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Non-muscle myosin IIA is post-translationally modified by interferon-stimulated gene 15 in breast cancer cells

Eduardo Cruz-Ramos[a], Marina Macías-Silva[b], Antonio Sandoval-Hernández[a], Angeles C. Tecalco-Cruz[a,*]

[a] Instituto de Investigaciones Biomédicas. Universidad Nacional Autónoma de México, Mexico City, 04510, Mexico
[b] Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Mexico City, 04510, Mexico

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ABSTRACT

ISG15 (interferon-stimulated gene 15) exists as free ISG15 or conjugated ISG15 modifying its target proteins via ISGylation. Few proteins have been identified and studied as ISGylation targets, and their relevance is not completely clear. Here, we isolated ISG15 from MDA-MB-231 breast cancer cells using immunoprecipitation and identified non-muscle myosin IIA (NMIIA) using mass spectrometry as endogenously associated with ISG15. The identification of NMIIA as an ISG15-interacting protein was important, because levels of NMIIA mRNA were not deregulated in all breast cancers, and because our *in silico* analysis indicated that NMIIA was the target of different posttranslational modifications and had an interactome associated with cytoskeletal remodeling. Furthermore, our experimental assays of co-immunoprecipitation and immunofluorescence confirmed that ISG15 was covalently associated with NMIIA in the cytoplasm of breast cancer cells and that interferon γ (IFN-γ) increased this association without alterations in the NMIIA levels. Thus, NMIIA ISGylation is regulated by IFN-γ, and this modification may modulate its interactions with proteins that remodel the cytoskeleton, participating in the growth and progression of mammary tumors.

1. Introduction

Interferon-stimulated gene 15 (*ISG15*) encodes a small l5 kDa protein made up of two ubiquitin-like domains and the amino acid sequence LRLRGG in the C-terminal domain. This sequence has been shown to covalently bind target proteins, resulting in ISGylation, a modification that requires a system similar to ubiquitination formed by the UBE1L (E1-activating), UBCH8 (E2-conjugating), and E3 ligase enzymes such as HERC5, EFP, and HHARI. Additionally, this ISGylation system contains an enzyme to remove ISGylation, the USP18 de-ISGylase enzyme, also named UBP43 (Basters et al., 2014; Dastur et al., 2006; Feng et al., 2008; Okumura et al., 2007; Wong et al., 2006; Zou and Zhang, 2006; Kim et al., 2004; Zhao et al., 2004). Nonetheless, ISG15 can be detected when it is not covalently bound to its target proteins, designated as free ISG15. The free ISG15 form can be secreted from lymphocytes and monocytes and can be recognized by the lymphocyte function-associated antigen 1 (LFA-1) receptor to induce interferon γ (IFN-γ) release from natural killer cells (D'Cunha et al., 1996; Knight and Cordova, 1991; Swaim et al., 2017). So far, it has been proposed that protein ISGylation can interfere or collaborate with the ubiquitination pathway and/or can affect the interactions between proteins. Moreover, the majority of ISGylated proteins seem to be monoISGylated (Burks et al., 2014; Desai et al., 2006; Fan et al., 2015; Huang and Bulavin, 2014; Jeon et al., 2009; Liu et al., 2003).

With respect to the regulation of ISGylation, ISG15 is upregulated in several cancer types (Chen et al., 2016; Mao et al., 2016; Padovan et al., 2002; Satake et al., 2010; Wan et al., 2013). Moreover, type I interferons (IFN-α and IFN-β) have been shown to be inducers of *ISG15* in several cell types, and, recently, we demonstrated that IFN-γ increased free ISG15 and ISGylation levels in ERα+ and ERα− breast cancer cells (Malakhova et al., 2003; Recht et al., 1991; Tecalco Cruz and Mejia-Barreto, 2017; Tecalco-Cruz and Cruz-Ramos, 2018). IFN-γ is a unique member of type II interferons that can transduce its signal via a specific heterotetrameric receptor that activates the Janus protein tyrosine kinase (JAK)/signal transducer and activator of transcription 1 (STAT1) system to modulate the transcription of its target genes. IFN-γ has immunoregulatory functions and dual roles in some cancer types. In breast cancer, IFN-γ seems to induce cycle arrest and apoptosis, and gene signatures for interferons have been associated with the resistance to chemotherapy or endocrine therapy (Gooch et al., 2000; Legrier

* Corresponding author.
E-mail address: atecalco@iibiomedicas.unam.mx (A.C. Tecalco-Cruz).

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et al., 2016; Ning et al., 2010; Niu et al., 2015).

Hence, the effects of IFN-γ on breast cancer cells are important as mammary tumors are highly heterogeneous, able to present or develop resistance to treatments, and progress to invade other tissues because of several deregulated molecular pathways, including nuclear receptors signaling (Tecalco-Cruz, 2018; Tecalco-Cruz and Ramirez-Jarquin, 2017). Furthermore, it has been suggested that free ISG15 has anti-tumor functions *in vivo*; however, downregulation of ISG15 decreases proliferation and migration of breast cancer cell lines (Burks et al., 2014, 2015; Desai et al., 2012). Nevertheless, ISGylation functions have yet to be elucidated, as only a limited number of proteins modified by ISG15 are known. Likewise, IFN-γ signaling and its relationship with specific protein modification by ISGylation in breast cancer have not been studied. Here, we identified non-muscle myosin IIA (NMIIA) as a cytoplasmic protein that is modified via ISGylation and modulated by IFN-γ in breast cancer cells.

2. Results

2.1. NMIIA is an endogenous ISG15-interacting protein in breast cancer cells

In order to identify endogenous ISGylation target proteins in breast cancer cells, we decided to use MDA-MB-231 cells as they had higher levels of conjugated ISG15 than free, as shown by western blotting (WB) against ISG15 using total extracts of these cells (Fig. 1A). These cells acted as an adequate model to identify proteins covalently conjugated to ISG15. Next, we used the total extracts from these cells to carry out an immunoprecipitation assay using a specific monoclonal antibody for ISG15. The endogenous ISG15 was immunoprecipitated and separated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie staining using IgG and a mock immunoprecipitation as negative controls. Among the detected bands, we selected one very dense band (approximately 250 kDa in size) to identify the ISG15-associated protein (Fig. 1B). Mass spectrometry (MS) revealed that this band corresponded to NMIIA (230 kDa in size) encoded by *MYH9*, a member of the non-muscle myosin family (NMII A, B, and C) (Fig. 1C and D). These proteins are comprised of a heavy chain encompassing a globular head domain that binds actin and adenosine triphosphate (ATP), a neck region that binds essential light chain (ELC), and a regulatory light chain (RLC) as well as a tail region that homodimerizes in a helical fashion. Light and heavy chains are conserved among NMII proteins; however, the end of the heavy chain tail is a sequence with considerable divergence between the NMII isoforms, as implicated in their regulation. NMII proteins associate with actin filaments, contracting them and forming the actomyosin complex that participates in cytoskeletal remodeling and regulation of shape, organization, polarity, and motility of the cell (Newell-Litwa et al., 2015; Ouderkirk and Krendel, 2014). Interestingly, our results indicate that only one of the NMII proteins described in humans, NMIIA, is endogenously associated with ISG15 in MDA-MB-231 breast cancer cells.

2.2. NMIIA mRNA is not deregulated in breast cancers, but NMIIA seems to be highly affected by posttranslational modifications and protein-protein interactions

Initially, we decided to explore the role of NMIIA expression in breast cancer and its regulation. We analyzed the expression of *MYH9* in normal mammary tissue and mammary tumors from patients. Using Oncomine, we selected the Curtis (2136 samples) database for this analysis and determined that *MYH9* expression was similar in breast cancers and normal conditions, as statistical analyses revealed that *MYH9* expression was not significantly different between breast cancer and normal mammary tissues (Fig. 2A). We also analyzed the reported and predicted posttranslational modifications for NMIIA using PhosphoSitePlus (PSP). Remarkably, NMIIA was a highly modified protein, mainly by phosphorylation but also through other modifications, including acetylation and ubiquitination (Fig. 2B). Using the Search Tool for the Retrieval of Interacting Genes (STRING) software, we also found proteins associated with cytoskeletal remodeling (RhoA, Ras, Cdc42, and ACTG1) in the interactome for NMIIA (Fig. 2C). Thus, *MYH9* expression was not deregulated in all mammary tumors from patients, suggesting that protein-protein interactions of NMIIA and its posttranslational modifications, including ISGylation, could be critical and interconnected in the regulation of the levels and functions of this protein.

2.3. Subcellular distribution of NMIIA and ISGylation mark in breast cancer cells

Because NMIIA has been reported as the central element of actomyosin, we first evaluated the subcellular distribution of this protein. We transfected the enhanced green fluorescent protein (EGFP)-NMIIA plasmid in AD293 cells (kidney cells with high transfection efficiency) and detected its localization in the cytoplasm using immunofluorescence (Fig. 3A). Next, we analyzed the localization of endogenous NMIIA protein in MDA-MB-231 and MCF-7 breast cancer cells using subcellular fractionation and immunofluorescence assays. NMIIA was detected mainly in the cytoplasmic fraction of these cells (Fig. 3B–D). Free ISG15 was localized in cytoplasm, and ISGylation was detected in both the nucleus and cytoplasm of these cells (Fig. 3B, C). We also performed immunofluorescence assays using anti-ISG15 and anti-NMIIA antibodies for the endogenous detection of these proteins in MDA-MB-231 and MCF-7 cells (Fig. 3D). Similarly, the results showed that NMIIA was mostly detected outside the nucleus, and ISG15 was detected in the cytoplasm and in the nucleus. Thus, NMIIA and ISG15 are principally co-localized in the cell cytoplasm as indicated by the merge of these images (Fig. 3D, E).

