Received: 17 September 2024 | Revised: 18 February 2025 | Accepted: 25 February 2025

DOI: 10.1002/ijc.35405

RESEARCH ARTICLE

Molecular Cancer Biology

IJC INTERNATIONAL JOURNAL of CANCER | UICC

The transcription factor RIP140 regulates interferon γ signaling in breast cancer

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Funding information

Groupement des Entreprises Françaises dans la lutte contre le Cancer

Abstract

RIP140 (receptor interacting protein of 140 kDa) is an important player in breast cancer (BC) by regulating key cellular pathways such as nuclear hormone receptor signaling. In order to identify additional genes specifically regulated by RIP140 in BC, we performed a transcriptomic analysis after silencing its expression in MCF-7 cells. We identified the interferon γ (IFNγ) signaling as being substantially repressed by RIP140 knockdown. Using the *GBP1* (guanylate binding protein 1) gene as a reporter of IFNγ signaling, we demonstrated its robust induction by RIP140 through an ISRE motif, leading to a significant reduction of its induction upon IFNγ treatment. Furthermore, we showed that low levels of RIP140 amplified the IFNγ-dependent inhibition of BC cell proliferation. In line with these data, reanalysis of transcriptomic data obtained in human BC samples revealed that IFNγ levels were associated with good prognosis only for BC patients exhibiting tumors expressing low levels of RIP140, thus confirming its effect on the anti-tumor activity of IFNγ provided by our experimental data. Altogether, this study identifies RIP140 as a new regulator of IFNγ signaling in breast tumorigenesis.

KEYWORDS

breast cancer, cell proliferation, GBP1, gene expression, interferon-γ, RIP140/NRIP1

What's New?

Interferon γ (IFNγ) is a key player in breast cancer, with the potential to induce apoptosis and to shift tumors into a dormant state. However, it also may promote carcinogenesis and angiogenesis. In this study, transcriptomic analysis identified the transcription factor RIP140 as a key regulator of IFNγ signaling in breast cancer. In different breast cancer cell lines, RIP140 was found to repress the expression of the IFNγ-dependent gene *GBP1* and to restrict the antiproliferative effects of IFNγ. In patient samples, elevated RIP140 levels abolished correlations between IFNγ expression and good prognosis, suggesting that RIP140 expression is also clinically relevant.

Abbreviations: BC, breast cancer; GBP1, guanylate binding protein1; IFNγ, interferon γ; NRIP1, nuclear receptor interacting protein 1; RIP140, receptor interacting protein of 140 kDa; SP100, SP100 nuclear antigen; VDR, itamin D receptor.

1 | INTRODUCTION

Breast cancer (BC) is the most common tumor for women in the world. In 2020, according to the World Health Organization, over two million women were diagnosed with a breast cancer and more than 600,000 died from that pathology. A diversity of signaling pathways, which strongly cross-talk, causes the survival and proliferation of BC cells. From a clinical point of view, the main molecular target is estrogen receptor α (ERα) which drives around 70% of BC.[1] These tumors also express the progesterone receptor (PR). Another important molecular target is epidermal growth factor 2 (ERBB2, formerly HER2 or HER2/neu), a transmembrane receptor amplified or overexpressed in approximately 20% of BC.[2] Finally, a third BC subtype, triple-negative breast cancers, characterized by the lack of expression of molecular targets ERα, PR, or ERBB2, accounts for approximately 15% of all breast tumors.[3]

Other pathways are implicated, including the interferon-γ/STAT1 (IFNγ/STAT1) signaling, which can induce tumor cell apoptosis and results in repression of BC cell growth.[4] In addition to prompt apoptosis and senescence, IFNγ can shift tumors to a dormant state.[5] It must also be underlined that this pathway is believed to be critical for the success of immune therapy.[6] However, IFNγ displays a yin and yang effect, involving repression of tumor growth by arresting the cell cycle, induction of tumor ischemia, inhibition of suppressive immune cells as well as promotion of carcinogenesis and angiogenesis.[7]

RIP140 (receptor interacting protein of 140 kDa),[8] also known as NRIP1 (nuclear receptor interacting protein 1), was shown to act both as a corepressor and a coactivator depending on interaction dynamics with other transcription cofactors.[9] It was shown to play an active role in many physiological or pathological processes. For instance, mice lacking the *Rip140* gene displayed a female infertility, a reduced body fat content, and severe cognitive defects.[10] RIP140 regulates mammary gland development by directly regulating the expression of the progesterone receptor, amphiregulin, and signal transducer and activator of transcription 5a.[9] In BC, we showed that a decreased expression of RIP140 activity was associated with the bad prognosis of basal-like tumors[11] while an increase in RIP140 mRNA was observed in overall breast tumors.[12] Very recently, our team provided evidence that RIP140 could repress the proliferation of BC cells by targeting glycolysis (more precisely GLUT3 expression)[13] and the phosphate pentose pathway through inhibition of glucose 6-phosphate dehydrogenase.[14]

In the present study, for a better understanding of RIP140 action in BC, we performed an RNA sequencing analysis of MCF-7 cells presenting a downregulation of *NRIP1*. We herein exhibited a large number of target genes upregulated by RIP140, as expected from its role as a transcriptional repressor, but others were significantly down-regulated, including, for instance, the vitamin D receptor. We documented the interferon signaling as a key target of RIP140 and further focused on GBP1, a main IFNγ-induced gene. We demonstrated that its IFNγ-dependent expression in three BC cell lines (MCF-7, T-47D and MDA-MB-231) was inhibited by RIP140. We showed that the regulation occurred at the transcriptional level through an IFN-response element and that reducing RIP140 levels in BC cells amplified the IFNγ response on gene expression and cell proliferation. Finally, reanalysis of transcriptomics data from BC patient samples validated the impact of low RIP140 levels on the prognostic value of the *IFNG* gene expression. Altogether, our data demonstrated that RIP140 acts as a key regulator of the IFNγ response in BC cells.

2 | MATERIALS AND METHODS

2.1 | Materials

Estradiol and Interferon γ were purchased from Sigma-Aldrich®.

2.2 | Plasmids

pEFcmyc-RIP140 was previously described.[15] Pro3757-hGBP1, pro237-hGBP1, and ΔISRE-hGBP1 were kindly provided by Dr. Elisabeth Naschberger.[16] pISRE-F-luc was a generous gift from Dr. Konstantin Maria Johannes Sparrer.[17,18]

2.3 | RNA-seq data generation and processing

Total RNA sequencing was performed on MELN cells, on two independent RNA extracts. The data we generated were already published[19] and are available from GEO (GSE143956).

