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Interplay between interferon-stimulated gene 15/ISGylation and interferon gamma signaling in breast cancer cells

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ABSTRACT

Interferon-stimulated gene 15 (ISG15) is a ubiquitin-like protein that conjugates to its target proteins to modify them through ISGylation, but the relevance of *ISG15* expression and its effects have been not completely defined. Herein, we examined the interplay between ISG15/ISGylation and the interferon-gamma (IFN-γ) signaling pathway in mammary tumors and compared it with that in normal mammary tissues. Our results indicated that mammary tumors had higher levels of *ISG15* mRNA and ISG15 protein than the adjacent normal mammary tissue. Furthermore, the expression of IFN-γ signaling components was altered in breast cancer. Interestingly, IFN-γ treatment induced morphological changes in MCF-7 and MDA-MB-231 breast cancer cell lines due to cytoskeletal reorganization. This cellular process seems to be related to the increase in ISGylation of cytoplasmic IQ Motif Containing GTPase Activating Protein 1 (IQGAP1). Interactome analysis also indicated that IFN-γ signaling and the ISGylation system are associated with several proteins implicated in cytoskeletal remodeling, including IQGAP1. Thus, ISG15 may present a potential biomarker for breast cancer, and IFN-γ signaling and protein ISGylation may participate in the regulation of the cytoskeleton in breast cancer cells.

1. Introduction

IFN-γ is a unique member of the type II interferon family, and possesses important immunomodulatory and antiviral properties but has ambiguous functions in several cancers. The canonical signaling of IFN-γ requires a specific heterotetrameric receptor complex formed by IFNGR1 and IFNGR2 subunits and the JAK-STAT1 system. In response to the binding of IFN-γ to its receptor complex, activated JAKs1/2 (associated with the receptor complex in the intracellular region) mediates STAT1 phosphorylation. Activated STAT1 homodimers bind to the gamma interferon activated site (GAS) localized in the regulatory sequences of IFN-γ target genes to modulate gene expression [1,2]. Genes induced by IFN-γ include *IRF-1/2* [3–5], *RIG-1* [6–9], *SOCS1/3* [10] *SMAD7* [11], and genes implicated in antigen presentation by major histocompatibility complex (MHC) class I and II [12,13] *IRF-1* mediates IFN-γ-induced expression of late genes, while *IRF-2* represses the transcription of these genes [5]; *RIG-1* has antiviral, immunomodulatory, and suppressor tumor activities and regulates the expression of some genes [6–9]. *SOCS1/3* inhibits IFNs-signaling, and *SMAD7* is a negative regulator of TGFβ signaling [10,11]. Interestingly, in breast cancer, it has been suggested that IFN-γ induces apoptosis and cell cycle arrest, and also that there is autocrine signaling by IFN-γ in these cells [14,15]. Additionally, a gene signature regulated by IFNs may be associated with sensitivity and/or resistance to chemotherapy and endocrine therapy observed in breast cancer cells [16,17].

There is a high rate of heterogeneity observed in breast cancers. Approximately 70% of the cases are estrogen receptor-α positive (ERα+), and the residual cases are ERα-. In both cases, mechanisms that modulate ERα activity and expression appear to be deregulated [18–20]. Previously, we demonstrated that IFN-γ increases ISG15 (Interferon-stimulated gene 15) protein levels in ERα + and ERα- breast cancer cells in addition to type I interferons (IFN-α and IFN-β) that had been previously considered to be the exclusive inducers of ISG15 in other cell types [21–24].

ISG15 is a 15 kDa ubiquitin-like protein that conjugates to its target proteins in a covalent manner through an enzymatic process similar to ubiquitination [25–28]. This process is known as ISGylation and the enzymes involved are the E1-activating (UBE1L), E2-conjugating (UBCH8) [29], and E3 ligase enzymes (HERC5, EFP, and HHARI) [30–33], as well as the de-ISGylase enzyme USP18 that removes

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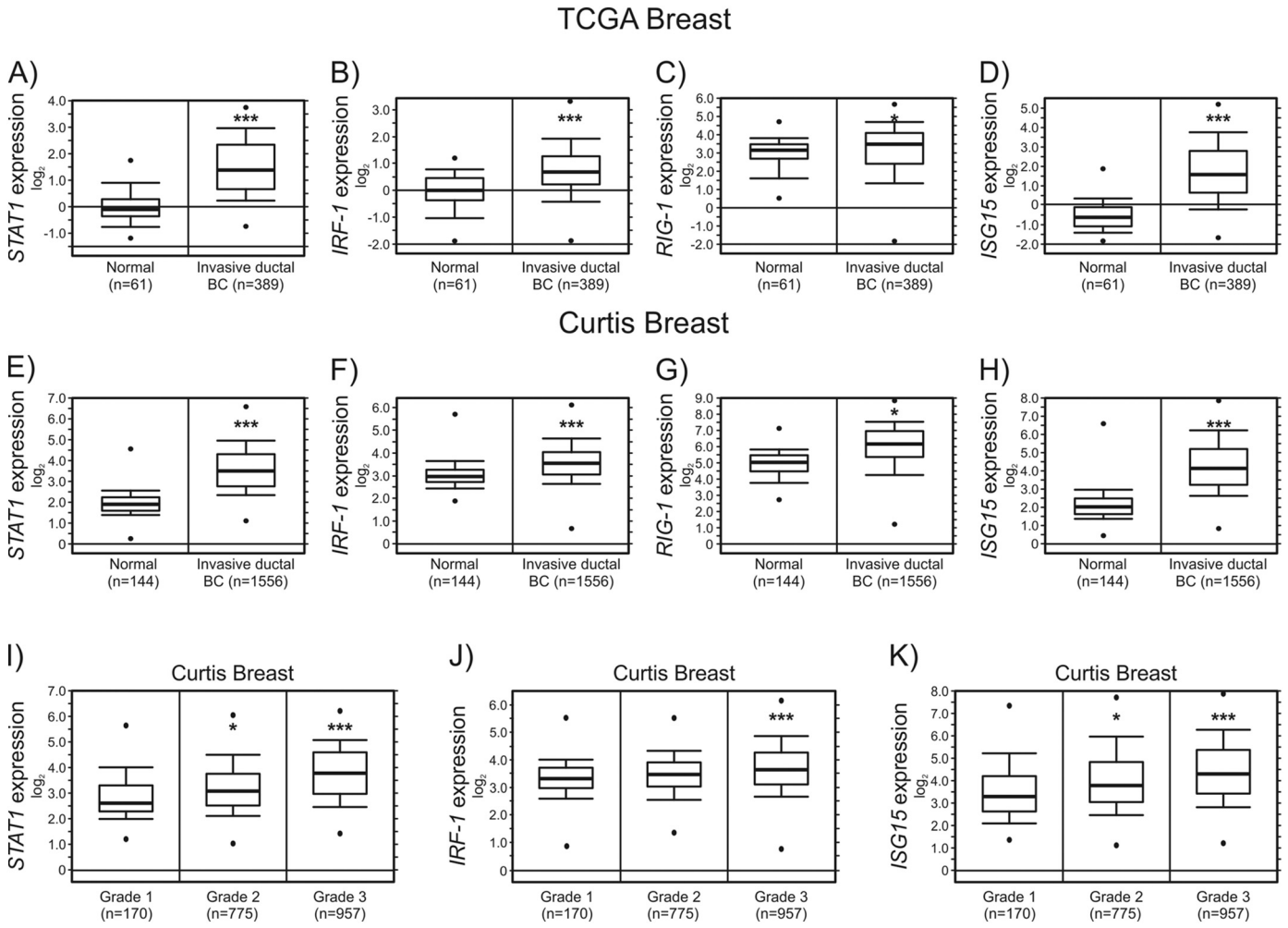

Fig. 1. Increased expression of *STAT1*, *RIG-1*, and *ISG15* in mammary tumors. A-D) TCGA (The Cancer Genome Atlas) and *E*-H) Curtis datasets from Oncomine were analyzed to evaluate *STAT1, IRF-1, RIG1*, and *ISG15* expression in human mammary tumors compared to those in normal tissue. I—K) Curtis dataset was analyzed to determine the *STAT1, IRF-1,* and *ISG15* expression in mammary tumors of grade 1, 2, and 3. Results are considered significant when $p < .05$ (*), $p < .01$ (**) and $p < .001$ (***).