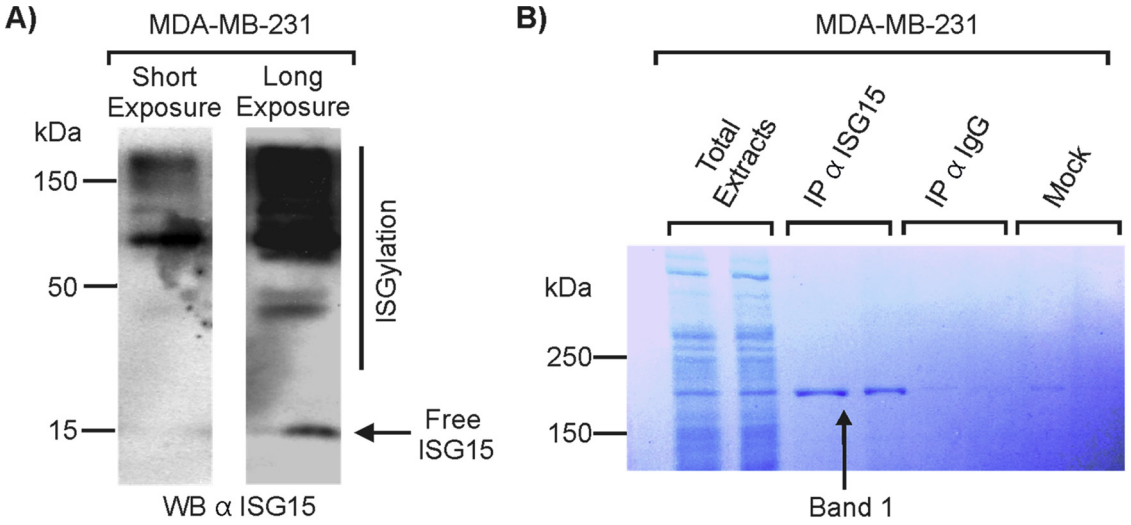
2.4. NMIIA is associated specifically with ISG15 in vivo

Based on our results, we evaluated whether ISG15 interacted specifically with NMIIA in *vivo*. We chose AD293 cells that had high levels of conjugated ISG15 and undetectable free ISG15 levels (Fig. 4A). Then, we used total extracts from AD293 cells transfected with EGFP-NMIIA and performed an immunoprecipitation assay to enrich endogenous conjugated ISG15 protein, followed by WB to detect EGFP-NMIIA. We determined that EGFP-NMIIA was contained in the immunoprecipitated ISG15 (Fig. 4B).

Similarly, AD293 cells were transfected with the EGFP-NMIIA plasmid. We performed an immunoprecipitation experiment for conjugated ISG15 and used anti-NMIIA antibody for the WB, resulting in detection of the exogenous (~260 kDa) and endogenous (~230 kDa) form of NMIIA in the transfected cells. Only endogenous NMIIA was observed in non-transfected cells, indicating a specific interaction between these proteins (Fig. 4C). Interestingly, ISG15-NMIIA interaction resulted in a shifted band compared to the band corresponding to NMIIA protein detected in total extracts by WB, suggesting that the association of these proteins occurred in a covalent manner.

2.5. NMIIA is an ISGylated protein in breast cancer cells

Based on the specific interaction between ISG15 and NMIIA, we verified this interaction in an endogenous context of breast cancer cells. We performed an immunoprecipitation assay using monoclonal antibody against ISG15 as well as IgG and/or mock immunoprecipitation as negative controls, followed by WB for NMIIA to demonstrate the specific endogenous binding between ISG15 and NMIIA in MDA-MB231 and MCF-7 cells (Fig. 5A). ISG15-NMIIA interaction (IP ISG15 WB NMIIA) resulted in a shifted band compared to the band of NMIIA protein detected by WB using total extracts (input) from breast cancer



C)

MAQQAADKYLYVDKNFINNPLAQADWAAKKLVWVPSDKSGFEPASLKEEVGEEAIVELVENGKKVKVNKDDIQKMNPPKFSKVEDMAELTCLNEASVLHNLK
ERYYSGLIYTYSGLFCVVINPYKNLPIYSEEIVEMYKGKKRHEMPPHIYAITDTAYRSMMQDREDQSILCTGESGAGKTENTKKVIQYLAYVASSHKSKKDQGELE
RQLLQANPILEAFGNAKTVKNDNSSRFGKFIRINFDVNGYIVGANIETYLLEKSRAIRQAKEERTFHIFYYLLSGAGEHLKTDLLLEPYNKYRFLSNGHVTIPGQQD
KDMFQETMEAMRIMGIPEEEQMGLLRVISGVLQLGNIVFKKERNTDQASMPDNTAAQKVSHLLGINVTDFTRGILTPRIKVGRDYVQKAQTKEQADFAIEALAK
ATYERMFRWLVLRINKALDKTKRQGASFIGILDIAGFEIFDLNSFEQLCINYTNEKLQQLFNHTMFILEQEEYQREGIEWNFIDFGLDLQPCIDLIEKPAGPPGILALL
DEECWFPKATDKSFVEKVMQEQGTHPKFQKPKQLKDKADFCIIHYAGKVDYKADEWLMKNMDPLNDNIATLLHQSSDKFVSELWKDVDRIIGLDQVAGMSETA
LPGAFKTRKGMFRTVGQLYKEQLAKLMATLRNTNPNFVRCIIPNHEKKAGKLDPHLVLDQLRCNGVLEGIRICRQGFPNRVVFQEFRQRYEILTPNSIPKGFMD
GKQACVLMIKALELDSNLYRIGQSKVFFRAGVLAHLEEERDLKITDVIIGFQACCRGYLARKAFAKRQQQLTAMKVLQRNCAAYLKLRNWQWWRLFTKVKPLLQ
VSRQEEEMMAKEEELVKVREKQLAAENRLTEMETLQSQLMAEKLQLQEQLQAETELCAEAEELRARLTAKKQELEEICHDLEARVEEEEERCQHLQAEKKK
MQQNIQELEEQLEEEESARQKLQLEKVTTEAKLKKLEEEQIILEDQNCKLAKEKKLLEDRIAEFTTNLTEEEEKSKSLAKLKNKHEAMITDLEERLRREEKQRQE
LEKTRRKLEGDSTDLSDQIAELQAQIAELKMQLAKKEEELQAALARVEEEAAQKNMALKKIRELESQISELQEDLESERASRNKAEKQKRDLGEELEALKTELED
TLDSTAAQQELRSKREQEVNILKKTLEEEAKTHEAQIQEMRQKHSQAVEELAEQLEQTKRVKANLEKAKQTLENERGELANEVKVLLQGKGDSEHKRKKVEAQ
LQELQVKFNEGERVRTELADKVTKLQVELDNVTGLLSQSDSKSSKLTKDFSALESQLQDTQELLQEENRQKLSLSTKLKQVEDEKNSFREQLEEEEEAKHNLEK
QIATLHAQVADMKKKMEDSVGCLETAEEVKRKLQKDLEGLSQRHEEKVAAYDKLEKTKTRLQQELDDLLVDLDHQRQSACNLEKKQKKFDQLLAEEKTISAKYA
EERDRAEAEAREKETKALSLARALEEAMEQKAELERLNKQFRTEMEDLMSSKDDVGKSVHELEKSKRALEQQVEEMKTQLEELEDELQATEDAKLRLEVNLQ
AMKAQFERDLQGRDEQSEEKKKQLVRQVREMEAELEDERKQRSMAVAARKKLEMDLKDLEAHIDSANKNRDEAIKQLRKLQAQMKDCMRELDDTRASREEIL
AQAKENEKKLKSMEAEMIQLQEELAAAERAKRQAQQERDELADEIANSSGKGALALEEKRRLEARIAQLEEELEEEQGNTELINDRLKKANLQIDQINTDLNLE
RSHAQKNENARQQLERQNKELKVKLQEMEGTVKSKYKASITALEAKIAQLEEQLDNETKERQAACKQVRRTEKKLKDVLLQVDDERRNAEQYKDQADKASTR
LKQLKRQLEEAEEEAQRANASRRKLQRELEDATETADAMNREVSSLKNKLRRGDLPFVVPRRMARKGAGDGSDEEVDGKADGAEAKPAE

Band	Accession	Name	MW	Abbreviation
1	P35579	Myosin heavy chain, non-muscle type A	227 kDa	NMIIA

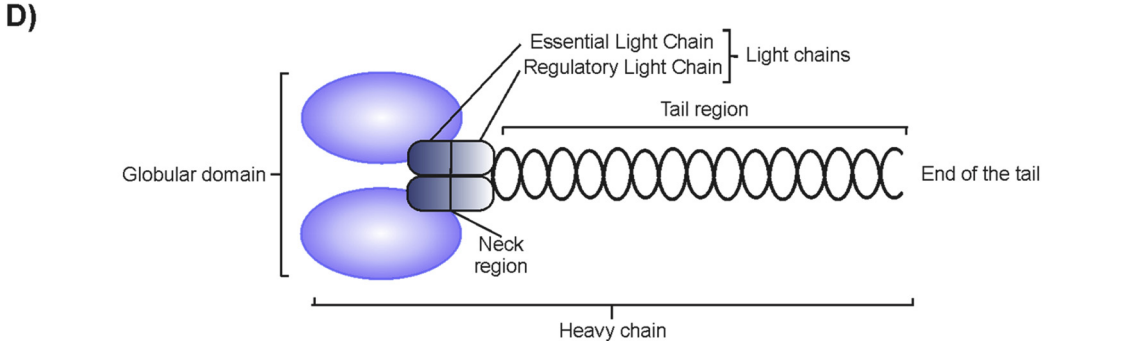


Fig. 1. Identification of non-muscle myosin IIA (NMIIA) as a putative interferon-stimulated gene 15 (ISG15)-associated protein.
A) Total extracts from MDA-MB-231 cells were analyzed by western blotting (WB) against interferon-stimulated gene 15 (ISG15). B) Coomassie staining of ISG15-immunoprecipitated complexes from MDA-MB-231 lysates. A band approximately 250 kDa in size was excised for analysis by mass spectrometry. Samples were analyzed in duplicate. C) ISG15-interacting protein identified by mass spectrometry is shown in the table. The identified peptides are indicated in green (bold) on non-muscle myosin IIA (NMIIA) protein sequence. NMIIA peptide coverage was 20%. D) Structural organization of NMIIA identified protein.

cells, indicating the covalent association between these proteins (Fig. 5A). The same assay was performed using cytoplasmic and nuclear extracts from these cells, and the interaction between ISG15 and NMIIA was strongly detected in the cytoplasm of these cells (Fig. 5B). Thus, we validated the endogenous interaction indicated by MS between ISG15 and NMIIA protein in breast cancer cells.

