site for in vitro translated full-length E2F1, we generated the corresponding RIP140 mutant (ΔEID1 deleted from amino acids 119–199) as a fusion with GFP. In transient transfection experiments, we found that this ΔEID1 mutant was significantly less efficient (P < 0.01) to repress E2F1/DP1 transactivation on the cyclin E promoter (Fig. 4B). This effect was specific of E2F transactivation because WT RIP140 and the ΔEID1 deletion mutant inhibited estrogen receptor transcriptional activity to the same extent (data not shown).

We also analyzed the effect of the ΔEID1 mutant on cell cycle (Fig. 4C). We found that its overexpression in MCF-7 cells led to an increase in the proportion of cells in G1 (compared with GFP alone), and this increase was significantly lower (P < 0.01) than that obtained with the WT GFP-RIP40 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 real-time PCR the steady-state levels of various mRNAs known to be transcribed from E2F target genes (4). As shown in Fig. 4D, we first noticed that in cells overexpressing the GFP-RIP140 expression vector, the mRNA levels of CCNE1, CCNB2, CDC2, and CDC6 were strongly decreased compared with cells that did not overexpressed GFP-RIP140. The negative regulation was, however, not general for all E2F-traget genes because, for instance, the dihydrofolate reductase mRNA levels were not significantly regulated (Fig. 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 versus tumors with high levels of RIP140 (the two groups including tumors with log2 RIP140 expression values lower and upper than 0, respectively). As shown in Fig. 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 (Fig. 4C).

The gene expression signature comprising RIP140 and the six selected E2F1 target genes was used to cluster the 170-point data set and is displayed as a condition tree in Fig. 5B. This map clearly showed two regions of gene correlation, 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 underepressed. As shown in Fig. 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 versus low levels of RIP140 mRNA (0.41 and 0.29, respectively; P > 0.05). Together, these data show that RIP140 deficiency is inversely correlated with a signature of E2F1 target genes in human breast cancer, thus strongly strengthening 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 because a proliferation gene cluster including E2F1 target genes is differentially expressed through
the breast tumor subtypes (15) and reported as a hallmark of a series of prognosis molecular signatures (22–24). 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 (Fig. 6A). Interestingly, as shown in Fig. 6B, the group of tumors with the lowest levels of RIP140 gene expression (left) 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) contained 82.1% of the luminal-subtype tumors and only 12.5% of the basal-subtype tumors. It should be noted that

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Fig. 5. Inverse correlation between RIP140 and E2F1 target gene expression in breast cancer. A, differential statistical analyses between two 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 log2-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 two 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 ≥1.45 between high and low RIP140 expression tumor groups were selected (boxed in A) and used for cluster analysis of the 170 tumor specimens. C, box plot analyses of the expression values for RIP140 and E2F1 target metagene into the two groups defined by RIP140 expression. The expression value of the E2F1 target metagene was defined in each tumor as the mean expression of the six E2F1 target genes selected in A. RIP140 and metagene expression values were visualized for the low and high RIP140 tumor groups using box plots.
75.2% of the tumors included in this high RIP140 expression group belonged to the luminal subtype, whereas 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 (Fig. 6C). Indeed, the RIP140 gene expression was 3.1-fold higher in luminal-like than in basal-like tumors, whereas the E2F1 target metagene level was 2.1-fold higher in basal-like than in luminal-like tumors. Using the same data, we also analyzed the partition of luminal- and basal-like tumors according to the expression of the RB gene or those of the E2F coactivators 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 (Supplementary Fig. 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 (see ref. 25 for a review). 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 show 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 on 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 seems 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 toward E2F family members. Indeed, pRB targets preferentially the activator E2Fs (E2F1, E2F2, and E2F3), 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 among E2F transcription factors, and our preliminary results indicate

5 M. Lapierre, unpublished data.
that RIP140 is not specific for E2F1, E2F2, and E2F3 and also interacts with repressor E2Fs (Supplementary Fig. S2).

Very interestingly, our data also identified RIP140 as a new regulator of cell proliferation with a significant effect on cell cycle progression because 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-ethyl-2'-deoxyuridine) was significantly increased in tissues from RIP140 knockout mice (27) compared with WT littermates.5 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 effect on cancer formation. Based on the data published on pocket proteins, this might not be obvious because, although RB−/− mice are prone to develop pituitary and thyroid tumors (28), no increase in tumor formation on p107 or p130 gene invalidation was observed, except in an RB−/− 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.

About 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 done to define prognosis classifiers (22–24), 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 showed that RIP140 mRNA levels discriminate between the different cancer subtypes classified as basal- or luminal-like based on 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 Fig. 6; Supplementary Fig. 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 compared with the different markers previously identified.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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