ISGylation of proteins [34]. Additionally, ISG15 can also be detected as free ISG15 when it is not covalently bound to a target protein [35–37]. The function of ISGylated proteins is unclear, as this modification can interfere with and also collaborate with the ubiquitination pathway [38–42].

In breast cancer, it has been shown *in vivo* experiments using athymic mice models demonstrate that depletion of ISG15 enhances tumor development. In contrast, downregulation of ISG15 has been shown to decrease proliferation and migration in breast cancer cell lines *in vitro* [38,43,44]. Although ISGylation functions are yet to be elucidated, since only a limited number of proteins modified by ISG15 are known, it is evident that expression of ISG15 affects the malignancy of breast cancer cells, and many proteins may be modulated by ISGylation under this condition.

In order to explore the significance of ISG15 expression in patients diagnosed with breast cancer, we analyzed the levels of ISG15 and the expression of IFN-γ signaling components in normal and mammary tumor tissues. We probed the effect of IFN-γ on viability, morphology, and protein ISGylation in breast cancer cells. Our data indicate that ISG15 can act as a potential biomarker for breast cancer. Our findings also suggest important implications for IFN-γ induced protein ISGylation in the biology of breast cancer cells.

2. Materials and methods

2.1. Reagents and cell lines

Recombinant human IFN-γ (285-IF-00) was obtained from R&D systems, culture media were obtained from Invitrogen, and reagents were obtained from Sigma, AMRESCO-VWR, and Bio-Rad. The MDA-MB-231 (ERα-, triple negative breast cancer cells), and MCF-7 (ERα + breast cancer cells) cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum and penicillin/streptomycin.

2.2. Subcellular fractionation and total extracts

Nuclear and cytoplasmic fractions were obtained by subcellular fractionation as described previously [23,45]. Using homogenization buffer (250 mM sucrose, 3 mM imidazole protease, and phosphatase inhibitors), the cells were homogenized and then passed 15 times through a 22-gauge needle. The homogenized cells were then centrifuged at 700 × *g* for 10 min to separate the supernatant (cytoplasmic fraction) from the pellet (nuclear fraction). Both fractions were lysed with RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% deoxycholic acid, 1% NP-40, and 0.1% SDS) containing protease and phosphatase inhibitors. For total extracts, the cells were directly

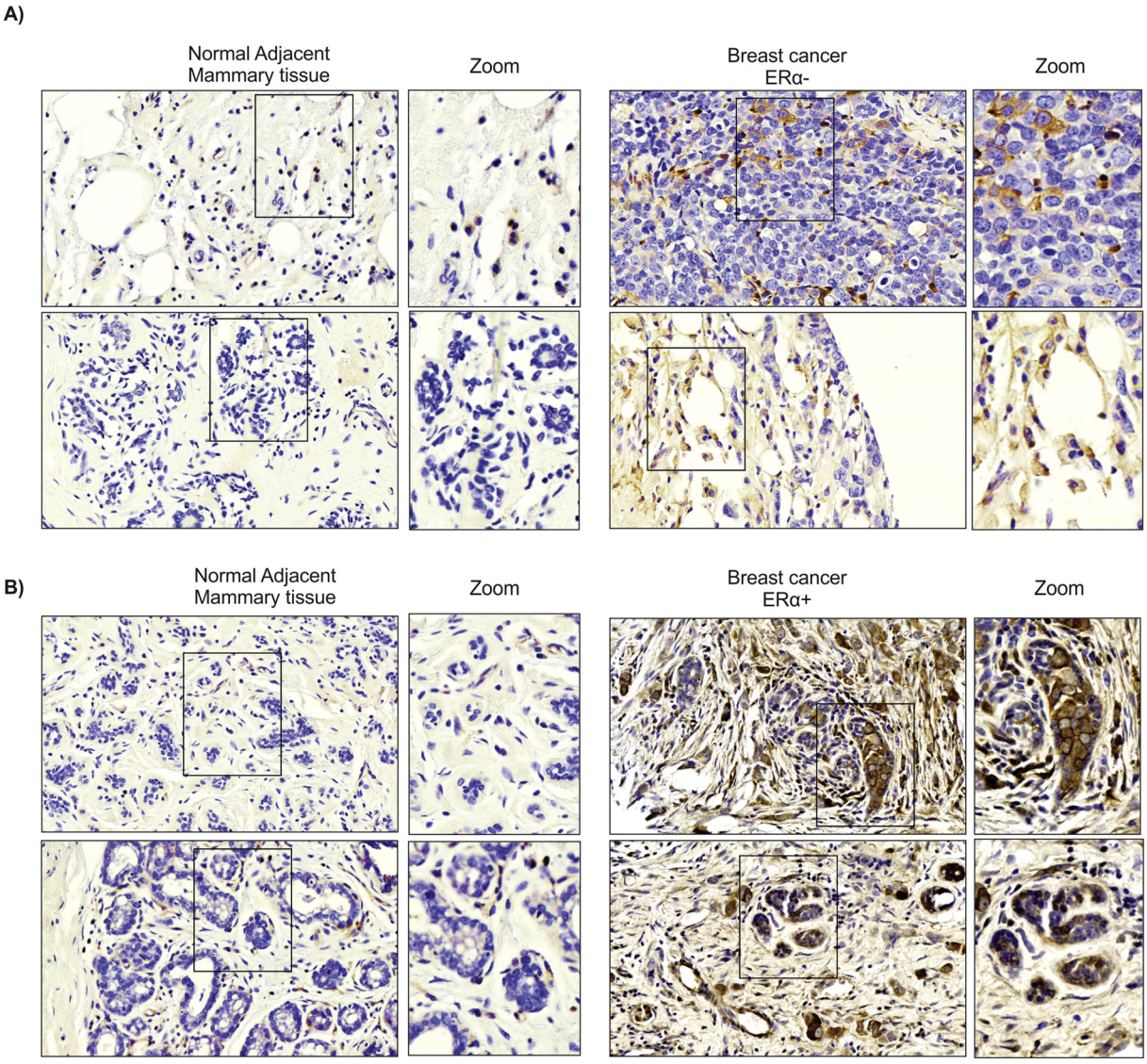

Fig. 2. ISG15 is detected at higher levels in mammary tumors than in adjacent normal mammary tissue.
A, B) Immunohistochemical (IHC) analysis of ISG15 protein expression in invasive ERα + and ERα- breast carcinomas. ISG15 protein expression is predominant in ERα + and ERα- breast cancer tissue in comparison with that in normal adjacent mammary tissue. 20× magnification of representative images. ISG15 protein is mainly detected in the cytoplasm of ERα + and ERα- mammary tumors from patients (A and B, zoom).