To discard the hypothesis of a non-covalent interaction between free ISG15 and NMIIA, we used MCF-7 cells, where both conjugated ISG15 and free ISG15 could be detected (Fig. 5C). We performed a reciprocal immunoprecipitation through the NMIIA protein immunoprecipitation, followed by a WB against ISG15 (Fig. 5D). Free ISG15 was detected in the total extracts (input); however, free ISG15 was not present in the immunoprecipitated NMIIA, suggesting that the interaction between ISG15 and NMIIA did not occur in a non-covalent manner but covalently via ISGylation. As control, the efficiency of NMIIA immunoprecipitation was tested, and the covalent interaction between ISG15 and NMIIA in these cells was confirmed (Fig. 5D bottom). These results indicate that ISG15 protein is covalently associated with NMIIA.

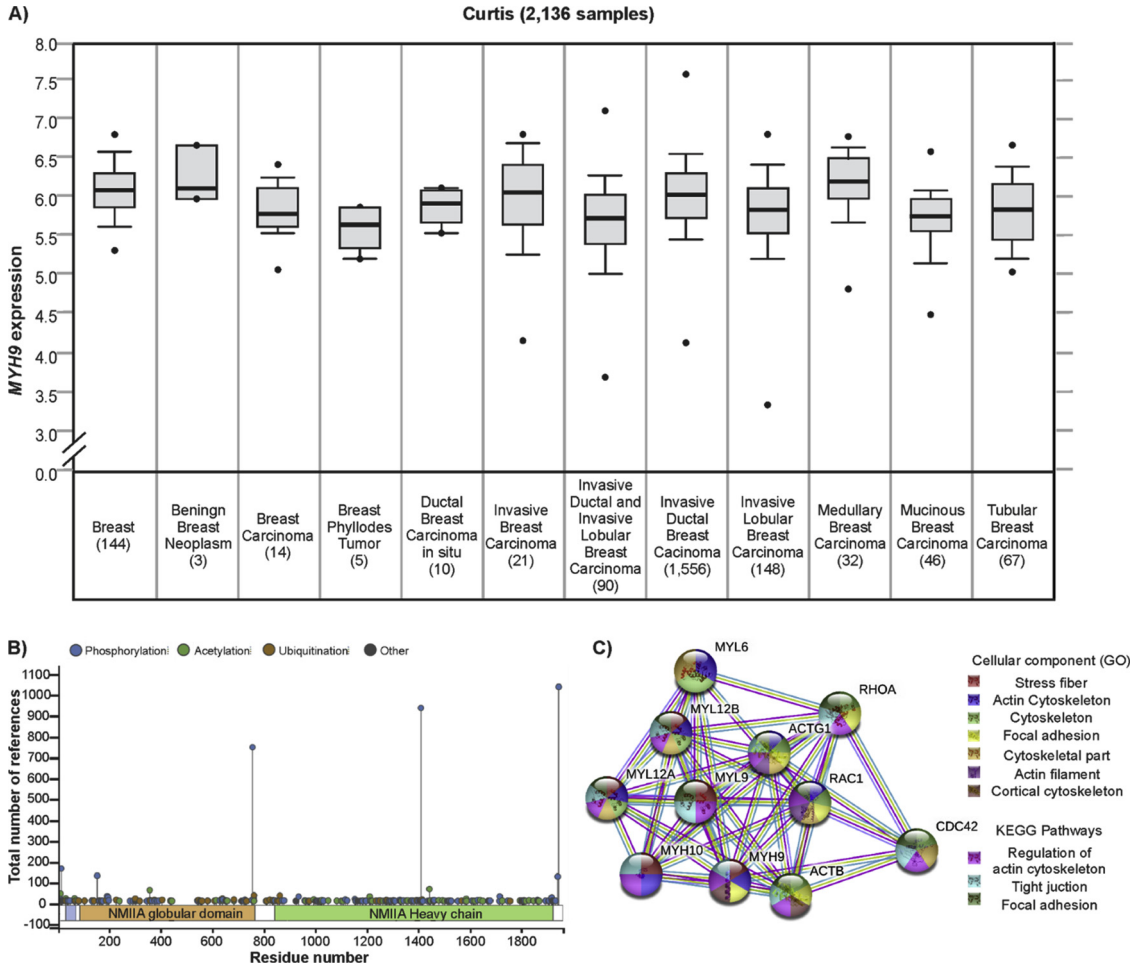


Fig. 2. Non-muscle myosin IIA (*MYH9*) mRNA levels in breast cancer and the modifications and protein-protein interactions for NMIIA.
A) Curtis datasets from Oncomine were analyzed to evaluate *MYH9* expression in human mammary tumors compared to normal tissue. B) Evaluation of sites target of posttranslational modification for non-muscle myosin IIA (NMIIA) using PhosphoSite. C) Analysis of NMIIA interactome using STRING. Known interactions from curated databases, text mining, experimental determination, and protein homology are indicated in blue, green, purple, and dark blue lines, respectively. Kyoto Encyclopedia of Genes and Genomes (KEGG). Gene Ontology (GO).

2.6. IFN-γ modulates NMIIA ISGylation in breast cancer cells

We previously showed that IFN-γ increased ISGylation levels in breast cancer cells; therefore, we sought to determine whether NMIIA ISGylation increased by treatment with IFN-γ. Using total extracts from MDA-MB-231 cells treated with IFN-γ or left untreated, we performed an ISG15 immunoprecipitation. The Coomassie staining showed that the band identified as NMIIA by MS was increased by IFN-γ treatment (Fig. 6A). Furthermore, we demonstrated that the specific interaction between ISG15 and NMIIA in MDA-MB-231 cells increased by IFN-γ treatment using WB with an anti-NMIIA antibody (Fig. 6B). This interaction was also enhanced by IFN-γ treatment in MCF-7 cells (Fig. 6C). Although IFN-γ enhanced the ISG15 and ISGylation levels after 24 h, no changes in the amount of NMIIA were detected in response to IFN-γ treatment in MDA-MB-231 and MCF-7 cells under these conditions (Fig. 6D and E)., suggesting that the availability of NMIIA was not affected by IFN-γ-induced ISGylation (Fig. 6F and G).

ISGylation has been reported to be associated with an increase or decrease in the protein stability and also in the protein-protein interactions. As NMIIA levels were not altered by IFN-γ-increased ISGylation, this modification could act affecting the interactions of NMIIA, many of them linked to cytoskeletal (Fig. 2C). For this reason, we performed an analysis using STRING software, evidencing an interconnection between the IFN-γ signaling pathway, ISGylation, and NMIIA, in which actin was the common protein that connected these pathways, similar to IQ motif containing GTPase activating protein 1 (IQGAP1), a cytoskeleton protein that has been reported to be a ISGylated target (Cerikan and Schiebel, 2017; Cerikan et al., 2016). Thus, our study demonstrated a new connection generated by the ISGylation of NMIIA modulated by IFN-γ. Moreover, our study suggests that IFN-γ may regulate the ISGylation of many other cytoskeleton proteins participating in the cytoskeletal dynamic in breast cancer cells (Fig. 7.

2.7. IFN-γ/ISG15-NMIIA-actin axis in cytoskeleton organization is important in modulation of the morphology of breast cancer cells

ISG15 conjugation to NMIIA had no effect on its stability (Fig. 6), but a functional interactome between NMIIA, cytoskeletal proteins and the IFN-γ/ISG15 route was suggested by our data (Fig. 7). Additionally, NMIIA interacts with F-actin to modulate cytoskeletal organization (Ouderkirk and Krendel, 2014). Based on these data, we considered that covalent interaction between ISG15 and NMIIA might have an effect on the cell morphology. Presently, immunofluorescence assays revealed greater elongation of MDA-MB-231 cells treated with IFN-γ, with an increasing co-localization of NMIIA with ISG15 in the cytoplasmic and perinuclear regions, in comparison with cells that were not treated with IFN-γ (Fig. 8A). Furthermore, co-localization of NMIIA and F-actin in the cytoplasm and at the edge of cells in response to IFN-γ was evident (Fig. 8B). Co-localization of NMIIA, ISG15, and F-actin occurred mainly in the cytoplasm of MDA-MB-231 cells treated with IFN-γ (Fig. 8C),

