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ISG15 is upregulated in respiratory syncytial virus infection and reduces virus growth through protein ISGylation

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Running title: ISG15 reduces RSV growth through protein ISGylation

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ABSTRACT

Human Respiratory Syncytial Virus (RSV), for which neither a vaccine nor an effective therapeutic treatment is currently available, is the leading cause of severe lower respiratory tract infections in children. Interferon stimulated gene 15 (ISG15) is an ubiquitin-like protein that is highly increased during viral infections and has been reported to play an antiviral or a proviral activity, depending on the virus. Previous studies from our laboratory demonstrated a strong ISG15 up-regulation during RSV infection *in vitro*. In this study, an in depth analysis of the role of ISG15 in RSV infection is presented. ISG15 overexpression and siRNA silencing experiments, along with ISG15 knockout cells ($ISG15^{-/-}$) revealed an anti-RSV effect of this molecule. Conjugation inhibition assays demonstrated that ISG15 exerts its antiviral activity via protein ISGylation. This antiviral activity requires high levels of ISG15 to be present in the cells before RSV infection. Finally, ISG15 is also up-regulated in human respiratory pseudo-stratified epithelia and in nasopharyngeal washes from infants infected with RSV, pointing to a possible antiviral role of this molecule *in vivo*. These results advance our understanding of the innate immune response elicited by RSV and open new possibilities to control infections by this virus.

IMPORTANCE

At present no vaccine or effective treatment against human respiratory syncytial virus (RSV) is available. This study shows that interferon-stimulated gene 15 (ISG15) lowers RSV growth through protein ISGylation. In addition, ISG15

43 accumulation highly correlates with RSV load in nasopharyngeal washes from
44 children, indicating that ISG15 may also have an antiviral role *in vivo*. These results
45 improve our understanding of the innate immune response against RSV and
46 identify ISG15 as a potential target for virus control.
47

INTRODUCTION

Human Respiratory Syncytial Virus (RSV) is the prototype of the *Pneumovirus* genus of the *Pneumovirinae* subfamily within the *Paramyxoviridae* family. It is an enveloped, single-stranded negative sense RNA virus whose genome of 15.2 kb contains 10 genes encoding a total of 11 proteins (1). These are: Three glycoproteins (F, G, SH) inserted in the viral envelope; four proteins (N, P, L and M2-1) associated to the ribonucleoprotein and which are required for RNA synthesis; an additional protein (M) which forms a protein shell underneath the viral membrane, and three nonstructural proteins, two of them implicated in modulating the host innate response to infection (NS1 and NS2) and the other (M2-2) involved in regulating the switch from RNA transcription to RNA replication (1, 2).

RSV is a leading cause of severe pediatric lower respiratory tract infections but also a significant cause of morbidity and mortality in the elderly and immunocompromised individuals (3). Symptoms vary from those of a common cold to bronchiolitis and pneumonia in serious cases (4). The mortality associated to acute lower respiratory RSV infections in children under the age of five is estimated to be 66,000-199,000 deaths per year worldwide (5).

The host response to RSV infection begins in the epithelial cells of the airways, where virus replication preferentially occurs. Cytokines such as type I interferons (IFN-α and IFN-β) are one of the first lines of defense against viral infections and stimulate the expression of a wide range of genes termed interferon-stimulated genes (ISGs) involved in the antiviral response (6). Many ISGs can also be induced by dsRNA or viruses in an IFN-independent manner (7, 8).