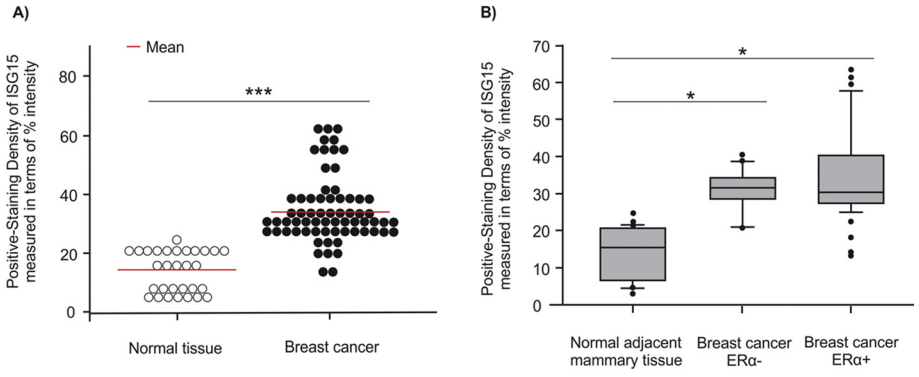

Fig. 3. Statistical analysis of immunohistochemical data from mammary tumors and adjacent normal mammary tissue. Immunohistochemical (IHC) analysis of ISG15 protein in invasive ERα + and ERα- breast carcinomas. A) Analysis of ISG15 protein signal in mammary tumors compared to signal in adjacent normal tissue. The Mann-Whitney rank sum test was performed, and the red line indicates the mean. B) Analysis of ISG15 protein signaling in ERα + and ERα- mammary tumors and in adjacent normal tissues. Kruskal-Wallis one way analysis of variance on ranks was performed to analyze the IHC data. Results are considered significant when $p < .001$ (***) and $p < .05$ (*). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

lysed with RIPA buffer. Lysis was performed at 4 °C for 1 h, and protein extracts were quantified using the Bradford method for WB and immunoprecipitation assays.

2.3. Cell viability assays

For the determination of cellular viability, calcein-AM (green) and propidium iodide or PI (red) assays were performed. Viable cells

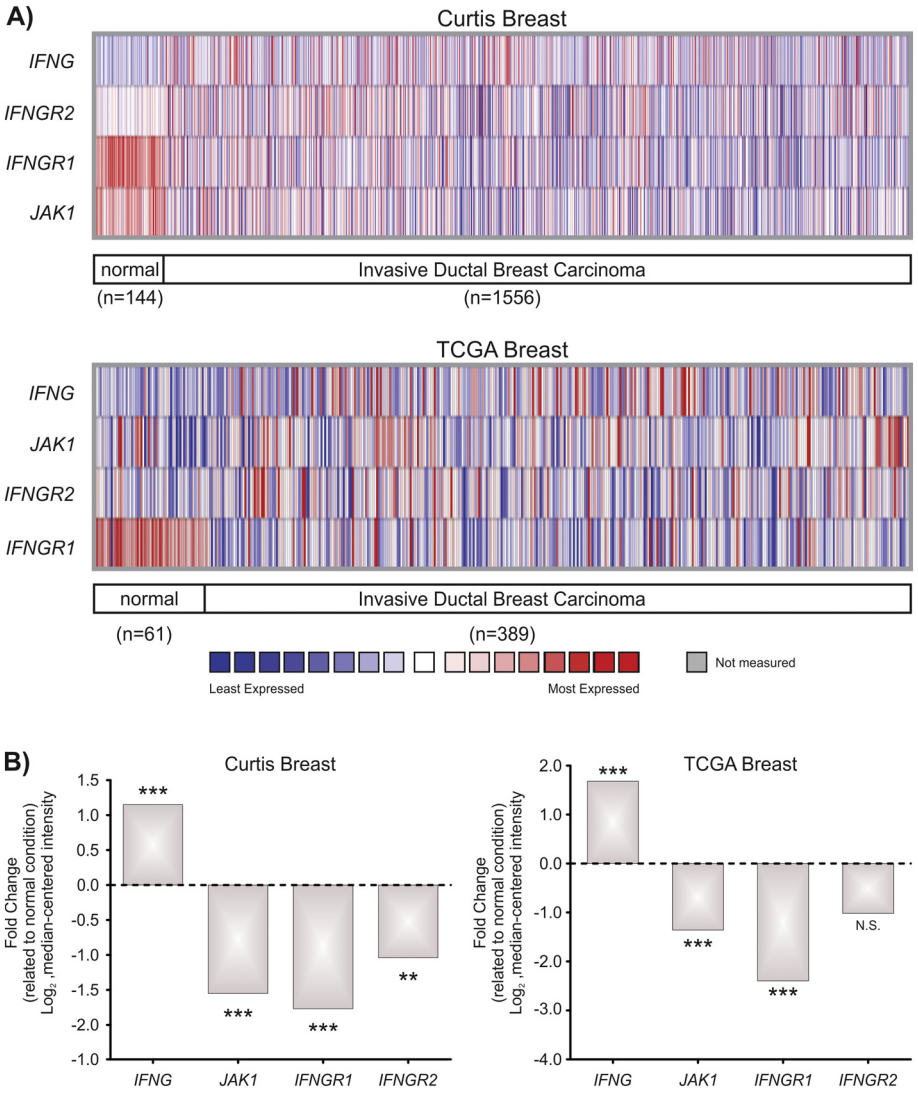

Fig. 4. Expression of IFN-γ signaling components in breast cancer. Heat maps for gene expression of IFN-γ signaling components. A) Curtis and TCGA (The Cancer Genome Atlas) datasets from Oncomine were analyzed to evaluate *IFNG, IFNGR2, IFNGR1* and *JAK1* expression in human mammary tumors and normal tissue. B) Analysis of *IFNG, JAK1*, *IFNGR1* and *IFNGR2* expression in mammary tumors compared to those in normal mammary tissue. Results are expressed as significant when $p < .01$ (**), and $p < .001$ (***). Non-significant differences are indicated by N.S.

possess an active metabolism, allowing intracellular esterases to convert non-fluorescent calcein-AM to green fluorescent calcein. In non-viable cells, a red fluorescence signal is generated when PI binds to DNA indicating that the nuclear membrane is disrupted. Cells treated with or without IFN-γ were incubated with calcein-AM (1 μM) and PI (5 mM) at 37 °C for 30 min, and the cells were then analyzed by fluorescence microscopy.

2.4. Co-immunoprecipitation assays and western blotting

Cytoplasmic and nuclear extracts or total extracts were used for immunoprecipitation assays as described previously [24]. Immunoprecipitation was performed using a specific anti-ISG15 (Santa Cruz Biotechnology) antibody. The immunoprecipitated complexes were separated by SDS-PAGE followed by immunoblotting with specific primary anti-IQGAP1 antibody (H-109, Santa Cruz Biotechnology). As input, cytoplasmic and nuclear extracts (25–100 μg of protein lysates) were used and separated by SDS-PAGE and then immunoblotted with anti-ISG15 (1:1000) (F-9, sc-166,755), anti-α-tubulin (1:3000) (B7, sc-5286) obtained from Santa-Cruz Biotechnology, and anti-lamin B1(1:2000) obtained from Cell Signaling (D4Q4Z). A secondary anti-rabbit IgG antibody was used (1:10000) against anti-lamin B1 (sc-2004; Santa Cruz Biotechnology), and peroxidase Affinipure goat anti-mouse antibody (1:10000) was used for anti-tubulin and anti-ISG15 (115–035-003, Jackson). Protein detection was performed using SuperSignal West Pico chemiluminescent substrates (Thermo Scientific) or Immobilon Western (Millipore) reagents. The experiments were performed in triplicate, and representative images are shown.