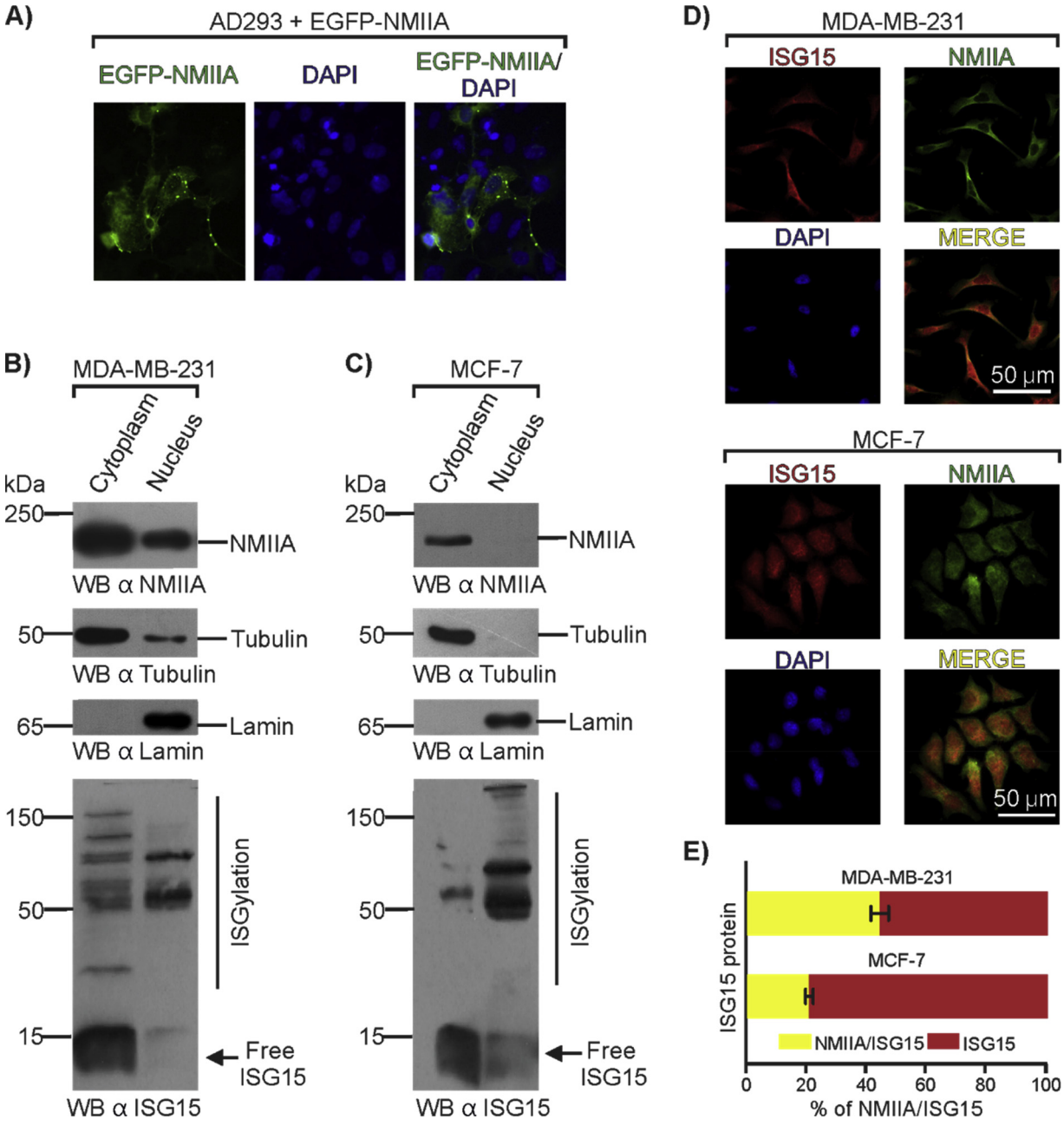


Fig. 3. Non-muscle myosin IIA (NMIIA) is a cytoplasmic protein associated with interferon-stimulated gene 15 (ISG15) in breast cancer cells.
A) Enhanced green fluorescent protein-non-muscle myosin IIA (EGFP-NMIIA) was detected in cytoplasm of AD293 cells. B) and C) Cytoplasmic and nuclear extracts from MDA-MB-231 and MCF-7 cells were analyzed by western blotting (WB) against NMIIA using tubulin and lamin as controls for cytoplasm and nucleus, respectively. D) Evaluation of subcellular distribution of interferon-stimulated gene 15 (ISG15) and NMIIA protein in MDA-MB-231 and MCF-7 using immunofluorescence assay. E) Analysis of co-localization for ISG15 and NMIIA in breast cancer cells.

suggesting that IFN-γ acting through ISG15 may be important to regulate the functional association of NMIIA and F-actin in the cytoplasm of breast cancer cells.

We decided to evaluate whether morphological changes induced by IFN-γ were associated with protein ISGylation (as ISGylated NMIIA), and whether this occurred in other breast cancer cell types. To this end, we examined MCF-7 cells, because they maintain an epithelial morphology, in contrast to MDA-MB-231 cells that present a mesenchymal-like morphology. Initially, we transfected these cells with EGFP-NMIIA or EGFP-NMIIA with ISG15, E1 (UBE1L), and E2 (UbcH8) ISGylation enzymes. ISGylation was increased when ISG15 and the ISGylation system was transfected (Fig. 8D). Under this condition, the immunoprecipitation of ISG15 and WB for NMIIA revealed the enhanced association of ISG15 with NMIIA, as well as EGFP-NMIIA (Fig. 8D bottom). Thus, ISG15-NMIIA interaction was increased when ISGylation enhanced, and MCF-7 cells displayed stress fibers with discrete morphological changes (Fig. 8E and 8F). Likewise, co-localization of NMIIA-ISG15 and NMIIA-F-actin was detected under the condition of ISGylation, in a manner similar to the co-localization of these proteins in MDA-MB-231 cells treated with IFN-γ (Fig. 8E and F). The results indicate that NMIIA ISGylation contributes to the changes in the morphology associated with F-actin organization in breast cancer cells.

2.8. NMIIA-ISG15 interaction occurs during spreading of breast cancer cells

Cell spreading is a critical event that involves actin polymerization

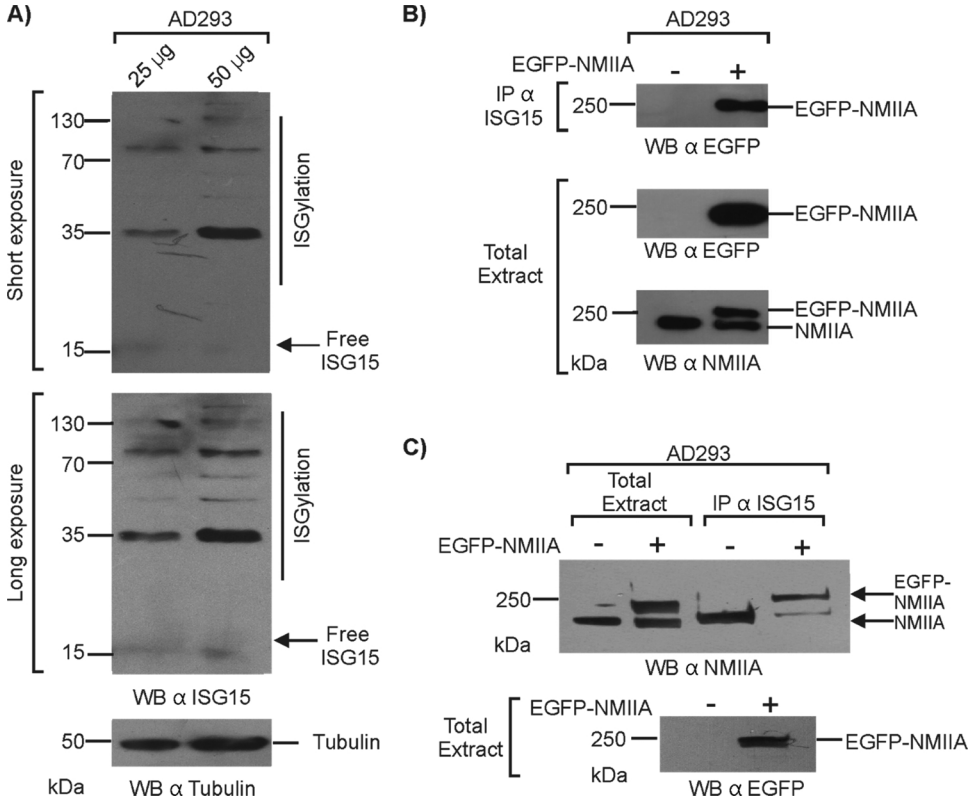


Fig. 4. Non-muscle myosin IIA (NMIIA) is specifically associated with interferon-stimulated gene 15 (ISG15).
A) Western blotting (WB) for interferon-stimulated gene 15 (ISG15) using 25 and 50 µg from AD293 cell total extracts. B) AD293 cells were transfected with or without enhanced green fluorescent protein-non-muscle myosin IIA (EGPF-NMIIA). After 48 h, the total extracts were used for ISG15 immunoprecipitation and WB for EGFP or C) WB for NMIIA.

to generate a cellular contact area with the extracellular matrix or substrate. NMII proteins participate differently in the generation of protrusive forces that regulate cell spreading for cell motility. NMIIA and NMIIB are recruited in the lamellar margin during the spreading of MDA-MB-231 breast cancer cells participating in the regulation of this process (Betapudi, 2010; Betapudi et al., 2006)

Since cell spreading is a fundamental NMII-regulated event, we examined whether NMIIA-ISG15 interaction can occur in the spreading