RSV has been regarded as a poor IFN inducer as well as a poor responder to IFN (9-12). In fact, NS1 and NS2 proteins inhibit both IFN production and signaling (13-19). Infected cells, however, express high levels of ISGs, including ISG15 (20). ISG15 is an ubiquitin-like protein encoded by the interferon-stimulated gene 15. Similarly to ubiquitination, ISG15 is conjugated to target proteins through a conserved C-terminal Gly-Gly motif in a process termed ISGylation (21, 22). This process is conducted through a sequential reaction catalyzed by E1-activating, E2-conjugating and E3-ligase enzymes which are also induced by type I IFN (23). UbE1L is the only described E1 enzyme for ISG15 while UbcH8 and HERC5 are the predominant E2 and E3 enzymes respectively (24-28). ISG15 can be removed from its target proteins by the ubiquitin-specific protease 18 (USP18), making the ISGylation process reversible (29, 30). Once ISG15 is conjugated to one of more than 300 target proteins described (31), it causes either a gain or a loss of function (23). Interestingly, ISGylation has been described as a co-translational process of both cellular and pathogen encoded proteins with little specificity (31). In addition to its conjugated form, free unconjugated ISG15 is present intracellularly and it is also released to the extracellular space (32, 33). Although the mechanism responsible for the ISG15 antiviral activity is not fully understood, various studies have reported an essential role of both conjugated and unconjugated ISG15 in the antiviral response (34-41). A proviral effect, however, has been described in some cases (42-44). In contrast, viruses have evolved mechanisms to counteract ISG15 antiviral role; for instance, vaccinia virus E3 protein and influenza B NS1 protein bind ISG15 antagonizing its activity (24, 45).

Despite intensive research, neither a vaccine nor an effective antiviral therapy against RSV is currently available. A better understanding of the complex interactions between the virus and the host responses that counteract virus infection is therefore of great importance (46, 47). While the role of ISG15 in RSV infection has not been investigated, previous studies from our laboratory demonstrated a strong induction of this gene as a result of RSV infection in A549 cells (20). In order to determine whether ISG15 may play a role in the innate immune response elicited by RSV, we characterized ISG15 expression and ISGylation during RSV infection. Experiments of overexpression of both wild-type and conjugation-deficient ISG15, silencing of ISG15, UbE1L or USP18 and infection of $ISG15^{-/-}$ cells demonstrated an antiviral activity of conjugated ISG15 against RSV. In addition, a strong correlation was found between viral infection and expression of ISG15 in relevant models of infection both *in vitro* and *in vivo*.

MATERIAL AND METHODS

Cells and virus.

Human lung carcinoma cells (A549) and human carcinoma HeLa derived cells (HEp-2) were maintained in Dulbecco´s modified Eagle´s medium (DMEM, Lonza) supplemented with 10% fetal bovine serum (FBS, Biowest), 4 mM L-Glutamine (Lonza), 100 U/ml penicillin (Lonza) and 100 U/ml streptomycin (Lonza). All cells were incubated at 37ºC in a 5% CO_2 atmosphere. To generate viral stocks, the RSV Long strain was propagated in HEp-2 cells and purified from clarified culture supernatants by polyethylene glycol precipitation and centrifugation in a discontinuous sucrose gradient as previously described (20, 48).

Viral infections and plaque assays.

A549 subconfluent monolayers were infected with RSV at a multiplicity of infection (MOI) of 3 plaque-forming units (pfu) per cell (as indicated in the figure legend, MOI of 30 or 0.3 was also used in some experiments). Cells were incubated with the viral inoculum in DMEM 2% FBS (DMEM2) for 90 minutes at 37ºC. After this time, the inoculum was removed and fresh DMEM2 was added. Cell supernatants for viral titration and cell pellets for RNA and protein extraction were collected at different hours post-infection (hpi). For cell-associated virus, cells were washed with DMEM2, scrapped off in fresh DMEM2, disaggregated by thoroughly pippeting and brief sonication in an ultrasonic bath, and virus titrated in the clarified supernatant.

To determine the viral titer, HEp-2 cell monolayers were incubated with serial dilutions of the cell supernatants for 90 minutes at 37ºC and then overlaid with 0.7% agarose in DMEM2. Five days post-infection (dpi), the cell monolayers

were fixed with 4% formaldehyde in PBS followed by methanol permeabilization. Cells were incubated with a mixture of monoclonal antibodies against RSV (20) and plaques were visualized using an anti-mouse IgG horseradish peroxidase linked whole antibody (Abcam) and 3-amino-9-ethylcarbazole (AEC, Sigma).

Quantitative RT-PCR and western blots.