2.5. Immunofluorescence assay

The cells were fixed in 4% paraformaldehyde for 10 min, permeabilized with 0.1% Triton X-100 for 10 min, and blocked with 1% albumin for 1 h. Cells were incubated overnight with anti-ISG15 (F-9, sc-166,755; 1:100), and then the cells were washed and incubated with Alexa Fluor 647 anti-mouse IgG secondary antibody (ab150107, Abcam; 1:750) for 1 h in the dark. The slides were prepared with ProLong Diamond Antifade Mountant containing DAPI (Invitrogen). For phalloidin assays, after blocking, the cells were incubated for 1 h with phalloidin (1:40) and mounted with DAPI solution. An Olympus IX2 confocal laser-scanning microscope was used to visualize the cells, and ImageJ software was used for imaging, processing, and analyzing the

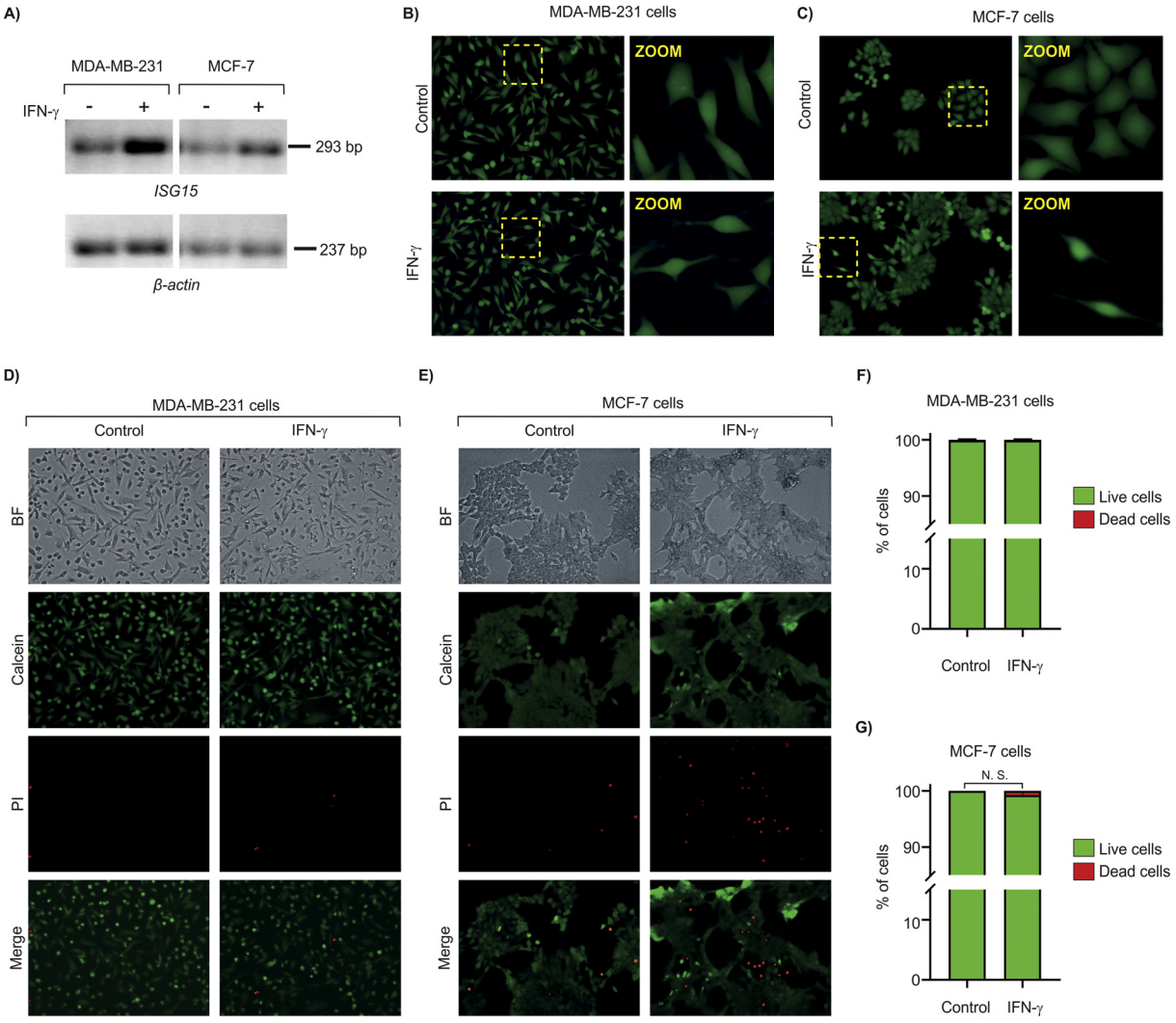

Fig. 5. IFN-γ affects the morphology of breast cancer cells. A) Total RNA was isolated from MDA-MB-231 and MCF-7 cells with or without IFN-γ (100 ng/ml) treatment and subjected to RT-PCR analysis. Specific primers for *ISG15* and *β-actin* were used. PCR products of 237 bp for *β-actin* and 293 bp for *ISG15* are shown. B, C) Calcein staining for MDA-MB-231 and MCF-7 cells with or without IFN-γ treatment for 24 h. D, E) Cellular viability assays using calcein/propidium iodide or PI on MDA-MB-231 and MCF-7 cells with or without IFN-γ treatment. Cells stained green represent viable cells and those stained red represent dead cells. F, G) Quantification of cell viability is shown. Non-significant differences are indicated by N.S. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

results.

2.6. Gene expression analysis

The cancer microarray database Oncomine (www.oncomine.org) was used to analyze gene expression in patient-derived mammary tumors and normal mammary tissue. The datasets used were The Cancer Genome Atlas (TCGA) and Curtis.

2.7. In silico analysis

The analysis of protein–protein interactions was carried out using STRING 10.5 version software (https://string-db.org/cgi/network.pl) [46].

2.8. Human breast cancer tissue microarray

Breast cancer and normal tissue arrays, along with corresponding control tissues derived from the same patient (16 cases/48 cores), were purchased from Biomax (BRC481, U.S. Biomax human breast cancer tissue microarray). This microarray was composed of formalin-fixed, paraffin-embedded tissue.

2.9. Immunohistochemical assay

The microarrays were examined by immunohistochemical (IHC) analysis using a monoclonal antibody specific for ISG15 (F-9, sc-166,755 from Santa-Cruz Biotechnology). Samples were initially deparaffinized in xylene followed by rehydration in graded alcohol. Endogenous peroxide activity was quenched using 3% hydrogen peroxide (5 min) and samples were blocked using horse serum solution. Samples were then incubated with anti-mouse IgG (peroxidase). Results were visualized after incubation using DAB solution. Slides were dehydrated and cleared in xylene baths, and then mounted. A counterstain with Hematoxylin was also performed. Positive and negative controls (negative control: lacking the ISG15 antibody) were used.