2.4 | Pathway analysis

Gene set enrichment analysis was conducted with the R BioConductor FGSEA library[20] using GO Biological Process terms and Reactome pathways as gene sets. FGSEA was run with 1,000,000 permutations, and we imposed minimum and maximum gene set sizes of 15 and 250, respectively.

2.5 | Cell culture and transfections

MELN (RRID:CVCL_WI91) cells are MCF-7 BC cells stably transfected with a luciferase gene driven by an estrogen-responsive element in front of the β-globin promoter (ERE-βGlob-Luc-SVNeo).[21] They were cultured in phenol red-free DMEM-F12 (Invitrogen) containing 5% dextran-charcoal-treated FCS (Invitrogen), MCF-7 (RRID:CVCL_0031) and MDA-MB-231 (RRID:CVCL_0062) in DMEM-F12 (Invitrogen) and T-47D (RRID: CVCL_0553) in RPMI (Invitrogen) supplemented with 10% fetal calf serum (FCS) and antibiotics (Gibco). All cell lines were authenticated using short tandem repeat profiling within the last 3 years. All experiments were performed with mycoplasma-free cells.

When needed, plasmids or siRNAs were transfected using either Jet-PEI or INTERFERin (Polyplus), respectively. When indicated, cells were treated with 100 nM E2 or 10 µg/mL IFNγ and then harvested 24 h later. The list and sequences of the siRNAs used in this study are indicated in Table S1. SiCtl is control siRNA, designed not to target any gene.

2.6 | mRNA quantification

RNA was isolated using the Zymo Research kit (Zymo research) and reverse transcription (RT)-qPCR assays were done using qScript (VWR) according to the manufacturer's protocol. Transcripts were quantified using SensiFAST SYBR (BioLine) on a LC480 instrument. The nucleotide sequences of the primers used for real-time PCR are indicated in Table S1.

2.7 | Immunoblotting assays

Western blot analyses were performed as previously described[12] using anti-VDR (Ozyme), anti-GBP1 (Origene) or anti-actin (Sigma) antibodies.

2.8 | Luciferase assays

MCF-7, MDA-MB-231, and T-47D cells were plated in 96-well plates 24 h prior to transfection with either JetPEI or INTERFERin according to the manufacturer's protocol. Firefly luciferase data were normalized to renilla luciferase activity and expressed as relative luciferase activities.

2.9 | Cell proliferation assay

MCF-7, MDA-MB-231, and T-47D cells were seeded at a density of 2000 cells per well. At the indicated time, 0.5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, St Louis, MO, USA) was added to selected wells, and cells were incubated at 37°C for 4 h. Formazan crystals were solubilized in DMSO, and the absorbance read at 560 nm on a spectrophotometer. Results were normalized to the cell density at day 1.

2.10 | Survival analysis

Using the KM-plot Private Edition (http://kmplot.com),[22] RNA sequencing data from the Cancer Genome Atlas (TCGA) were analyzed using Cox proportional hazards regression, as previously described.[23] The Kaplan–Meier method was used to estimate overall survival calculated from diagnosis to death. Patients lost to follow-up were censored at the time of last contact.

2.11 | Statistical analysis

All experiments were conducted independently at least three times. Results were expressed as the mean ± standard deviation (SD). Comparisons of two independent groups were performed using the Mann–Whitney U test. A probability level (p value) of 0.05 was chosen for statistical significance. Statistical analyses were performed using StatEL (AdScience).

3 | RESULTS

3.1 | Identification and validation of RIP140 target genes in BC cells

In order to get a comprehensive survey of RIP140 target genes in BC cells, we performed RNA sequencing using MCF-7 BC cells stably transfected with a luciferase gene driven by an estrogen-responsive element (MELN cells).[21] We first validated RIP140 silencing using small interfering RNA (siRIP140) transfection at the mRNA level (Figure 1A, left panel) and at the protein level (Figure 1A, right panel). RIP140 knockdown was also validated by a significant 2-fold increase in estradiol-dependent regulation of luciferase expression (Figure 1B).

An RNA sequencing analysis on two independent extracts of these BC cells transfected or not with siRIP140 allowed us to identify 116 genes differentially expressed. On Table 1 are shown the genes ranked according to the best p value and a negative (top panel) or positive (bottom panel) amplitude fold. Interestingly, downregulation of RIP140 expression only induced small variations of target gene levels, a phenomenon we long experienced.

The RNA sequencing results were then validated by RT-qPCR assays. As shown in Figure 1C, we validated several genes down-regulated under siRIP140 transfection, including the vitamin D receptor (*VDR*), acyl-CoA binding domain containing 5 (*ACBD5*) and macrophage erythroblast attacher (*MAEA*). Ectopic expression of RIP140 in MCF-7 cells induced the opposite effect, that is, an up-regulation of the three above-mentioned genes (Figure 1D). Similar results were obtained in the T-47D luminal BC cell line (data not shown). Concerning VDR, we also demonstrated at the protein level the effect of either siRIP140 (Figure 1E) or the ectopic expression of RIP140 (Figure 1F). Altogether, these data validated the robustness of our RNA sequencing analysis.

3.2 | Interferon γ signaling is a main target of RIP140

The gene set enrichment analysis (GSEA) of our sequencing data allowed us to identify a series of over-represented genes, significantly enriched or depleted, associated with a phenotype. Accordingly, IFNγ signaling, a major pathway in breast carcinogenesis, appeared on top of the list (Table 2). More precisely, five genes belonging to this