Total RNA from mock-infected or infected cells was purified with the RNeasy Mini Kit (Qiagen) and was reverse transcribed with the High-Capacity cDNA Archive Kit (Applied Biosystems) following the manufacturer´s instructions. Gene expression was measured by quantitative RT-PCR (qRT-PCR) with a Step One instrument (Applied Biosystems) and performed in triplicate following the manufacturer´s protocols. PCR primers and TaqMan MGB probes (FAM dye-labeled) for the following genes were used: β-actin (Hs99999903_m1), ISG15 (Hs00192713_m1), UbE1L (Hs00163295_m1), USP18 (Hs00276441_m1), IFIT1 (Hs00356631_g1), RIG-I (Hs00204833_m1) and RSV nucleoprotein (forward primer: 5´CATGATTCTCCTGATTGTGGGATGA3´, reverse primer: 5´TCACGGCTGTAAGACCAGATCTAT3´, probe: 5´CCCCTGCTGCCAATTT3´) (Applied Biosystems). Gene expression was normalized to the β-actin expression and the comparative CT (ΔΔCT) method was used for relative quantifications.

Protein expression was analyzed by western blot. Cell pellets were resuspended in sodium-deoxycholate lysis buffer and protein concentration was determined using a Bradford protein assay (Biorad). A total of 10μg of each protein sample was separated in 10% or 15% SDS-PAGE gels and subsequently transferred to an immobilon-P membrane (Milipore). Primary antibodies for

155 detection of the following proteins were used: ISG15 (H150sc-50366, Santa Cruz),
156 β-actin (8224-100, Abcam), RSV phosphoprotein (67P), RSV G glycoprotein
157 (021/1G), RSV fusion protein (476-510) and RSV nucleoprotein (79N) (49, 50).
158 Horseradish peroxidase-linked anti-rabbit or anti-mouse Ig (Abcam) were used as
159 secondary antibodies. Proteins were visualized by chemiluminiscence using Clarity
160 Western ECL Substrate (Biorad) in a Gel Logic 1500 Imaging System instrument
161 (Kodak). The intensity of the protein bands was quantified by using Image J
162 software (http://rsb.info.nih.gov/ij/index.html) and standardized against β-actin.

163 **ISG15 overexpression assays.**

164 Total RNA from A549 RSV-infected cells was reverse transcribed with the
165 High-Capacity cDNA Archive Kit using an oligo-dT primer (Applied Biosystems)
166 and ISG15 was amplified using the following primers: forward
167 (5´AAAAGCGGCCGCGGTGCTGCCTGCCGAAG3´) and reverse
168 (5´AAAAGCGGCCGCTCTTTACAACAGCCTTTATTTCCG3´). The PCR product
169 was cloned into the mammalian vector pCMV6-Neo (Origene) and the resulting
170 plasmid pCMV6-Neo-ISG15 was sequenced in order to verify that the ISG15
171 sequence was correct. Synthesis of the non-conjugative ISG15 plasmid pCMV6-
172 Neo-ISG15-LRAA from pCMV6-Neo-ISG15 was performed by directed
173 mutagenesis using the Phusion Site-directed Mutagenesis kit (Thermo Scientific)
174 following the manufacturer´s instructions with the following primers: forward
175 (5´CCTGCGGGCAGCCGGCACAGAGCCTGGCGGGCGGAGC3´) and reverse
176 (5´GGCTGCCCGCAGGCGCAGATTCATGAACACGGT3´).

For overexpression assays, 5 x 10^4 A549 cells were plated in each well of a 12-well plate and incubated for 60 hours before transfection. Cells were then transfected with 1 μg of purified plasmid (EndoFree Plasmid Maxi Kit, Qiagen) and 4μl of Lipofectamine 2000 (Invitrogen) per well. Twenty-four hours after transfection, the cells were infected with RSV at a MOI of 3. Cell supernatants for viral titration and cell pellets for RNA and protein extraction were collected at different hpi.

siRNA silencing.