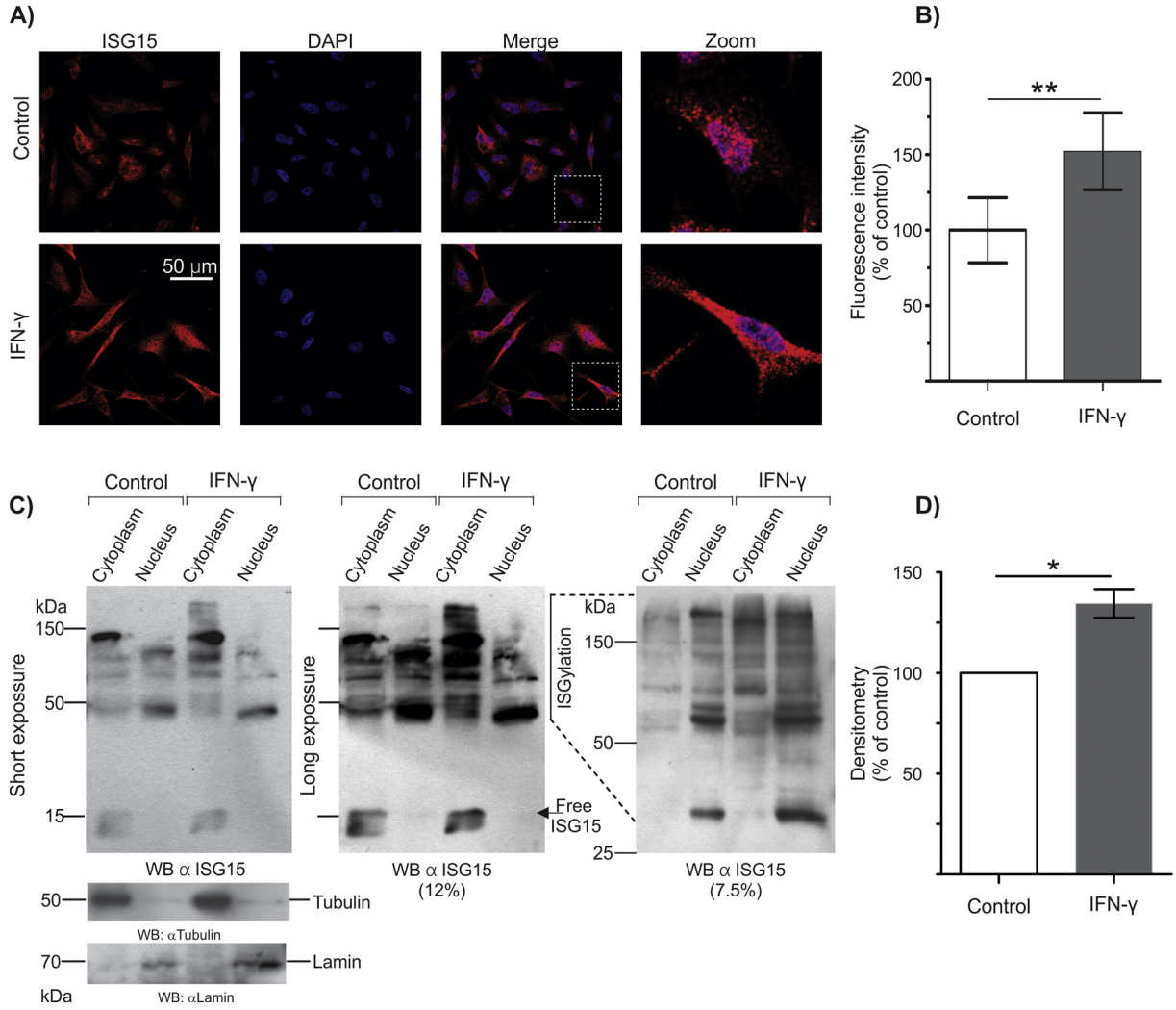

Fig. 6. IFN-γ increases cytoplasmic ISGylation marks in MDA-MB-231 cells. A) Subcellular localization of ISG15 in MDA-MB-231 cells with or without IFN-γ (100 ng/ml) treatment were evaluated by immunofluorescence. Changes in morphology are visible in the zoomed image. B) Quantification of ISG15 fluorescence intensity in MDA-MB-231 cells with or without IFN-γ treatment. C) Cytoplasmic and nuclear extracts taken from MDA-MB-231 cells with or without IFN-γ (100 ng/ml) treatment were used for WB analysis of ISG15 (gels 12%, short and long exposure). Cytoplasmic and nuclear fractions were also separated in 7.5% acrylamide gels for WB against ISG15. Lamin and tubulin were used as controls for the cytoplasmic and nuclear fractions, respectively. D) Densitometry analysis of the cytoplasmatic levels of protein ISGylation. Results are considered significant when $p < .05$ (*), and $p < .01$ (**).

2.10. Immunohistochemical analysis

2.10.1. Image acquisition

IHC photomicrographs were captured using an AxioScan.Z1 microscope (Zeiss, Germany) equipped with an AxioCam MRc5 camera (Zeiss, Germany). Light and camera settings were operated by ZEN2.3 software (blue edition, Carl Zeiss microscopy GmbH, 2011). Images were captured under 20× and 40× objective lenses.

2.10.2. Digital Image Analysis

IHC photomicrographs were used for developing a semi-automated analysis protocol called IHC profiler [47]. Quantitative analysis was performed using photomicrographs of 4 randomly selected fields taken from each representative tumor tissue on the microarray.

2.11. RT-PCR assay

ISG15 and *β-actin* mRNA levels were detected by RT-PCR. TRIzol (Invitrogen) was used to isolate total RNA from MCF-7 and MDA-MB-231 cells treated with or without IFN-γ (100 ng/ml) for 24 h. Total RNA (2 μg) was used for cDNA synthesis, and random hexamers and Moloney murine leukemia virus RT (Invitrogen) were used to promote the reaction. PCR was performed using a *Taq* PCR Master Mix kit (Qiagen) and primers specific for *ISG15* (Sense 5′-3′: GGT GGA CAA ATG CGA CGA AC and antisense 5′-3′: ATG CTG GTG GAG GCC CTT AG) and *β-actin* (Sense 5′-3′: GGG TCA GAA GGA TTC CTA TG and antisense 5′-3′: GGT CTC AAA CAT GAT CTG GG). PCR products were analyzed by agarose gel electrophoresis.

2.12. Statistical analysis

The data presented in our figures are representative of 3 or more independent experiments. Densitometry for WB, quantification of cell viability assays and fluorescence intensity percentages were performed using Fiji-ImageJ. Data were shown as percentages. Unpaired student's *t*-tests were performed using *GraphPad Prism* 5 software (GraphPad). Results were considered significant when $p < .05$ (*), $p < .01$ (**), and $p < .001$ (***). Non-significant differences were by N.S. Mann-Whitney rank sum test, and Kruskal-Wallis One Way Analysis of Variance on Ranks were performed to analyze IHC data.

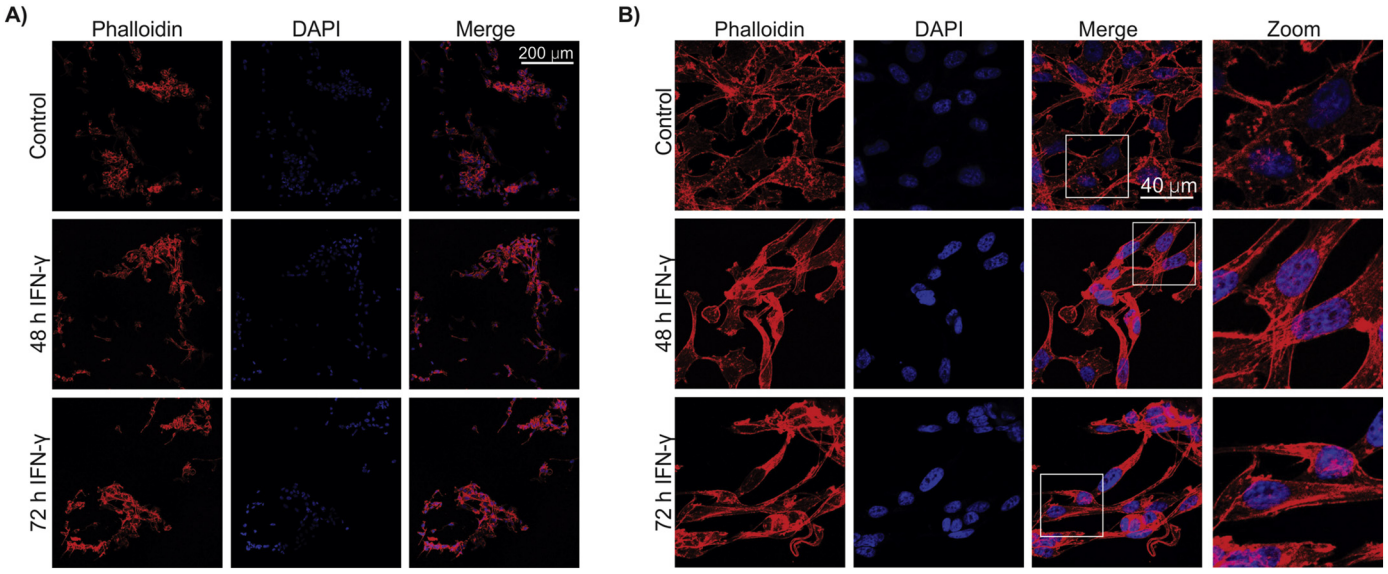

Fig. 7. IFN-γ modulates cytoskeletal remodeling of breast cancer cells. A, B) Phalloidin staining for MDA-MB-231 cells with or without IFN-γ (100 ng/ml) treatment for 48 and 72 h.