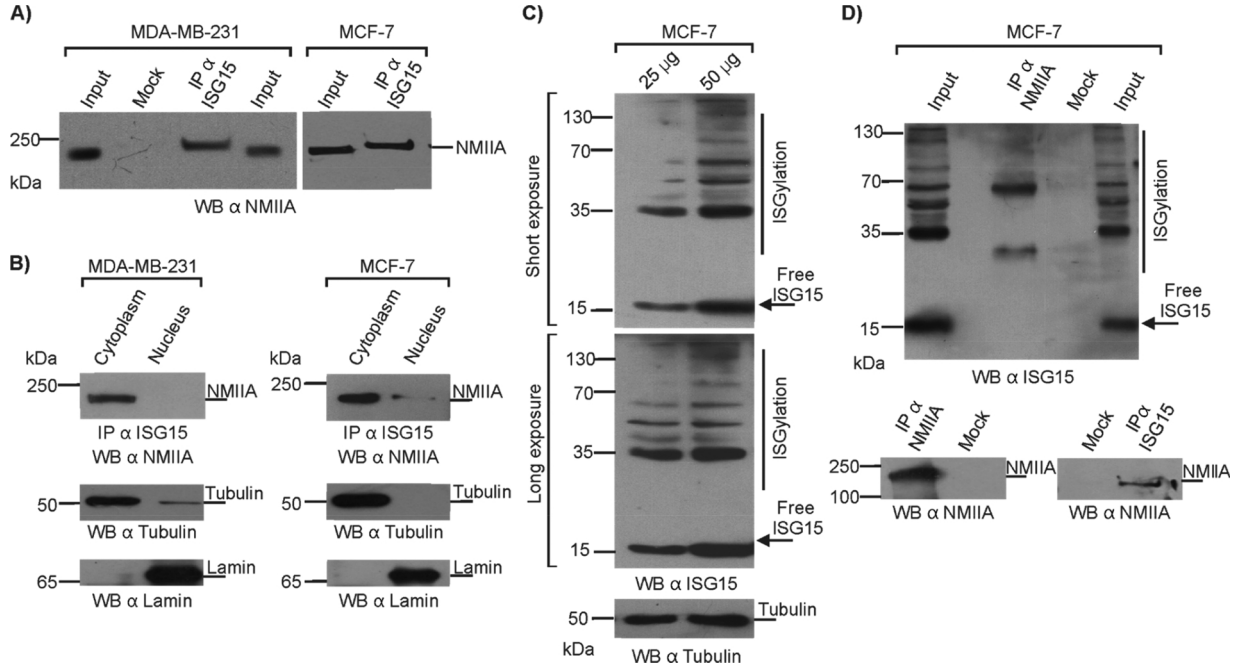


Fig. 5. Interferon-stimulated gene 15 (ISG15) is covalently associated with non-muscle myosin IIA (NMIIA) in breast cancer cells.
A) Interferon-stimulated gene 15 (ISG15) was immunoprecipitated from MDA-MB-231 and MCF-7 cell total extracts, and western blotting (WB) with anti-non-muscle myosin IIA (NMIIA) was performed. B) Cytoplasmic and nuclear fractions from MDA-MB-231 and MCF-7 cells were used for ISG15 immunoprecipitation followed by WB for NMIIA. Anti-tubulin and anti-lamin were used as controls for the cytoplasmic and nuclear extracts. C) WB for ISG15 using 25 and 50 µg from MCF-7 cell total extracts. D) Endogenous NMIIA immunoprecipitation from MCF-7 followed by WB for ISG15 was analyzed on 12% gels. Bottom: endogenous NMIIA and ISG15 immunoprecipitation followed by WB for NMIIA using total extract from MCF-7 cells.

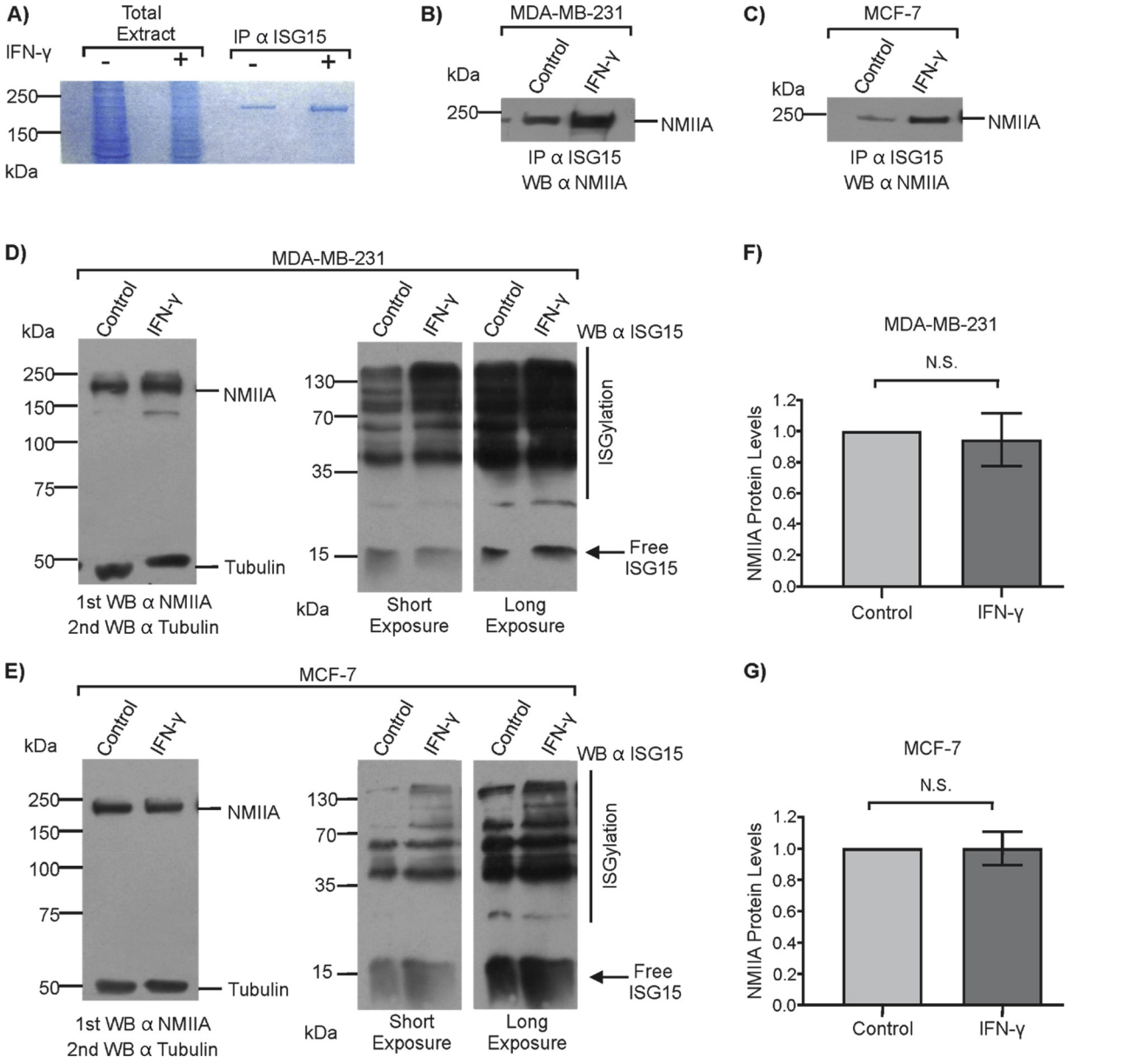


Fig. 6. Association between interferon-stimulated gene 15 (ISG15) and non-muscle myosin IIA (NMIIA) is increased by interferon γ (IFN-γ) treatment in breast cancer cells.

A) Coomassie staining of interferon-stimulated gene 15 (ISG15) immunoprecipitated complexes from MDA-MB-231 lysates treated or not treated with interferon γ (IFN-γ) (100 ng/mL) for 24 h. The band approximately 230 kDa in size that corresponds to NMIIA is enhanced in response to IFN-γ treatment. B) Endogenous ISG15 immunoprecipitation followed by western blotting (WB) for non-muscle myosin IIA (NMIIA), using total extract from MDA-MB-231 and C) MCF-7 cells treated or not treated with IFN-γ (100 ng/mL). D) and E) Total extract MDA-MB-231 and MCF-7 cells treated or not treated with IFN-γ (100 ng/mL) were analyzed by WB for NMIIA and ISG15, using tubulin as a loading control. F) and G) Densitometry of NMIIA protein levels in MDA-MB-231 and MCF-7 treated or not treated with IFN-γ. Not significant was indicated as N.S.

of MDA-MB-231 cells. We seeded cells in wells that were uncoated or coated with the fibronectin (FN) extracellular protein for 30 and 120 min. The early stage of spreading was detected at 30 min, whereas a well-defined spreading was observed at 120 min in the MDA-MB-231 cells seeded on FN in comparison with those seeded in uncoated wells (Fig. 9A and B). Since the later stages of cell spreading involve actin polymerization and myosin contraction, we analyzed the co-localization of NMIIA and ISG15, 120 min after seeding of MDA-MB-231 cells on glass treated with FN. ISG15 displayed mainly an extranuclear distribution that included its presence in lamellar margin, whereas NMIIA was detected completely as an extranuclear protein enriched in the lamellar margin. The collective results indicated that both proteins can co-localize during cell spreading, suggesting that ISG15-NMIIA association can occur in the marginal spreading lamellar region of MDA-MB-231 cells, as well as in more central regions in the cytoplasm of these cells during this event (Fig. 9C, arrowheads). Our results indicate that ISG15 and, NMIIA co-localize in the cytoplasm and lamellar region, and suggest that the association between these proteins may be critical in NMIIA-driven protrusive spreading events, in addition to the formation of focal adhesion complexes with the extracellular matrix.