A549 cells were plated 24 hours before transfection at a density of 5 x 10^4 cells per well in 24-well plates. Cells were transfected with 6 pmol of control small interfering RNAs (siRNAs) or specific siRNAs against ISG15, UbE1L or USP18 (Ambion) and 1 μl of Lipofectamine RNAiMAx reagent (Invitrogen) per well. Twenty-four hours after transfection, cells were infected with RSV at a MOI of 3. In the case of IFN-β treatment, culture medium was replaced four hours after transfection with fresh medium containing 500 U/ml of IFN-β (pbl assay science) and maintained during the whole infection period. Cell supernatants for viral titration and cell pellets for RNA and protein extraction were harvested at different hpi, as indicated in the figure legends.

ISG15 knockout A549 cells.

Two clones of ISG15 knockout ($ISG15^{-/-}$) A549 cells were generated using the Transcription Activator-Like Effector Nucleases (TALENs) technology. This technology allows generating knockout cells by using sequence-specific DNA-

cleaving enzymes against specific target genes (51). Cells were transfected as described above with the purified plasmids Human-H36698_TALEN_L1 and Human-H36698_TALEN_R1 (Talen Library Resource, Seoul National University). Three days after transfection, cells were trypsinized and cloned by limiting dilution in 96-well plates at a density of one cell per well. Single cell clones were selected and expanded to generate stocks. Cell DNA was extracted with the Cyclo-Prep Genomic DNA Purification kit (Amresco) following the manufacturer´s instructions. Screening for ISG15$^{-/-}$ clones was conducted by PCR amplification and DNA sequencing using the following primers: forward (5´GAGCAGCTCCATGTCGGTGTC3´) and reverse (5´ACACGGTGCTCAGGGGCTTG3´). ISG15$^{-/-}$ clones were confirmed by western blot using an ISG15 specific antibody. All selected clones were grown, cloned a second time by limiting dilution, and checked again by sequencing and western blot to ensure that they did not express ISG15. Two wild-type clones that underwent the same process of transfection and cloning were selected as controls.

Virus growth was monitored in wild-type and ISG15$^{-/-}$ cells treated or not with 500 U/ml of IFN-β 20 hours before infection. Cell supernatants were collected for virus titration at 48 hpi.

Pseudo-stratified epithelia.

Human lung tissue samples were obtained from patients who underwent surgery for lung carcinoma. Experiments were approved by the local ethics committee and informed consent was obtained. Human bronchial epithelial cells were obtained from normal tissue and differentiated to multilayered epithelia as

previously described (52). Samples were mock-infected or infected with RSV at $7x10^6$ pfu/cm^2 and cells were collected at different dpi, RNA was extracted with TRIZOL reagent (Invitrogen) and further purified and reverse-transcribed as described above. Samples were analyzed by qRT-PCR using specific RSV nucleoprotein and ISG15 probes.

Nasopharyngeal wash samples.

Nasopharyngeal wash samples from 19 children up to 24 months old infected with RSV were harvested at admission and discharge by instillation of 2.5 ml of a an isotonic saline solution into each nostril (NaCl 0.9%) as described elsewhere (53). In all cases, an informed consent was requested from the parents or legal guardians prior to the inclusion of the child in the study. Approval of the Committee for Ethics in Clinical Research of the “Hospital Clínico Universitario” in Valladolid (Spain) was obtained prior to the beginning of recruitment. Total RNA was extracted, reverse transcribed and gene expression quantified as described above.

RESULTS

RSV infection induces ISG15 expression and protein ISGylation.

To investigate if ISG15 expression is up-regulated after *in vitro* RSV infection, A549 cells were infected and the levels of ISG15 and RSV nucleoprotein RNAs were analyzed by qRT-PCR at different hpi. Fig. 1A shows a large increase of ISG15 RNA in RSV infected A549 cells in a time-dependent manner. The increase started between 6 to 16 hpi, reaching maximum level 48 hpi. ISG15 RNA increase showed a delay of 4-5 hours with respect to RSV nucleoprotein RNA (Fig. 1A). A high correlation between ISG15 and nucleoprotein RNA expression was observed (R^2=0.97, P<0.0001) (Fig. 1B).