3. Results

3.1. Expression of STAT1, IRF-1, RIG-1, and ISG15 is increased in breast cancer

Approximately 80% of breast cancers develop in the mammary ducts and are invasive [48]. In order to explore the expression of factors activated by IFN-γ signaling (*STAT1, IRF-1,* and *RIG-1*) and *ISG15*, we evaluated their mRNA levels in tumors of patients diagnosed with invasive ductal breast cancer and compared them with their corresponding levels in normal mammary tissue using TCGA (450 patients) and Curtis (1700 patients) datasets. Our analysis of both datasets showed that the expression of all four genes (*STAT1, IRF-1, RIG-1*, and *ISG15*) were significantly increased in breast cancer (Fig. 1A-1H). Additionally, using the Curtis dataset (1902 patients), we found that the mRNA levels of *STAT1, IRF-1* and *ISG15* increased in a tumor-grade dependent manner. Expression levels were lower in grade 1 tumor tissues (well differentiated, tumor tissues appear similar to normal mammary tissues) and increased in grade 2 tumors (moderately differentiated) that grow and spread slowly. The mRNA levels of *STAT1, IRF-1*, and *ISG15* were highest in grade 3 tumors, which were poorly differentiated with a rapid growth and spread (Fig. 1I-1K). These results indicated that the expression of *STAT1*, *IRF-1*, *RIG-1*, and *ISG15* were increased in mammary tumors, and in particular, *STAT1, IRF-1*, and *ISG15* expression seemed to be critical in the progression of breast cancer.

Interestingly, other IFN-γ target genes were also upregulated in mammary tumors when compared to normal mammary tissue. These included *SOCS1* and *SMAD7*, whereas other IFN-γ regulated genes such as *SOCS3* and *IRF-2* were downregulated in breast cancer when compared to healthy breast tissue (Supplementary fig. 1). Furthermore, genes associated with antigen presentation by MHC class I and II were generally upregulated in breast cancer (Supplementary fig. 2). These results indicate that the IFN-γ-driven transcriptome may be deregulated in breast cancer.

3.2. ISG15 protein levels are enhanced in mammary tumor samples compared to normal adjacent tissue

Because *ISG15* is one the upregulated genes in breast cancer, we first decided to analyze the protein levels of ISG15 in tumors of patients with breast cancer. We evaluated a breast cancer microarray that contained 16 cases of invasive breast cancer in duplicates with adjacent uninvolved tissue from the same patients, and we detected ISG15 protein by immunohistochemistry (IHC). A strong signal was detected for ISG15 protein in different cases of ERα- and ERα + mammary tumors compared to signals detected in normal mammary tissue (Fig. 2A and B). The ISG15 signal was mostly detected in the cytoplasm in all cases. However, in some cells, ISG15 was also detected in the nucleus (Fig. 2A and B, zoom). Our statistical analysis demonstrated that ISG15 protein expression was significantly higher in breast cancer samples than in the normal mammary tissue (Fig. 3A). We performed the same analysis by classifying the tumors as ERα- and ERα + , and our results indicated that these tumors also have higher levels of ISG15 protein than the adjacent normal mammary tissue and was independent of ERα status (Fig. 3B). These results indicate that the ISG15 modifier protein is upregulated in breast cancers, and this upregulation may lead to the ISGylation of target proteins to modulate their activities.

3.3. Expression of the IFN-γ signaling pathway elements IFNGRs, JAK2, and IFNG is also altered in breast cancer

Expression of *STAT1* and *ISG15* is positively associated with tumor grade, and ISG15 protein levels are increased in breast cancer cells. Since STAT1 and ISG15 are linked to IFN-γ signaling, we analyzed the expression of the elements of this pathway including *IFNGR1*, *IFNGR2*, and *JAK1*, as well as the expression of the gene that encodes for IFN-γ cytokine (*IFNG*) in mammary tumors. These analyses were performed using Curtis and TCGA datasets derived from Oncomine. The analysis of these two datasets indicated that the IFN-γ signaling pathway is deregulated in mammary tumors, as the mRNA levels of *JAK1*, *IFNGR2*, and *IFNGR1* were decreased in these tumors in comparison to those found in normal tissue, and the expression of *IFNG* was increased (Fig. 4A, B). Taken together, these results suggest that IFN-γ production and secretion may be enhanced in mammary tumors, and the activation of the IFN-γ pathway and some of its effectors may be altered in comparison with normal mammary tissue.

3.4. IFN-γ affects the morphology of breast cancer cells

Previously, we demonstrated that the protein levels of free ISG15 and protein ISGylation are increased in response to IFN-γ treatment in MCF-7 and MDA-MB-231 breast cancer cells [24]. To examine if *ISG15* is an IFN-γ-induced gene in these breast cancer cells, we performed an