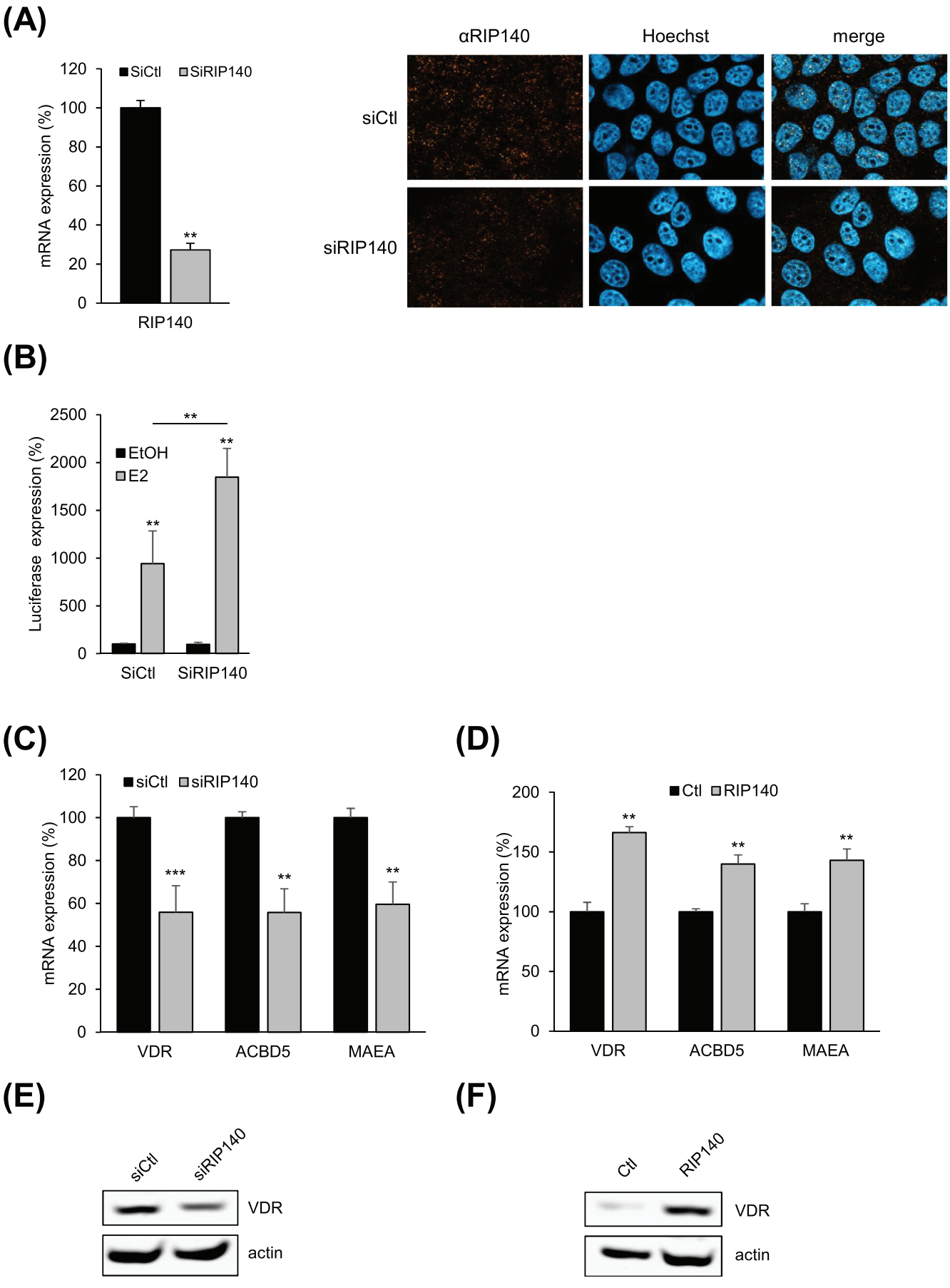


FIGURE 1 Legend on next page.

pathway were identified as being regulated by RIP140: guanylate binding protein 1 (GBP1), SP100 nuclear antigen (SP100), major histocompatibility complex, class I, G (HLA-G), major histocompatibility complex, class II, DP alpha 1 (HLA-DPA1) and interferon beta 1 (IFNB) (data not shown). GBP1, being described as one of the most significantly induced proteins in cells exposed to IFNγ,[24] and shown to play a major role in BC,[25] prompted us to further investigate the regulation of this gene by RIP140.

TABLE 1 Top genes down-regulated (upper panel) or up-regulated (lower panel) after RIP140 mRNA knock-down.

Genes	Name	logFC	*p* value
MATN2	matrilin 2	−1.009	8.067E−07
MAEA	macrophage erythroblast attacher	−0.830	7.930E−06
ACBD5	acyl-CoA binding domain containing 5	−0.843	6.339E−05
MLX	MLX, MAX dimerization protein	−0.657	2.901E−04
VDR	vitamin D receptor	−0.647	1.166E−03
RFX5	regulatory factor X5	−0.569	1.254E−03
ENO2	enolase 2	−0.596	1.288E−03
VCP	valosin containing protein	−0.526	3.749E−03
OR2B6	lfactory receptor family 2 subfamily B member 6	−1.162	4.559E−03
GTF2IRD2	GTF2I repeat domain containing 2	−0.899	5.094E−03
TIMP3	TIMP metallopeptidase inhibitor 3	−0.567	5.381E−03
CSF1	colony stimulating factor 1	−0.785	5.416E−03
ATF3	activating transcription factor 3	−0.738	5.514E−03
DPP4	dipeptidyl peptidase 4	−0.909	7.599E−03
CXCR4	C-X-C motif chemokine receptor 4	−0.820	9.427E−03
Genes	**Name**	**logFC**	***p* value**
ARMT1	acidic residue methyltransferase 1	0.681	4.137E−04
SLC9B1	olute carrier family 9 member B1	1.567	6.278E−04
GCSH	glycine cleavage system protein H	1.216	1.296E−03
WDR93	WD repeat domain 93	1.495	1.429E−03
GALNTL6	polypeptide N-acetylgalactosaminyltransferase like 6	0.946	2.964E−03
ID1	inhibitor of DNA binding 1, HLH protein	0.650	4.329E−03
SAMD13	sterile alpha motif domain containing 13	1.194	5.618E−03
AARD	alanine and arginine rich domain containing protein	1.049	5.834E−03
DYRK4	dual specificity tyrosine phosphorylation regulated kinase 4	0.948	6.614E−03
MAPK10	mitogen-activated protein kinase 10	1.012	7.211E−03
KLB	klotho beta	0.805	8.566E−03
RPL39	ribosomal protein L39	0.839	8.795E−03
FKBP7	FK506 binding protein 7	1.027	9.174E−03

Note: Genes are listed from lower to higher *p* values.

FIGURE 1 Validation of RNA sequencing results. (A) Left panel. MELN cells were transfected with either control siRNA (siCtl), or siRIP140. Total RNAs were subjected to RT-qPCR assays, and quantification of RIP140 mRNA was expressed relatively to the control condition (siCtl). Right panel. MCF7 cells were analyzed by immunofluorescence after transfection of siCtl (control) or siRIP140, using anti-RIP140 antibody (ab42126, Abcam) and Hoechst labeling. Images were acquired with a Zeiss Imager M2 microscope with the Apotome system and a Plan Apochromat 40×/1.3 DIC (oil) controlled by the ZEN software (Carl Zeiss). (B) MELN cells were transfected as in A and then treated or not with estradiol (10^{-8} M). Luciferase mRNA was quantified and results expressed as above described. MCF-7 cells were transfected with siCtl or siRIP140 (C) or with pEF-RIP140 or the empty vector (D). Quantification of mRNAs was done as described in (A). (E) MCF-7 cells were transfected as in (C) and western-blot assays were done with either an anti-VDR (upper panel) or an anti-actin (lower panel) antibody. (F) MCF-7 cells were transfected as in (D) and western-blot assays were performed as in (E). Statistical analyses were performed using the Mann–Whitney test. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

TABLE 2 Interferon signaling is a target of RIP140.