To confirm the above results and to determine whether the RNA levels of ISG15 and RSV nucleoprotein were translated to the protein levels, samples from a parallel infection were analyzed by western blot at various hpi. RSV nucleoprotein started to be detected 4 hpi (Fig. 1C) and increased continuously until 48 hpi. RSV infected A549 cells expressed large amounts of both free ISG15 and ISG15 conjugates. Accumulation of free ISG15 was time-dependent, becoming apparent 16 hpi, while the increase of ISG15 conjugates was not evident until 30-36 hpi. As described for RNAs, ISG15 protein increase had a delay of 4-5 hours with respect to the RSV nucleoprotein accumulation (Fig. 1C).

Finally, the protein ISGylation patterns obtained after 48 hours of RSV infection or IFN-β stimulation were compared by western blot. The results obtained revealed common bands being labelled but, additionally, some specific bands

appearing only in RSV infected cells or IFN-β stimulated cells were apparent (Fig. 1D); i.e., RSV induced ISGylation differs to some extent from that of IFN-β.

RSV titer as well as viral proteins and RNA levels are increased in ISG15 knockdown or ISG15$^{-/-}$ cells stimulated with IFN-β.

To analyze whether or not ISG15 has any anti-RSV activity, A549 cells were transfected with control siRNAs or specific ISG15 siRNAs before being infected with RSV. No differences were observed in viral titer between ISG15-silenced cells and control cells (Fig. 2A, -IFN-β). It was hypothesized that this lack of antiviral effect could be related to the fact that ISG15 expression and formation of ISG15 conjugates is delayed with respect to virus replication (Fig. 1A and 1C). Hence, ISG15 expression was induced before RSV infection by stimulation of cells with IFN-β. As expected, a decrease in viral titer was observed in IFN-β treated cells when compared with non-treated controls (Fig. 2A). However, an increase of 2.9 times in virus titer was observed in the ISG15-silenced cells with respect to control cells, indicative of an ISG15 assisted anti-RSV effect (Fig. 2A, +IFN-β).

In addition to virus titers, the amount of accumulated viral nucleoprotein and RNA was quantified at 24 and 48 hpi in control and ISG15-silenced cells previously stimulated with IFN-β. As expected, a clear inhibition of free ISG15 and ISG15 conjugates was observed in ISG15-silenced cells by western blot (Fig. 2B). At the same time, ISG15-silenced cells showed an increase in the amount of the RSV nucleoprotein at 24 and 48 hpi compared with control cells (Fig. 2B). Furthermore,

281 a significant increase on the amount of RSV nucleoprotein RNA was observed in
282 ISG15-silenced cells when compared with control cells at the same hpi (Fig. 2C).

283 To confirm the above results, ISG15$^{-/-}$ cells were generated using TALEN
284 nucleases. Two wild-type and two ISG15$^{-/-}$ cell clones were selected for further
285 studies (Fig. 3A). These four clones, along with the uncloned wild-type cells, were
286 infected either in the absence or presence of IFN-β at a MOI of 3 and viral titers
287 were determined 48 hours later. As expected, no significant differences were found
288 among the untreated wild-type or ISG15$^{-/-}$ cells. However, in the IFN-β treated
289 cells, a significant increase (from 1.9 to 4.6-fold) in viral titer was found in ISG15$^{-/-}$
290 cells, compared with wild-type cells (Fig. 3B). Together, these results demonstrate
291 that ISG15 has an anti-RSV activity in cells previously stimulated with IFN-β.

292 When RSV infections were carried out at MOI 30 or 0.3, an increase in virus
293 titer was also observed in ISG15$^{-/-}$ cells previously treated with IFN-β (Fig. 3C). The
294 magnitude of this effect seemed to decrease as MOI increased, suggesting that
295 ISG15 inhibition was more effective with lower input virus, as otherwise might be
296 expected for a partial block in virus replication.