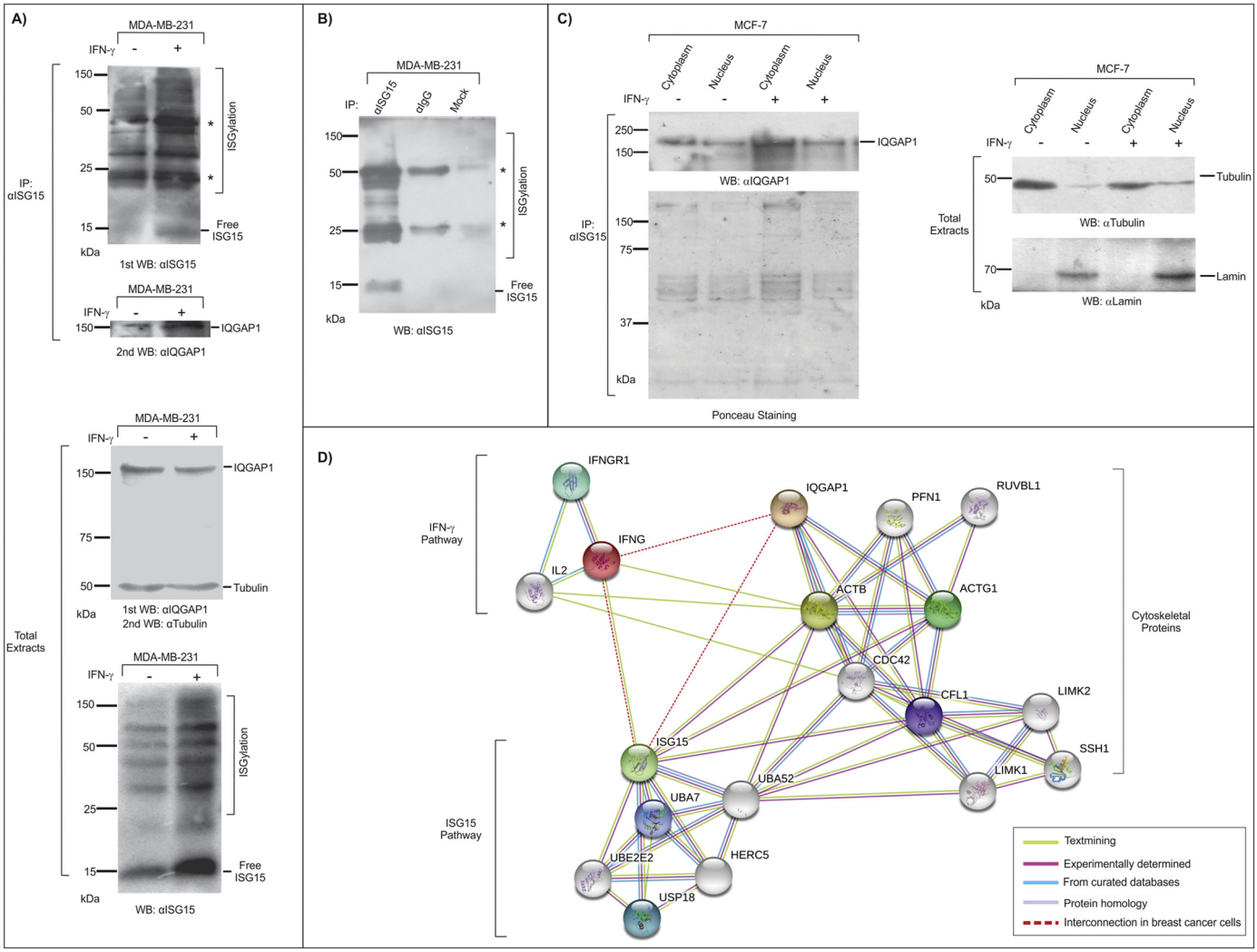

Fig. 8. Association between ISG15 and IQGAP1 is increased by IFN-γ treatment of breast cancer cells. A) Endogenous ISG15 immunoprecipitation (IP) followed by WB for ISG15, and then for IQGAP1, using total extracts from MDA-MB-231 cells with or without IFN-γ (100 ng/ml) treatment. IQGAP1 and ISG15 levels were also evaluated by WB, and tubulin was used as a loading control. B) Mock and IgG were used as controls to evaluate the specificity of ISG15 IP (*IgG). C) Cytoplasmic and nuclear extracts derived from MCF-7 cells with or without IFN-γ (100 ng/ml) treatment were immunoprecipitated using the ISG15 antibody and analyzed by WB for IQGAP1. Lamin and tubulin were used as loading controls for nuclear and cytoplasmic fractions, respectively. Ponceau staining was used on IP membrane. E) Interactome analysis using STRING software for IFN-γ signaling, ISGylation, and cytoskeletal remodeling associated proteins. The association between ISG15, IQGAP1, and IFN-γ is shown as a dashed red line. Known interactions from curated databases, text mining, experimental findings, and protein homology are indicated in blue, green, purple, and dark blue lines, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

RT-PCR assay using RNA obtained from cells treated or untreated with IFN-γ for 24 h. Our results indicated that *ISG15* mRNA levels are up-regulated by IFN-γ (Fig. 5A), suggesting that these cell lines may provide a useful system to study the effects of IFN-γ/ISG15 signaling. Since IFN-γ signaling pathway seems to be deregulated in breast cancer cells, we decided to examine the effect of IFN-γ treatment on the morphology and viability of MCF-7 and MDA-MB-231 human breast cancer cells. Initially, the cells were treated with IFN-γ for 24 h, followed by calcein staining, and as a result we detected morphological changes in MCF-7 and MDA-MB-231 cells, since some of the cells were more elongated in comparison with cells without IFN-γ treatment (Fig. 5B, C, zoom). We then evaluated the viability of these cells after IFN-γ treatment for 48 h using cell viability assays (Calcein-AM/propidium iodide (PI)). Calcein emits a green fluorescence when it is released from Calcein-AM by esterases enzymatic activity of viable cells. PI emits a red fluorescence when it passes the membranes of dead cells to intercalate with nuclear DNA. As a result, Calcein-AM stains viable cells green and PI stains dead cells red. Our results indicated that MDA-MB-293 cells with or without IFN-γ treatment were viable, as the majority stained green for calcein (Fig. 5D and F). Similarly, the viability of MCF-7 cells treated with IFN-γ was not decreased by IFN-γ stimulation (Fig. 5E and G). Our statistical analysis, determined that the percentage of dead cells was not significantly different between the IFN-γ treated and untreated cells (Fig. 5F and G). Thus, our assays revealed that MCF-7 and MDA-MB-231 breast cancer cells undergo morphological changes in response to IFN-γ, and IFN-γ treatment does not appreciably affect the viability of these cells.

3.5. IFN-γ increases the marks of ISGylation in the cytoplasm of MDA-MB-231 breast cancer cells

We have evidenced changes in the morphology of breast cancer cells when they were treated with IFN-γ. For this reason, we performed an immunofluorescence assay to evaluate the subcellular distribution of ISG15 in response to IFN-γ treatment and its possible relation with the changes in morphology of MDA-MB-231 cells in this condition. As a

result, ISG15 signal was detected in the cytoplasm and nucleus of breast cancer cells without IFN-γ treatment. After IFN-γ treatment we also detected changes in the morphology of some of these cells (cell shape more elongated) in comparison with untreated cells (Fig. 6A, zoom). We also observed that IFN-γ treatment had no effect on ISG15 subcellular distribution in these cells, but a stronger signal for ISG15 was detected (Fig. 6A). Our analysis demonstrated that fluorescence intensity for ISG15 was significantly higher in MDA-MB-231 cells treated with IFN-γ compared with that detected in untreated cells (Fig. 6B).

Additionally, we performed a western blot (WB) to specifically detect the distribution of ISGylation marks between the cytoplasmic and nuclear compartments in MDA-MB-231 cells treated with or without IFN-γ (Fig. 6C short/long exposure, 12% and 7.5% gels). We detected statistically significant increase in ISGylation marks within the cytoplasm in response to IFN-γ (Fig. 6D). Hence, IFN-γ induces changes in the morphology of breast cancer cells and also increases ISG15 levels, while exerting no effects on subcellular distribution of this protein. ISGylation marks were mainly detected in the cytoplasm, suggesting that this interferon promotes the conjugation of ISG15 to several cytoplasmic proteins to modify them and modulate their functions.

3.6. *IFN-γ induces changes in the actin cytoskeleton and ISGylation of some cytoplasmic proteins could be implicated*

To further explore if IFN-γ could modulate the actin cytoskeleton of breast cancer cells, we performed phalloidin staining of actin filaments (also known as F-actin) in IFN-γ treated and untreated MDA-MB-231 cells. We observed changes in the actin cytoskeleton that were promoted by IFN-γ treatment. F-actin was concentrated in various subcellular areas but was reorganized into stress fibers following 48 h and 72 h of IFN-γ treatment (Fig. 7A and B). A similar effect was observed in MCF-7 cells expressing the ISGylation system (Supplementary Fig. 3). Given these observations, modifications in the morphology of breast cancer cells are clearly linked to cytoskeletal remodeling in response to IFN-γ treatment.