Pathways	NES	*p* value	*p* adjusted
Interferon gamma-mediated signaling pathway // BP	−2.27	2.4e−05	7.0e−03
Type I interferon signaling pathway // BP	−2.27	2.4e−05	7.0e−03
Interfon alpha/beta signaling // Reactome	−2.26	2.4e−05	7.0e−03
Interferon gamma signaling // Reactome	−2.21	2.4e−05	7.0e−03
Interferon signaling // Reactome	−2.09	2.7e−05	7.0e−03
Aromatase activity // MF	−2.09	2.1e−05	7.0e−03
T cell costimulation // BP	−2.07	7.0e−05	1.2e−02
Establishment of skin barrier // BP	−2.03	1.9e−04	2.3e−02
Positive regulation of Wnt signaling pathway // BP	−2.01	3.3e−04	3.4e−02
Actomyosin structure organization // BP	−2.00	3.9e−04	3.6e−02
Keratinization // BP	−2.00	3.8e−04	3.6e−02
Chromatin DNA binding // MF	−1.99	1.8e−04	2.3e−02
PKMTs methylate histone lysines // Reactome	−1.94	3.9e−04	3.6e−02
Z disc // CC	−1.93	9.7e−04	1.6e−02
Keratinization // Reactome	−1.93	1.4e−04	1.9e−02
Extracellular matrix organization // Reactome	−1.93	2.8e−04	7.0e−03
Viral mRNA Translation // Reactome	1.92	5.1e−04	1.0e−02
Cytokine-mediated signaling pathway // BP	−1.92	2.8e−04	7.0e−03
Response to hypoxia // BP	−1.92	2.6e−04	7.0e−03
Neuropeptide signaling pathway // BP	1.92	4.8e−04	4.2e−02
Extracellular matrix // CC	−1.89	5.1e−04	1.0e−02

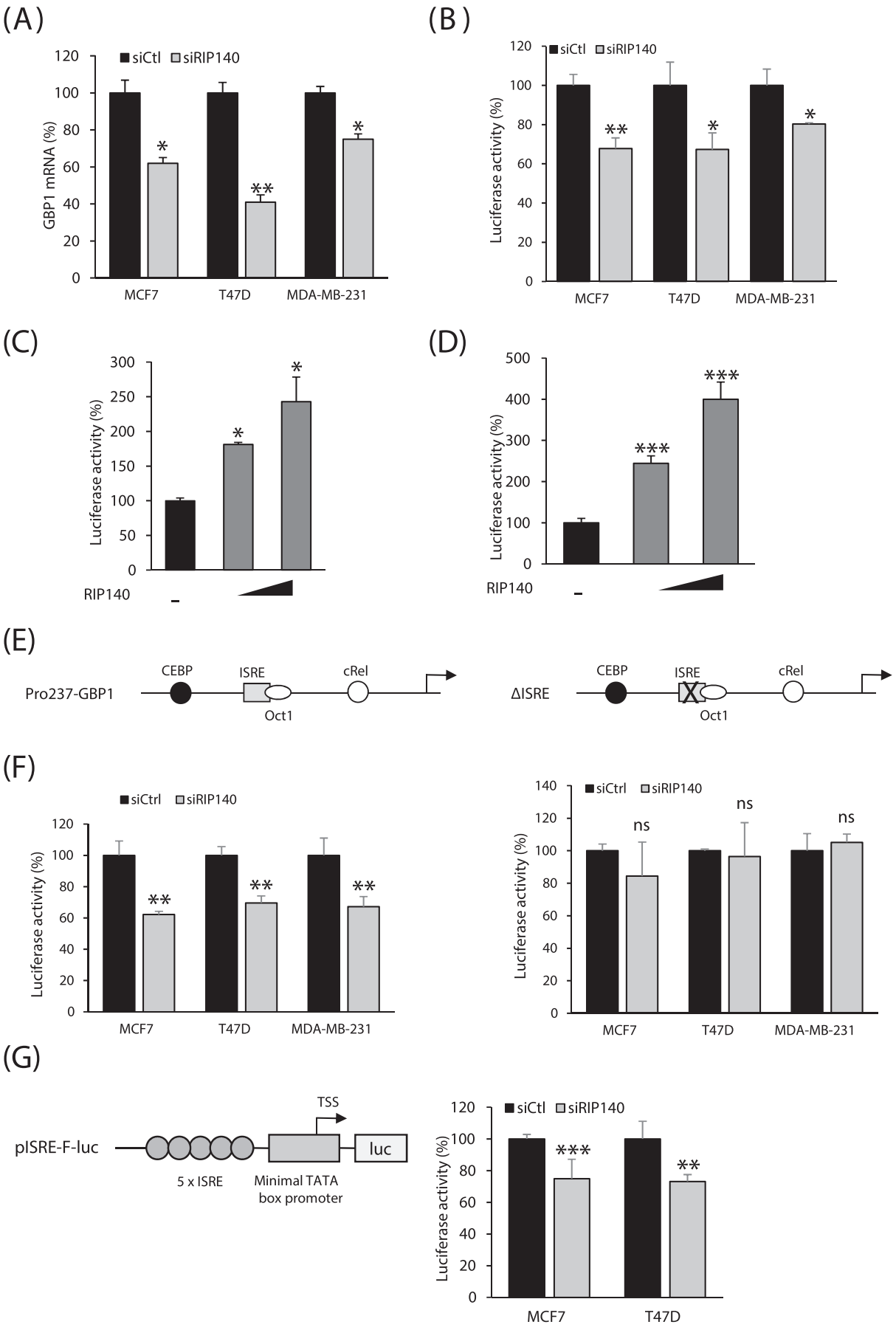
Note: Gene set enrichment analysis of RIP140 knockdown consequences under basal conditions, combining GO Biological Process (BP), Reactome, Molecular Functions (MF) and Cellular Component (CC) pathways.