3. Discussion

In contrast to other posttranslational modifications, ISGylation has not been extensively studied. This modification occurs when ISG15, an ubiquitin-like protein, is covalently bound to target proteins through an

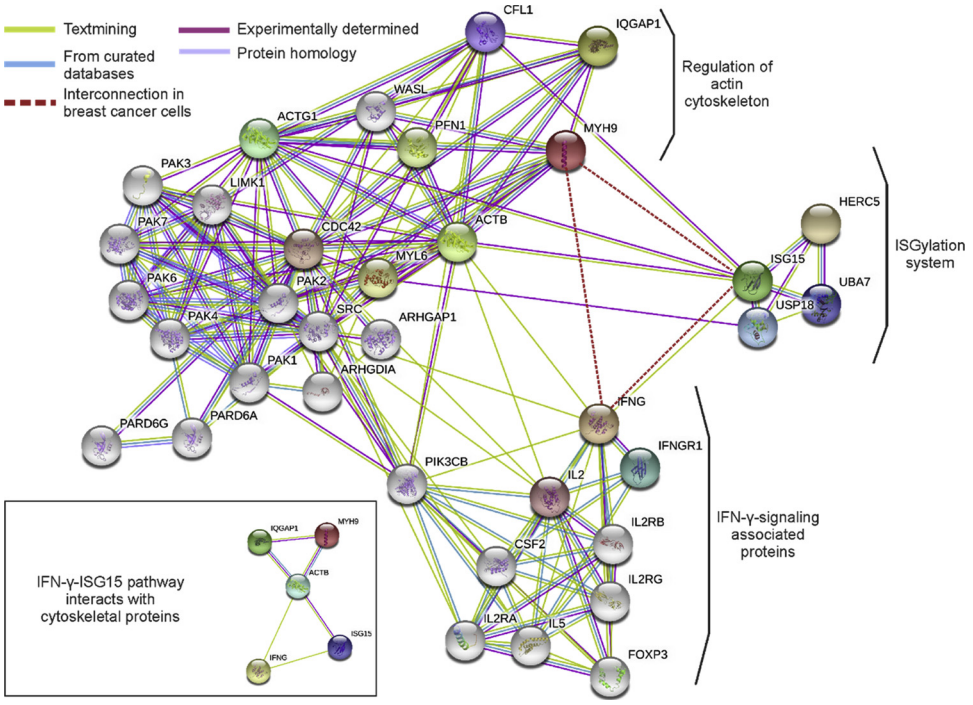


Fig. 7. Interconnection between interferon γ (IFN-γ) pathway, ISGylation system, and non-muscle myosin IIA (NMIIA).
Relationship between interferon γ (IFN-γ), interferon-stimulated gene 15 (ISG15), NMIIA. Analysis using STRING. Known interactions from curated databases, text mining, experimental determination, and protein homology are indicated in blue, green, purple, and dark blue lines, respectively. Connection of functional interactomes for IFN-γ, ISGylation system, NMIIA, and other proteins linked to cytoskeleton dynamic, is shown. Dashed red line indicates the interconnections suggested by our data.

enzymatic system similar to ubiquitination. A small number of candidate proteins have been proposed as ISGylation targets, and an even smaller number of ISGylated proteins have been studied mechanistically and functionally, including FILAMIN B, proliferating cell nuclear antigen (PCNA), IQGAP1, PARKIN, and C-terminus of Hsc70-interacting protein (CHIP). ISG15-dependent regulation of these proteins has important biological implications through modulating the stability and/or interactions of these proteins. Moreover, poly-ISGylation is not common as most proteins are mono or di-ISGylated (Jeon et al., 2009; Cerikan and Schiebel, 2017; Im et al., 2016; Park et al., 2014; Yoo et al., 2018).

ISG15 is upregulated in several cancer types, including breast cancer that has a characteristic profile of free ISG15 and ISGylation levels increased by IFN-γ (Tecalco Cruz and Mejia-Barreto, 2017; Tecalco-Cruz and Cruz-Ramos, 2018). However, proteins identified as ISGylation targets in this cancer are currently not studied. Here, we showed that NMIIA was endogenously associated with ISG15 in breast cancer cells using MS. This protein of high molecular weight (230 kDa) is important, as it is an element of actomyosin, participating in the cytoskeletal organization. Interestingly, our analysis indicated that *MHY9* expression was not altered in all mammary tumors compared to normal tissue, suggesting that posttranslational modifications might play a central role in regulating the NMIIA function. Bioinformatics analyses also showed many target sites in NMIIA for several different modifications that appeared to be critical in breast cancer. For example, NMIIA phosphorylation has been related to the contraction of actin filaments in the formation of protrusions and migration (Dulyaninova et al., 2007).

We identified NMIIA as an ISG15-interacting protein. This interaction occurred covalently in breast cancer cells. The results showed a migration for the band corresponding to ISG15-NMIIA interaction, revealing a band discretely higher than the detected band for NMIIA in total extracts. Our result was also validated by several co-immunoprecipitation assays using cell lines with high levels of conjugated ISG15. However, free ISG15 was not detectable, suggesting that proteins associated with ISG15 in these cells were ISGylated. In addition, our results are reinforced in part by a previous report that suggests that one of the several candidate proteins of ISGylation is NMIIA, confirming this covalent interaction in a non-endogenous system in 293T cells (Giannakopoulos et al., 2005). Thus, we showed an endogenous covalent association between ISG15 and NMIIA in breast cancer cells and demonstrated that this ISGylation of NMIIA was positively regulated by IFN-γ.

What is the function of ISGylation? It is a question not easily to understand so far, since this modification has been related to increased or decreased protein stability, and with stability-independent actions as the modulation of protein-protein interactions. Importantly, it has been reported that the NMIIA modification by monoubiquitination regulates its protein-protein interactions more than its stability or other functional aspects (Li et al., 2016). This is relevant because we show that the interaction between ISG15 ubiquitin-like protein and NMIIA in response to IFN-γ treatment has no effect on the abundance of this myosin but may affect its association with its protein partners. This observation is also supported by the fact that ISGylation alters some protein-protein interactions. For instance, FILAMIN B that binds actin and connects actin filaments to the cell membrane is also a scaffold for IFN-β signaling. Interestingly, IFN-β induces FILAMIN B ISGylation, and as a result, Ras-related C3 botulinum toxin substrate 1 (RAC1), MEKK1, and MKK4 are dissociated from FILAMIN B, thus blocking the activation of c-Jun N-terminal kinases (JNK) signaling. This occurs without any changes in FILAMIN B stability (Jeon et al., 2009). Moreover, ISGylation seems to be an important regulator of cytoskeleton dynamic as other proteins that participate in this process like IQGAP1 are modulated by this modification (Cerikan and Schiebel, 2017; Cerikan et al., 2016).

Hence, ISGylation is associated with stability and degradation of some proteins but also acts as a regulator of interactions between proteins. Therefore, NMIIA ISGylation may participate in modulating protein-protein interactions. The interactome analysis of NMIIA showed that many of its protein partners were related to cytoskeletal proteins and/or signaling and regulation of the actin cytoskeleton. Thus, NMIIA ISGylation induced by IFN-γ may affect the association between NMIIA and other cytoplasmic proteins involved in cytoskeletal remodeling. Furthermore, our data suggest that IFN-γ increases ISG15 levels and the ISGylation of its target proteins, including NMIIA. For this reason, the transfection of the ISGylation system also promoted an increase in the ISG15-NMIIA association. This association was apparent in the cytoplasm and perinuclear region of breast cancer cells. The increase of

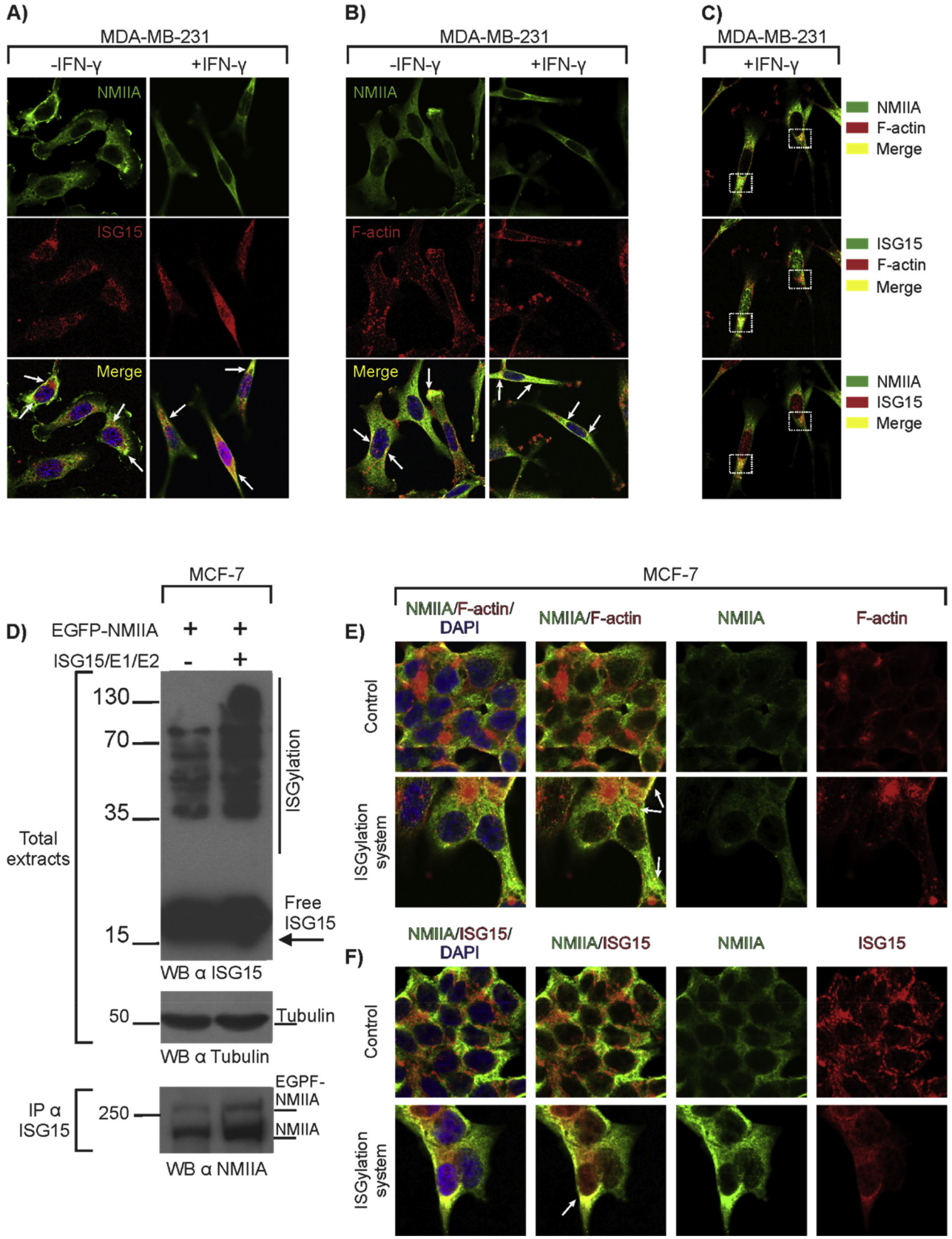


Fig. 8. Association induced by IFN-γ and the ISGylation system of non-muscle myosin IIA (NMIIA) and interferon-stimulated gene 15 (ISG15) is implicated in the morphology of breast cancer cells.