The changes in the cytoskeleton of breast cancer cells promoted by IFN-γ treatment could be linked to ISGylation of cytoplasmic proteins, but few ISGylation-target proteins have been identified to date. IQGAP1 is a protein that participates in the regulation of cytoskeletal dynamics, and interestingly, is one of the few proteins that appears to be ISGylated [49–51]. Based on this, we evaluated the effect of IFN-γ treatment on ISGylation of IQGAP1. Using total extracts obtained from MDA-MB-231 cells that were untreated or treated with IFN-γ, we performed an immunoprecipitation assay to enrich the modifier protein ISG15. A first WB against ISG15 was performed to probe the efficiency of immunoprecipitation, displaying a pattern of ISG15-conjugated proteins that increased in response to IFN-γ treatment (Fig. 8A, 1st WB:αISG15). Then, a second WB against IQGAP1 showed that IFN-γ also increased association between ISG15 and IQGAP1 (Fig. 8A, 2nd WB:αIQGAP1). Furthermore, the detection of tubulin from total extracts was used as a loading control, and it was observed that the IQGAP1 levels were not affected by IFN-γ. In contrast, ISGylation levels increased when the cells were treated with IFN-γ (Fig. 8A, bottom). Additionally, the use of IgG and mock controls were used to evaluate the specificity of ISG15 immunoprecipitation (Fig. 8B). IFN-γ treatment also increased this association between ISG15 and IQGAP1 in the cytoplasm of MCF-7 breast cancer cells (Fig. 8C). This ISG15 immunoprecipitation was probed using Ponceau staining. Therefore, proteins such as IQGAP1 that are involved in cytoskeletal remodeling may associate with ISG15 *via* ISGylation, and be modulated by IFN-γ in breast cancer cells.

To investigate the relationship between the IFN-γ signaling pathway, ISG15, and the ISGylation system in the context of the modulation of cytoskeletal reorganization, we performed an interactome analysis using STRING software. Our analysis showed a connection between these pathways, where the actin proteins (ACTB, ACTG1) are key players along with other proteins that regulate the cytoskeleton. Importantly, we observed a novel association between IFN-γ, ISGylation, and IQGAP1 in the cytoskeletal dynamics of breast cancer cells (Fig. 8D).

4. Discussion

Our study revealed increased expression of *STAT1, IRF-1, RIG-1* (factors that mediate IFN-γ signaling), and *ISG15* genes in breast cancer cells compared to their expression in normal mammary tissues, suggesting a possible interplay between IFN-γ and ISG15-regulated molecular pathways. We also observed increase in the expression of *STAT1, IRF-1*, and *ISG15* in grade 3 mammary tumors in comparison to grade 1 tumors, demonstrating the relevance of the expression of these genes in the progression of breast cancer.

Additionally, the observation that the levels of the modifier ISG15 are elevated in mammary tumors in comparison with those in normal adjacent tissue suggests that protein ISGylation may be a highly active modification in breast cancer cells. Under this condition, the function of several proteins may be affected by ISGylation, and some of them may be linked to proliferation and metastasis. As ISG15 expression and protein levels are increased in breast cancer, this may provide a potential biomarker for this cancer type. It must be noted that ISG15 upregulation has also been reported in other cancer types [52–56], but the mechanism of action is unclear.

Breast cancer cells exhibit a characteristic profile of free ISG15 and ISGylation that is upregulated by IFN-γ [23,24] and not exclusively by IFN-α/β as in some cell types. Given this, IFN-γ signaling may also play a critical role in breast cancer by mediating the modification of proteins by ISGylation and the gene expression characteristic of these cells. For example, it has also been reported that patient-derived mammary tumor xenografts in athymic mice can secrete IFN-γ, and a gene signature regulated by interferons may be associated with the grade of sensitivity to chemotherapy. Additionally, IFN-γ autocrine signaling has been associated with sensitivity to fulvestrant treatment [14–17].

Accordingly, IFN-γ may be a key component of the mammary tumor microenvironment and may function to generate autocrine and paracrine signals to induce protein ISGylation. Here, our results suggest that the *IFNG* gene that encodes for IFN-γ is increased in mammary tumors relative to control tissue, and as a consequence the production and secretion of IFN-γ may be increased in these tumors. Our analysis also indicated, however, that expression of IFN-γ signaling elements such as receptors and JAK1 is decreased in breast cancer. It is unclear if the deregulation of these IFN-γ signaling components is a compensatory mechanism to regulate this signaling, but this deregulation may have serious implications in for intercellular communication within the tumor microenvironment. Additionally, the deregulation of IFN-γ signaling in breast cancer may affect the expression of IFN-γ target genes, including those that allow for recognition of tumor cells by the antitumor immunity system (Supplementary fig. 2), and may also affect the pattern of proteins regulated by ISGylation.

Although there are alterations in the expression IFN-γ signaling elements in mammary tumors from patients, MCF-7 and MDA-MB-231 breast cancer cell lines are responsive to this molecule. Cellular outcomes, however, may be different due to crosstalk with other signaling pathways such as ERα and other nuclear receptor pathways that possess altered regulatory mechanisms that affect signaling in certain cancer types [57]. MCF-7 cells are ERα+, and the viability of these cells is not altered in a statistically significant manner when treated with IFN-γ. Additionally, MDA-MB-231 cells are ERα- and their viability is also unaffected by IFN-γ stimulation. Interestingly, in both cell types, IFN-γ treatment induced changes in cellular morphology, and our results suggest that these changes are linked to cytoskeletal reorganization. A deregulation of the IFN-γ signaling pathways may be associated with breast cancer progression.

The changes in the morphology of breast cancer cells induced by IFN-γ can, in part, be mediated *via* ISGylation of cytoplasmic proteins,

as IFN-γ increases ISGylation mark mainly in the cytoplasm. Here, we analyzed IQGAP1, a protein that is associated with cytoskeletal remodeling that has also been identified as an ISGylation target. Our findings suggest that the interaction between ISG15 and IQGAP1 is increased by IFN-γ treatment.

An important challenge is to identify other ISG15-interacting proteins that modulate the cytoskeletal organization and determine the specific function of this modification. To date, protein ISGylation has been determined principally as monoISGylation, regulating the protein stability either by inhibiting or promoting the ubiquitination pathway. Also, functions unrelated to protein stability have been proposed, in which ISGylation appears to modulate protein–protein interactions. For example, filamin B is another protein associated with actin that connects actin filaments to the cell membrane. When filamin B is not ISGylated, it acts as a scaffold for RAC1, MEKK1, and MKK4 proteins allowing IFN-α/β induced JNK signaling for the induction of apoptosis. When filamin B becomes ISGylated, however, its interactions with RAC1, MEKK1, and MKK4 proteins are lost, resulting in the inhibition of JNK signaling. Therefore, filamin B ISGylation blocks IFN-α/β induced JNK signaling [58]. In our study, we observed that IQGAP1 levels were not altered by IFN-γ stimulus (Fig. 8 A (bottom) and Supplementary fig. 4), and it is likely that ISGylation modulates the interaction of IQGAP1 with other proteins. Additionally, our interactome study indicated a close relationship between IFN-γ signaling, the ISGylation system, and proteins, such as IQGAP1, present in the cytoskeleton that may participate in cytoskeletal organization. Many of these proteins may be regulated by IFN-γ-induced ISGylation and/or modulated at the transcriptional level by IFN-γ. Thus, our findings may provide insight between these pathways in breast cancer cells. More studies may help to demonstrate functional and mechanistic implications of these pathways in the growth and progression of mammary tumors, as well as, their potential for the diagnosis, prognosis, and treatment of breast cancer.

5. Conclusion

In conclusion, our results indicate that the expression of elements involved in IFN-γ signaling pathway, IFN-γ target genes, and protein ISGylation are deregulated in mammary tumors. IFN-γ in breast cancer cells increases *ISG15* expression and subsequent protein ISGylation, and induces changes in cellular morphology associated with the cytoskeleton. ISG15 can be a potential biomarker for breast cancer, and the target of ISGylation, as IQGAP1, may present important implications in this context.

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Declaration of interest

The authors report no conflicts of interest in this work.

Author contributions

A.C.T.-C. designed research, performed some WB, conducted cellular viability assays, performed the *in silico* analysis, and wrote the manuscript. C.C. performed the IHC assays and analyzed data. E.C.-R. performed the IF and Co-IP experiments and prepared some figs. J.O.R.-J. prepared some figures and improved the manuscript. A.R.-M. assisted in the IHC assays. M.S-G performed the RT-PCR assay.

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Appendix A. Supplementary data

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