3.3 | RIP140 increases GBP1 expression in BC cells

When transfected with siRIP140, MCF-7 cells as well as T-47D and MDA-MB-231 cells displayed a significant decrease (between 20 and 40%) in GBP1 mRNA expression (Figure 2A), confirming the data obtained with MELN cells (Figure S1A, left panel). Transfection of an additional siRIP140 allowed us to validate these observations (Figure S1A, right panel). Using pro-GBP-1, a reporter construct with the GBP1 promoter driving the luciferase gene,[16] we demonstrated that silencing RIP140 activity in MCF-7, T-47D, and MDA-MB-231 cells reduced GBP1 promoter activity (Figure 2B). Conversely, when transfecting increasing doses of RIP140 expression plasmid, we observed that activity augmented both in MCF-7 (Figure 2C) and T-47D (Figure 2D) cells, indicating a transcriptional regulation of GBP1 expression by RIP140. In parallel, we also investigated the regulation of Sp100. As exhibited in Figure S1B, a transfection of siRIP140 or siRIP140#1 also decreased Sp100 mRNA expression in MELN, MCF-7, and T-47D cells, while ectopic expression of RIP140 significantly augmented it (Figure S1C). From these two examples, also consolidated by other IFNγ-regulated genes (such as *HLA-G*, *HLA-DPA1* or *IFNB1*) (data not shown), we concluded that, in various BC cells, RIP140 increased the expression of IFNγ signaling pathway actors.

3.4 | RIP140 regulates GBP1 expression through an ISRE binding site

In order to unveil the RIP140 mechanism of action on the regulation of GBP1 expression, we used a shorter version of the GBP1 promoter, that is, pro237-GBP1 (Figure 2E, left panel), together with a ΔISRE mutated form, where the interferon specific response element (ISRE) is mutated[16] (Figure 2E, right panel). As shown in Figure 2F (left panel), transfection of siRIP140 induced a very similar decrease in pro237-GBP1 activity in MCF-7, T-47D, and MDA-MB-231 cells as the one observed with pro-GBP-1 (Figure 2B), indicating the two constructs behaved similarly. The same result was also obtained when using siRIP140#1 (Figure S1D, left panel). Interestingly, transfection of siRIP140 (Figure 2F, right panel) or siRIP140#1 (Figure S1D, right panel) induced no change in ΔISRE activity, revealing the importance of the response element for RIP140-dependent regulation of the GBP1 promoter. Furthermore, we used a reporter plasmid consisting of five repeated ISRE sequences upstream of the luciferase gene (Figure 2G, left panel). As shown in Figure 2G (right panel), both MCF-7 and T-47D transfected with the vector and siRIP140 displayed a significant reduction in luciferase activity. At this stage, it is not known whether RIP140 can act directly through binding to ISRE or indirectly via titrating a transcriptionally repressive protein or regulating the expression of a transcription factor able to bind to the



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response element. However, our results indicate a need for a valid ISRE to mediate the RIP140 transcriptional response on the GBP1 promoter.

3.5 | RIP140 knock-down increases the IFNγ response on GBP1 expression

Upon treatment of MCF-7, T-47D, and MDA-MB-231 cells with IFNγ (Figure 3A), expression of GBP1 mRNA was robustly stimulated (especially in T-47D cells), further confirming GBP1 as a major target of IFNγ. A milder induction, but still significant, was also observed when the same cell lines were transfected with the pro-GBP-1 plasmid and treated with the same doses of IFNγ (Figure 3B).

We next wondered whether RIP140 could affect IFNγ-induced GBP1 expression. After depletion of RIP140 mRNA and treatment with increasing doses of IFNγ, we observed that the difference in GBP1 expression between siRIP140 and siCtl augmented with the dose of IFNγ in both MCF-7 (Figure 3C) and T-47D cells (Figure S2A). At the protein level, while the expression of GBP1 in MCF-7 cells was low in basal conditions (transfection with siCtl and DMSO treatment) (Figure 3D, left panel), a decrease was observed under siRIP140 transfection. Remarkably, IFNγ induced a strong GBP1 expression that is not affected when lowering RIP140 expression (Figure 3D, right panel). Likewise, IFNγ induction of GBP1 expression was stronger when depleting RIP140 mRNA in all three cell lines, with a stronger effect in T-47D (Figures 3E and S2B). As a control, raw data obtained for T-47D, in the absence and presence of IFNγ are shown in Figure S2C. We also observed a significantly stronger IFNγ-induced activity of pro-GBP-1 under transfection of siRIP140 in MCF-7, T-47D, and MDA-MB-231 (Figure 3F). Conversely, when ectopically expressing increasing doses of RIP140, IFNγ-induced activity of pro-GBP-1 decreased both in MCF-7 (Figure 3G) and T-47D (Figure 3H) cells.

In MCF-7, T-47D, or MDA-MB231 cells transfected with the RIP140, ΔISRE plasmid could no longer be stimulated by IFNγ (Figure S2D), further demonstrating the role of the ISRE element in mediating both RIP140 and IFNγ signaling.

In parallel, focusing on Sp100 expression, we showed that IFNγ significantly stimulated its mRNA expression in MCF-7, T-47D, and MDA-MB-231 cells (Figure S2E). We also demonstrated that IFNγ induction of Sp100 expression was stronger when transfecting siRIP140 (MCF-7 and T-47D cells, Figure S2F, left panel) or siRIP140#1 (MCF-7 and MDA-MB-231, Figure S2F, right panel). This series of experiments demonstrated that low levels of RIP140 allow a full stimulatory effect of IFNγ on gene expression.