A-C) Evaluation of the subcellular distribution of NMIIA, and F-actin and/or interferon-stimulated gene 15 (ISG15) in MDA-MB-231 cells that were untreated or treated with IFN-γ using an immunofluorescence assay. D) Enhanced green fluorescent protein-non-muscle myosin IIA (EGFP-NMIIA) was expressed with or without ISG15 and E1 and E2 enzyme in MCF-7 cells. ISG15 and Tubulin were detected by western blotting (WB). Endogenous ISG15 immunoprecipitation was followed by WB for non-muscle myosin IIA (NMIIA), using total extract from MCF-7 cells. E and F) Immunofluorescence assay of the subcellular distribution of NMIIA and F-actin or interferon-stimulated gene 15 (ISG15) in control MCF-7 cells or cells expressing ISG15 and ISGylation system. Arrowheads, co-localization of NMIIA, ISG15 or F-

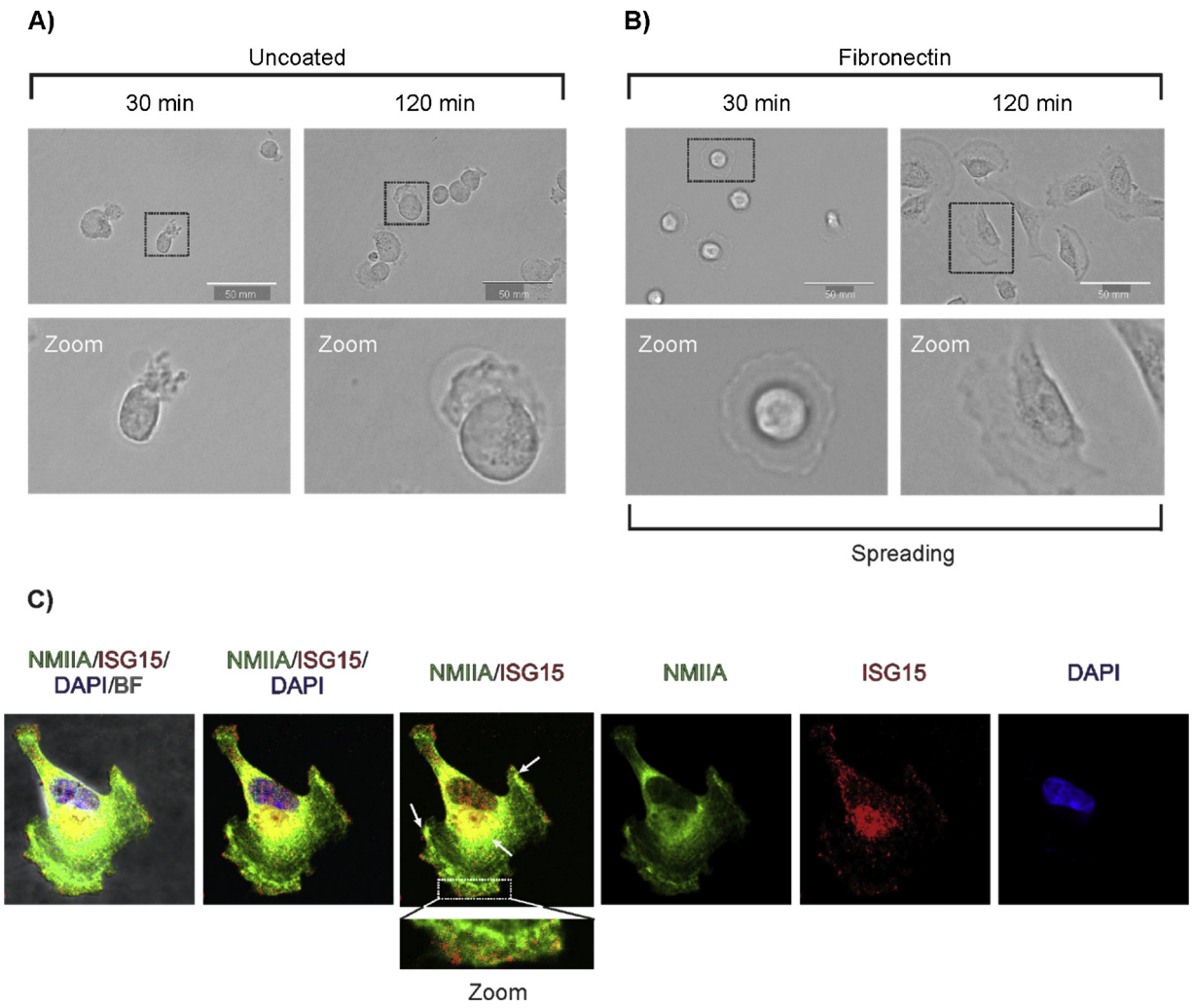


Fig. 9. Association of non-muscle myosin IIA (NMIIA) and interferon-stimulated gene 15 (ISG15) is implicated in the spreading of breast cancer cells induced by fibronectin (FN).

A and B) MDA-MB-231 cells were seeded in uncoated wells or wells coated with fibronectin (FN) and analyzed after 30 or 120 min. C) Immunofluorescence assay of the distribution and co-localization of NMIIA and ISG15 in MDA-MB-231 cells seeded in FN-coated wells after 120 min. Arrowheads and zoom image show co-localization regions.

ISG15-NMIIA association was accompanied by a more elongated morphology of the MDA-MB-231 cells (Fig. 8A–C), as well as with the formation of stress fibers of F-actin in MCF-7 cells (Fig. 8E). Thus, ISGylation of NMIIA may have implications in actin-cytoskeleton remodeling.

The dynamics of the cytoskeleton is also important in cell spreading, which is modulated by myosin proteins, and represents a critical step for the attachment and detachment of the cells with the substrate, a central event in cell migration. NMIIA and NMIIB are enriched in the lamellar margin. However, their functions are unclear. It has been suggested that they may have an opposite effect on spreading, with NMIIB promoting cell spreading (Betapudi, 2010; Betapudi et al., 2006; Cai et al., 2006). Interestingly, the ISG15-NMIIA association seems to occur in the spreading of breast cancer cells induced by FN, and this association is specific for NMIIA, but not for NMIIB. In addition, FN may induce *ISG15* expression and protein ISGylation via integrin-dependent signaling (Hermann et al., 2016).

Our findings are relevant since they open new avenues to understand ISG15 as a regulator of the NMIIA as a motor of the F-actin, and its effects in the spreading and migration of breast cancer cells, and its modulation by interferons and proteins of extracellular matrix as FN.

NMIIA has a high identity percentage with NMIIB and NMIIC isoforms (79% and 65%, respectively). Because NMIIA is a protein with high molecular weight, containing 10.56% lysine residues, there are many possible lysine residues for ISGylation. Our MS results identifying NMIIA suggest that lysines only in NMIIA but not in NMIIB and NMIIC may be involved in this covalent interaction. The generation of mutants of NMIIA to block its ISGylation may help to understand the function of this modification in NMIIA in breast cancer cells.

In conclusion, we have identified NMIIA as an endogenously and covalently ISG15-associated protein in the cytoplasm of breast cancer cells. NMIIA is modified by ISGylation, and this modification may regulate its protein-protein interactions, including its association with F-actin for cytoskeletal reorganization. IFN-γ treatment increases ISGylation of NMIIA and promotes changes in the morphology of breast cancer cells with the formation of stress fibers. The FN extracellular matrix protein may induce ISG15 expression and increases the ISGylation of NMIIA. This ISG15-NMIIA association seems also occur in the FN-induced and NMII-modulated spreading of MDA-MB-231 cells (Fig. 10). This new link between IFN-γ, ISGylation, and cytoskeletal proteins may be important in the progression of some cancers such as breast cancer.