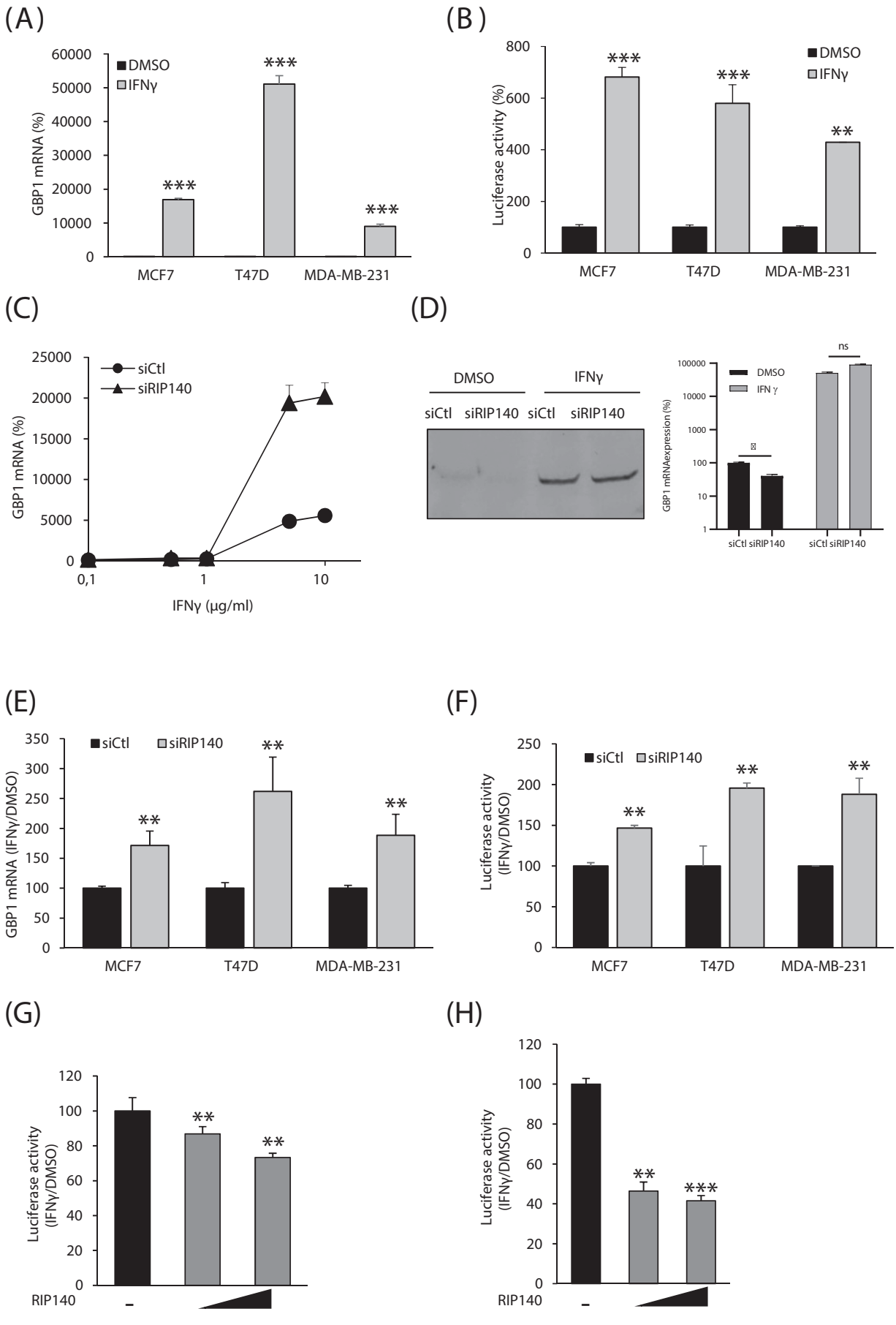
3.6 | Low levels of RIP140 increase IFNγ-driven BC cell proliferation

Based on the effect of RIP140 on IFNγ-target genes, we then wondered whether RIP140 could modulate IFNγ-dependent BC cell proliferation. Using MTT assays, we first confirmed that IFNγ inhibited the growth of MCF-7 and MDA-MB-231 cells (Figure 4A, left and right panels), with a more marked effect on the latter. Using the same means, the two BC cell lines transfected with siRIP140 exhibited a gain in cell proliferation (Figure 4B, left and right panels), validating that RIP140 displays anti-proliferative properties in BC cells. When down-regulating RIP140 expression, we observed that IFNγ acted as a stronger repressor of either MCF-7 (Figure 4C, left panel) or MDA-MB-231 (Figure 4C, right panel) cell proliferation, indicating that RIP140 prevents the cytokine from fully exerting its antiproliferative effect.

3.7 | RIP140 expression determines the prognostic value of the *IFNG* gene in BC

The experimental data shown in Figure 4C strongly suggested that high levels of RIP140 expression should inhibit the antiproliferative activity of IFNγ. We therefore hypothesized that, in breast cancers, low RIP140 levels could be a marker of such an IFNγ anti-tumor activity and, consequently, that *IFNG* gene expression might be associated with an increased overall survival for patients with tumors expressing a reduced level of RIP140. Using Cox proportional hazard regression,[26] we analyzed RNAseq data obtained from 1068 breast tumor samples from the TCGA dataset as described previously[27] (Figure 4). We first checked the prognostic values of the expression of each gene by analyzing patient overall survival at 60 months. High *IFNG* expression was correlated with good prognosis (HR 0.59, $p = 0.0011$) (Figure S3A). Then we used the median as a cutoff value for classification of patients into two groups of tumors with low and high RIP140 expression, respectively. Using Kaplan–Meier plots, we

FIGURE 2 RIP140 stimulates GBP1 expression. (A) MCF-7, T-47D and MDA-MB-231 cells were transfected with either siCtl or siRIP140. RNAs were subject to RT-qPCR assays, and quantification of GBP1 mRNA was expressed relatively to siCtl condition. (B) MCF-7, T-47D and MDA-MB-231 cells were transfected with pRL-CMV-renilla and pro3757-GBP1 plasmids together with siCtl or siRIP140. Luciferase values were normalized to the renilla luciferase control and expressed relatively to the siCtl condition. MCF-7 (C) and T-47D (D) were transfected with pRL-CMV-renilla and pro3757-GBP1 plasmids together with increasing amounts of RIP140 expressing plasmid. Luciferase values are expressed as above described. (E) Schematic representation of pro237-GBP1 (left panel) and ΔISRE (right panel). (F) MCF-7, T-47D and MDA-MB-231 cells were transfected with pRL-CMV-renilla and either pro237-GBP1 (left panel) or ΔISRE (right panel) plasmids, siCtl and siRIP140. Luciferase values were normalized to the renilla luciferase control and expressed relatively to the siCtl condition. (G) Left panel: schematic representation of pISRE-F-luc. Right panel: MCF-7 and T-47D cells were transfected with siCtl and siRIP140 together with pISRE-F-luc and pRL-CMV-renilla. Luciferase values were expressed as in (F). Statistical analyses were performed using the Mann–Whitney test. $^{*}p < 0.05$, $^{**}p < 0.01$; $^{***}p < 0.001$.



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investigated whether *IFNG* expression correlated with OS at 60 months within both groups using the same cutoff values. Interestingly, *IFNG* (Figure 4D) expression, as well as *GBP1*, *HLA-G*, and *HLA-DPA1* expression (Figure S3B), was significantly associated with increased overall survival in the low RIP140 expression group ($p = 0.0017$) but not in the high RIP140 expression group ($p = 0.47$). Similar results were obtained when the analysis was performed using disease-free survival and a cutoff of *IFNG* expression based on the first and fourth quartiles (data not shown). Altogether, these results further reinforce the link between RIP140 expression and the IFNγ signaling in breast cancers.

4 | DISCUSSION

In this study, we performed a transcriptomic analysis after silencing RIP140 in MCF-7 cells. We confirmed by means of qPCR the RIP140-dependent regulation of some genes, including VDR. Analysis of the sequencing data allowed us to identify IFNγ targets as being substantially modulated by RIP140. Accordingly, we identified GBP1 as a robustly RIP140-regulated gene, and we described how RIP140 drives IFNγ-driven inhibition of breast cancer cell proliferation.

GBPs are a family of proteins described as IFN-inducible GTPases that can induce antimicrobial immunity and cell death.[28] GBP1 was also associated with a bad prognosis for triple-negative breast cancers.[25] In this latter study, it must be underlined that in proliferation assays, GBP1 was shown to be oncogenic in MDA-MB-231 cells, whereas we described it as having anti-proliferative activity. Moreover, GBP1 expression is very heterogeneous depending on the type of cancer.[29] It is oncogenic in prostate cancer[30]; its expression correlates with worse survival in lung cancer[31,32]; it is associated with treatment resistance in ovarian cancer[26] and overexpression of GBP1 is correlated with a poor prognosis and promotes tumor growth in glioblastoma.[27] Conversely, GBP1 was described as a tumor suppressor in colon cancer.[33] These observations remind us of RIP140, which has a protective role in colon[34] or triple-negative breast cancer[12,35] whereas a high expression is associated with stomach cancer[36] as well as a shorter overall survival of cervical cancer patients.[37] GBP1 and RIP140 are a good illustration of the tissue specificity of gene activity.

In this study, we described cross-talks between RIP140 and IFNγ on common transcriptional targets, mainly GBP1 and Sp100. However, we can also highlight other examples, especially the E2F1 transcription factor whose expression is down-regulated by IFNγ in T-47D cells[38] while RIP140 represses E2F1 activity.[11] A similar cross-talk involves the androgen receptor (AR) since IFNγ is described to increase the receptor expression in prostate[39] while we describe a repressive activity of RIP140 on agonist-liganded AR in prostate cancer cells.[15] We also provided evidence that RIP140 was able to repress estradiol-induced AP1 activity[40] which was also described to induce IFNγ expression.[41]

IFNγ is described to have anti-tumorigenic effects, noticeably by promoting apoptosis through the induction of caspases expression (caspase 8 and caspase 9).[42] Interestingly, our RNA sequencing data together with qPCR assays indicated a positive effect of RIP140 on the expression of the two caspases (data not shown). Alongside, IFNγ is able to repress metastasis progression through the induction of fibronectin 1 (FN1) expression.[43] Again, we also found that RIP140 was able to induce FN1 expression. Together with our cell proliferation assay, the effect of RIP140 on apoptosis and metastasis markers expression argues for a protective role of RIP140 on tumor development. By sharp contrast, low doses of IFNγ may have pro-tumorigenic effects by inducing tumor stemness through the induction of ICAM1 expression.[44] Like IFNγ, RIP140 can also induce ICAM1 (data not shown), participating in a potential pro-tumorigenic effect. Interestingly, RIP140 has the same action as IFNγ on many genes, including the yin and yang effect on carcinogenesis.

IFNγ also participates in cancer evasion by promoting carcinogenesis and angiogenesis. It is well characterized for papillary thyroid cancer cells where IFNγ induces epithelial-mesenchymal transition (EMT) and increases migration and invasion of the cancer cells.[45] Even though RIP140 was not shown to promote EMT, we demonstrated that its cytoplasmic expression in breast cancer cells correlates with the expression of N-cadherin, a marker of EMT.[46] Accordingly, together with IFNγ, RIP140 could be envisaged to participate in cancer evasion.

FIGURE 3 RIP140 decreases IFN-dependent GBP1 gene expression. (A) MCF-7, T-47D and MDA-MB-231 cells were either treated with vehicle or IFNγ (10 μg/mL) during 24 h. RNAs were subjected to RT-qPCR assays, and quantification of GBP1 mRNA was expressed relatively to siCtl condition. (B) MCF-7, T-47D and MDA-MB-231 cells were transfected with pRL-CMV-renilla and pro3757-GBP1 plasmids and treated as in (A). Luciferase values were normalized to the renilla luciferase control and expressed relatively to the DMSO condition. (C) MCF-7 cells were transfected with either siCtl or siRIP140 and then treated with increasing doses of IFNγ. RNAs were subject to RT-qPCR assays, and quantification of GBP1 mRNA was expressed relatively to siCtl condition. (D) MCF-7 cells were transfected with either siCtl or siRIP140 and treated with either vehicle or IFNγ (10 μg/mL) during 24 h. Protein extracts were submitted to western-blotting using an anti-GBP1 antibody (left panel). The image is representative of three independent experiments. Relative intensity of the bands is determined by the ratio between GBP1 specific and total protein bands (right panel). (E) MCF-7, T-47D and MDA-MB-231 cells were transfected with either siCtl or siRIP140. Cells were then treated as in (A). GBP1 was quantified by RT-qPCR and results are expressed as the ratio between IFNγ and vehicle treatment, relatively to siCtl condition. (F) MCF-7, T-47D and MDA-MB-231 cells were transfected with siCtl or siRIP140 and pRL-CMV-renilla and pro3757-GBP1 plasmids and treated as in (A). MCF-7 (G) and T-47D (H) cells were transfected with increasing doses of pEF-RIP140, pRL-CMV-renilla and pro3757-GBP1 plasmids. Luciferase activity (F), (G) and (H) was measured and results expressed as in (E). Statistical analyses were performed using the Mann–Whitney test. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