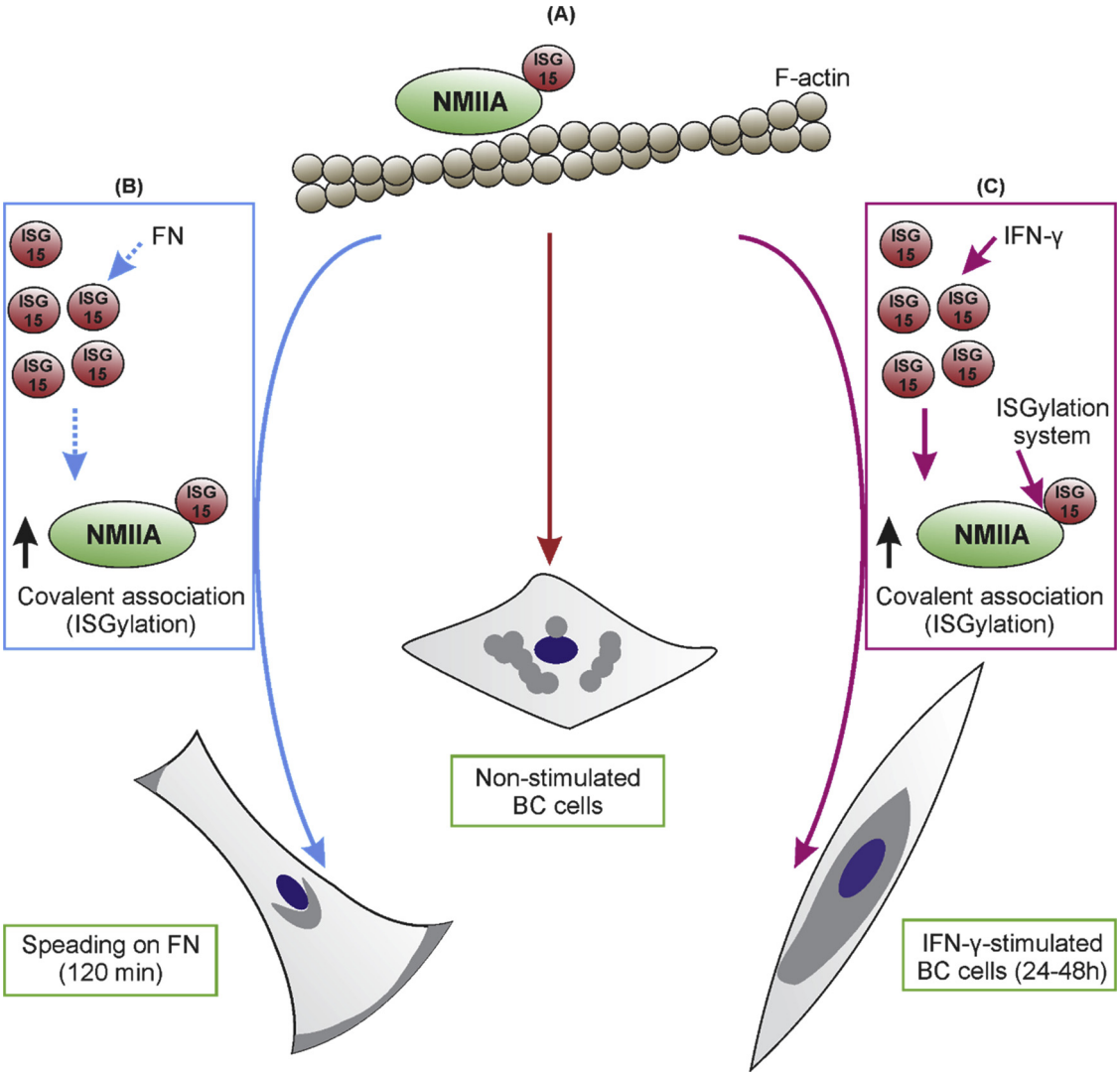


Fig. 10. Proposed model.
NMIIA is covalently associated with ISG15 in breast cancer cells. A) This modification of NMIIA by ISGylation may modulate the *actomyosin complex* (NMIIA-F-actin), which affects the reorganization of cytoskeleton and consequently the morphology of the cells. B) Extracellular matrix proteins like FN seem to induce *ISG15* expression, with subsequently increased NMIIA ISGylation. These events may be critical in FN-induced cell spreading, in which actomyosin activity is also required. C) In response to IFN-γ, *ISG15* expression and NMIIA ISGylation are increased, and changes in the cell morphology associated with F-actin organization are produced. These events are also generated by the overexpression of the ISGylation system. Thus, NMIIA ISGylation may have central implications in the proliferation and migration of mammary tumor cells. BC: breast cancer. In gray: ISG15, NMIIA and actin co-localization.

4. Materials and methods

4.1. Cell lines and reagents

The AD293 human embryonic kidney cell line and the MDA-MB-231 (ERα−, triple negative) and MCF-7 (ERα+) breast cancer cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum and penicillin/streptomycin. 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-Aldrich, AMRESCO-VWR, and Bio-Rad.

4.2. Plasmids and transfections

The pEGFPMIIAC3 plasmid was a gift from Dr. Anne R. Bresnick (Albert Einstein College of Medicine, USA) and plasmids pCAGGS-5HA-mISG15, pFlagCMV2-UbcH8, and pCAGGS-HA-hUBE1L were a gift from Dr. Dong-Er Zhang (Department of Pathology, Moores Cancer Center University of California, San Diego). AD293 or MCF-7 cells were seeded and grown to 80% confluence for transient transfection with plasmid DNA using the Lipofectamine 2000 system, according to the manufacturer's instructions (Invitrogen).

4.3. Total, cytoplasmic, and nuclear extract preparation

To obtain total extracts, cells were lysed with TNTE buffer (50 mM Tris−HCl, 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), and 0.5% Triton-X-100) or radioimmunoprecipitation assay (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. Subcellular fractionation was performed to obtain nuclear and cytoplasmic fractions, as described previously (Tecalco Cruz and Mejia-Barreto, 2017; Grewal et al., 2000). Briefly, cells were homogenized in homogenization buffer (250 mM sucrose, 3 mM imidazole, and protease and phosphatase inhibitors), passed through a 22−gauge needle 15 times for MDA-MB-231 cells and 12 times for MCF-7 cells, and then centrifuged at 700 × *g* for 10 min. The supernatant (cytoplasmic fraction) was separated from the pellet

(nuclear fraction). Both fractions were lysed with RIPA buffer at 4 °C for 1 h. Protein extracts were quantified using the Bradford method for the WB and immunoprecipitation assays.

4.4. Immunoprecipitation, Coomassie staining, and MS

Immunoprecipitation assays were performed with 0.6 or 1 µg of anti-ISG15 and anti-NMIIA antibodies, respectively. Immunoprecipitated complexes in Laemmli solution were separated by SDS-PAGE, followed by Coomassie Blue G staining for the selection of bands for MS analysis. The MS service from Instituto Nacional de Medicina Genómica (INMEGEN), Unidad de Proteómica, was utilized. The visible band was manually cut and reduced with dithiothreitol (DTT) 10 mM, alquilated with iodoacetamide 100 mM and destained with acetonitrile (ACN) (NH_4HCO_3 50 mM, 50:50 v/v). Protein digestion was carried out for 18 h at 37 °C with trypsin (Promega V528 A). The obtained peptides were extracted (ACN:H2O:formic acid, 50:45:5), the volume was reduced in a concentrator, and the sample was desalinated using a column C18 (ZipTipC18). The sample was loaded 6-fold using alpha-cyano as a matrix and analyzed using a MALDITOF/TOF 4800. A search was performed with the obtained MS/MS spectrums, using the Paragon algorithm from the software ProteinPilot.

4.5. Co-immunoprecipitation assays and WB

Cells were lysed using RIPA or TNTE lysis buffer as described previously (Tecalco-Cruz and Cruz-Ramos, 2018). Proteins were immunoprecipitated with specific anti–ISG15 (Santa Cruz Biotechnology), anti–NMIIA (Sigma-Aldrich), or anti–GFP (Clontech) antibodies. The immunoprecipitated proteins were separated by SDS-PAGE, followed by immunoblotting with specific primary antibodies (anti–GFP, anti-NMIIA, or anti-ISG15). The experiments were performed in triplicate, and representative images were shown. For analysis of total, cytoplasmic, and nuclear extracts, 25–100 µg of protein lysates were used. Protein lysates were separated by SDS-PAGE and then subjected to immunoblotting with anti-ISG15 (1:1000) (F-9, sc-166755), anti-α-tubulin (1:3000) (B7, sc-5286) obtained from Santa-Cruz Biotechnology, anti-lamin B1 (1:2000) obtained from Cell Signaling (D4Q4Z), anti-NMIIA (1:3000) (M8064), and anti-EGFP (1:3000) (632592) obtained from ClonTech. A secondary anti-rabbit IgG antibody was used (1:10,000 for anti-lamin and anti-GFP, 1:25,000 for anti-NMIIA) (sc-2004; Santa Cruz Biotechnology) as well as peroxidase AffiniPure goat anti-mouse (1:10,000 for anti-tubulin and anti-ISG15) (115-035-003, Jackson). Protein detection was performed using SuperSignal West Pico Chemiluminescent Substrates (Thermo Fisher Scientific) or Immobilon Western (Millipore). All experiments were performed in triplicate.

4.6. Immunofluorescence assay

Cells were seeded on glass coverslips coated with poly-L-lysine in 6-well plates. The cells were fixed in 4% paraformaldehyde for 10 min and then permeabilized with 0.1% Triton X-100 for 10 min. After blocking with 1% albumin for 1 h, cells were incubated overnight with anti-ISG15 (F-9, sc-166755; 1:100) and anti-NMIIA (M8064). After primary incubation, cells were washed and incubated with Alexa Fluor 647 anti-mouse IgG secondary antibody (ab150107, Abcam; 1:750) and Alexa Fluor 488 anti-rabbit IgG secondary antibody (ab150073, Abcam; 1:500) for 1 h in the dark. The slides were prepared with ProLong Diamond Antifade Mountant with 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen). For phalloidin assays (F-actin detection), after blocking, the cells were incubated for 1 h with phalloidin (1:40) and mounted with DAPI solution. An Olympus IX2 and FV10i confocal laser-scanning microscopes were used to visualize the cells, and ImageJ software was used for the imaging, processing, and analysis of results.

4.7. Spreading assays

MDA-MB-231 cells were seeded on wells or glass coverslips in 6-well plates treated with FN (25 mg/mL) for 1 h. After 30 or 120 min following seeding, cells were fixed and visualized, or were prepared for immunofluorescence assay. Cell morphology was evaluated using an Invitrogen EVOS FL Cell Imaging System. Co-localization was analyzed using an Olympus FV10i confocal laser scanning microscope.

4.8. Bioinformatics

The cancer microarray database Oncomine (www.oncomine.org) was consulted to analyze *MYH9* expression in patient-derived mammary tumors and normal mammary tissue. The analysis of post-translational modifications for NMIIA was performed with PhosphoSite (Hornbeck et al., 2015) (www.phosphosite.org), and analysis of protein-protein interactions for NMIIA was carried out using STRING 10.5 version (https://string-db.org/cgi/network.pl) (Szklarczyk et al., 2017).

4.9. Statistical analysis

Statistical analysis was performed with Student's *t*-test using GraphPad Prism 5 software (GraphPad). Densitometry for WB assays were performed using Fiji-ImageJ. Results were expressed as significant when $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***). Not significant is described as N.S.

Author contributions

E.C.-R. performed the experiments and prepared some figures. M.M.-S. participated in the data analysis. A. S-H performed a colocalization analysis and prepared the figures. A.C.T.-C. designed research, the bioinformatics analysis, some WB, and wrote the manuscript.

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