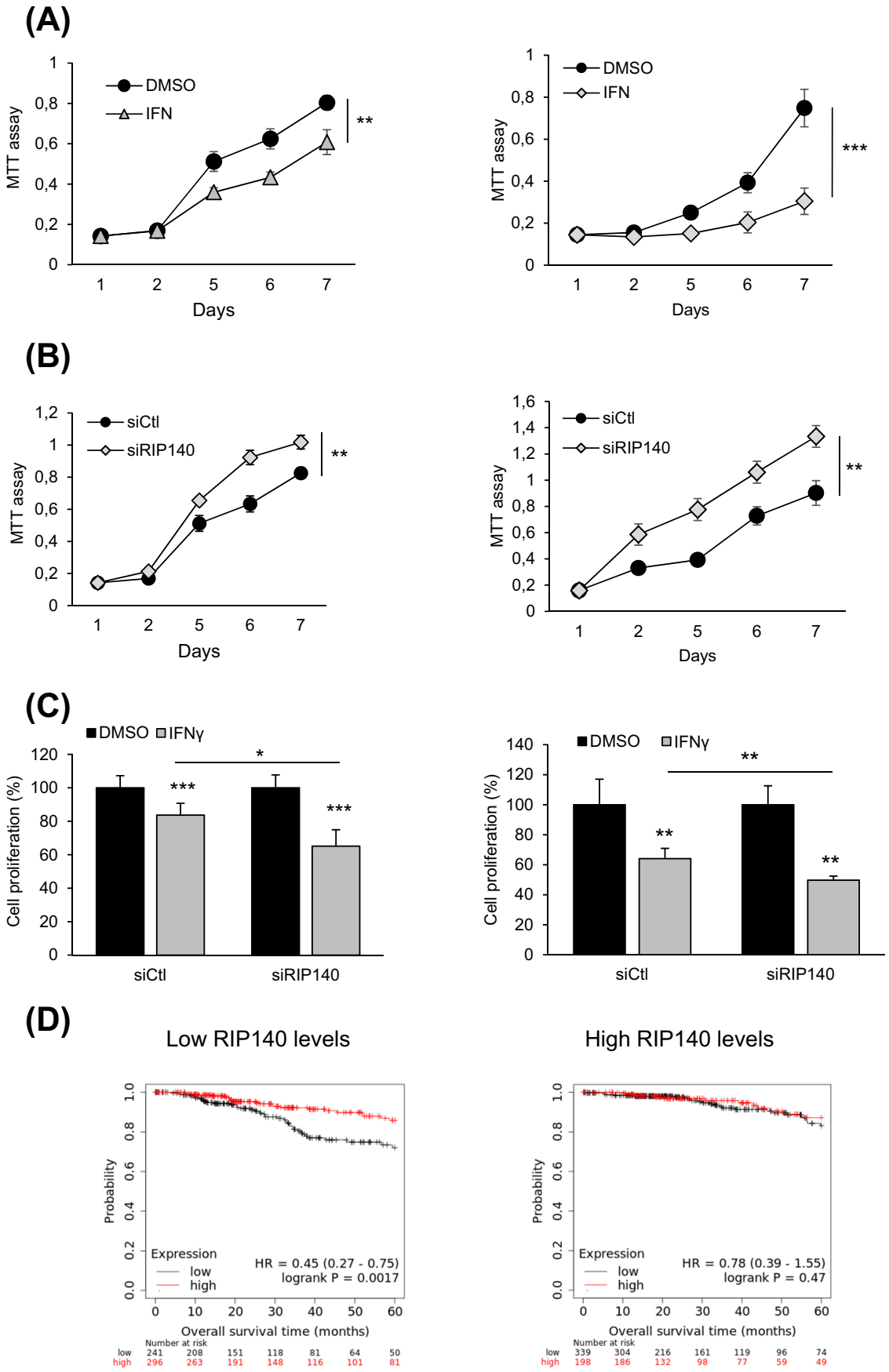

FIGURE 4 Legend on next page.

Finally, it has long been accepted that IFNγ plays a central role in host antitumor immunity.[7] Indeed, tumor-infiltrating lymphocytes, essential for cancer immune surveillance, are the main source of IFNγ in tumors.[7] Non-small cell lung cancer and melanoma patients treated with a PD-1 inhibitor display an increase in IFNγ protein expression accompanied by a longer progression-free survival,[47] which could indicate the cytokine as a biomarker for the prediction of response to immune checkpoint blockade. Since we describe RIP140 as a repressor of IFNγ-induced target genes, its own expression could also be hypothesized as a biomarker of the checkpoint blockade.

In conclusion, identifying IFNγ as a new target of RIP140 reinforces the major role of this transcription factor in carcinogenesis, not only within the tumor but also potentially in the tumor microenvironment.

AUTHOR CONTRIBUTIONS

Stéphan Jalaguier: Conceptualization; formal analysis; investigation; methodology; writing – original draft. **Axel Kuehn:** Investigation. **Chloé Petitpas:** Investigation. **Arnaud Dulom:** Investigation. **Rémy Jacquemont:** Investigation. **Cindy Assi:** Investigation. **Sophie Sixou:** Formal analysis. **Udo Jeschke:** Formal analysis. **Jacques Colinge:** Conceptualization; writing – original draft; funding acquisition. **Vincent Cavaillès:** Conceptualization; funding acquisition; writing – original draft.

ACKNOWLEDGEMENTS

We would like to thank Dr. Elisabeth Naschberger for the kind gift of pro3757-GBP1, pro237-GBP1, and ΔISRE plasmids, as well as Dr. Konstantin Maria Johannes Sparrer for the generous gift of pISRE-F-luc. We are grateful to Pr. Balázs Győrffy for the use of the Kaplan–Meier plotter private edition. We are grateful to Dr. Catherine Teyssier for the critical reading of the manuscript.

FUNDING INFORMATION

This work was supported by Groupement des Entreprises Françaises dans la Lutte contre le Cancer (GEFLUC).

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data sources and handling of the publicly available datasets used in this study are described in the Materials and Methods and in Table 2. Further details and other data that support the findings of this study are available from the corresponding authors upon request.

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FIGURE 4 RIP140 and GBP1 regulate MCF-7 and MDA-MB-231 cell proliferation. (A) MCF-7 (left panel) and MDA-MB-231 (right panel) cells were treated with IFNγ (10 μg/mL) or vehicle. Cell proliferation was measured over 7 days. Absorbance of Formazan crystals was read on a spectrophotometer. (B) MCF-7 (left panel) and MDA-MB-231 (right panel) cells were transfected with either siCtl or siRIP140. Cell proliferation was quantified as in (A). Cell proliferation was quantified as in (A). (C) MCF-7 (left panel) and MDA-MB-231 (right panel) cells were transfected with siCtl or siRIP140. Cell proliferation was monitored as in (A) and results from day 6 were expressed relative to treatment with vehicle (DMSO). (D) The Kaplan–Meier method was used to estimate overall survival of patients from the TCGA dataset. The analysis was done on patients presenting low levels (left panel) or high levels (right panel) of *NRIP1* gene expression. Statistical analyses were performed using the Mann–Whitney test. $^{**}p < 0.01$; $^{***}p < 0.001$.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Jalaguier S, Kuehn A, Petitpas C, et al. The transcription factor RIP140 regulates interferon γ signaling in breast cancer. *Int J Cancer*. 2025;157(1):170-182. doi:10.1002/ijc.35405