297 **ISG15 overexpression before virus infection reduces RSV titer as well as**
298 **viral proteins and RNA accumulation.**

299 The results from previous sections indicated that the high levels of ISG15
300 induced by RSV infection had no antiviral effect. However, this antiviral effect was
301 revealed when ISG15 was overexpressed before virus infection by stimulation of
302 cells with IFN-β. ISG15 might need the collaboration of other proteins induced by

303 IFN-β for its antiviral activity or it may be just a matter of the time period at which
304 IS15 is expressed in relation to virus infection. To distinguish between these two
305 possibilities, A549 cells were either transfected with a plasmid overexpressing
306 ISG15 or with the same empty vector as a negative control, and then infected with
307 the RSV. Cell supernatants were harvested at 24 and 48 hpi and viral titers
308 determined by plaque assay. A significant reduction of the viral titer (4.8-fold) was
309 observed in the ISG15 overexpressing cells when compared with control
310 transfected cells at 48 hpi (Fig. 4A). Western blot analysis of four viral proteins
311 revealed decreased accumulation in the ISG15 transfected cells compared with
312 control cells. This decrease was observed at 24 and 48 hpi, with the most evident
313 effect at 24 hours (Fig. 4B). In addition, the RSV nucleoprotein RNA was analyzed
314 by qRT-PCR at the same hpi. The results showed a significant RNA reduction in
315 the ISG15 overexpressing cells compared to control cells at both 24 and 48 hpi
316 (Fig. 4C).

317 Therefore, these findings support the conclusions reached with ISG15-
318 silenced or ISG15$^{-/-}$ cells and demonstrated that ISG15 has an anti-RSV activity
319 when overexpressed before virus infection, either alone or in the context of the
320 antiviral response induced by IFN-β.

321 **Antiviral activity of ISG15 against RSV is due to protein ISGylation.**

322 In order to determine if ISG15 accomplishes its antiviral activity against RSV
323 in a conjugated or unconjugated form, experiments were carried out using three
324 different approaches:

First, since a C-terminal Gly-Gly motif is required for ISG15 conjugation (21, 22), a plasmid was generated by site-directed mutagenesis in which those two residues were mutated to Ala. This plasmid therefore expresses an ISG15 protein that cannot be conjugated to target proteins. A549 cells were transfected with the plasmid expressing either wild-type ISG15, the plasmid expressing non-conjugative ISG15 or an empty vector. Twenty-four hours later, cells were infected with RSV and virus titers were measured at 48 hpi. As expected, a significant decrease (3.9-fold) was found in the viral titer of cells overexpressing wild-type ISG15 when compared with control cells transfected with the empty vector. However, no differences were found between these control cells and cells transfected with the non-conjugative ISG15 plasmid (Fig. 5A). Similar results were obtained after transfection/infection experiments of $ISG15^{-/-}$ cells, demonstrating that their phenotype can be reconstituted by wild-type ISG15 but not by non-conjugative ISG15 (Fig. 5B).

A second approach to inhibit the formation of ISG15 conjugates was knocking down the only identified E1 enzyme for ISG15, UbE1L (24). A549 cells were either transfected with control siRNAs or specific UbE1L siRNAs and then infected with RSV in either the absence or presence of IFN-β. IFN-β-stimulated UbE1L-silenced cells expressed high levels of free ISG15, as did control cells, but failed to form ISG15 conjugates (Fig. 5C). Besides, similarly to what happened with ISG15-silenced cells, UbE1L inhibition led to an important increase in the amount of RSV nucleoprotein at 24 and 48 hpi (Fig. 5C). Also, resembling the results of infecting ISG15-silenced cells with RSV (Fig. 2A), no differences were found

between the UbE1L-silenced cells and control cells in the absence of IFN-β stimulation (Fig. 5D, -IFN-β). In contrast, a significant increase (3.9-fold) in viral titer was observed in the UbE1L-silenced cells and treated with IFN-β when compared with control cells (Fig. 5D, +IFN-β).

The third approach consisted in silencing of USP18, an ISG15-specific deconjugating protease that removes ISG15 from its protein targets (29, 30). As expected, the results were the opposite of those obtained from UbE1L silencing experiments: in IFN-β treated cells, USP18 silencing increased protein ISGylation, decreased RSV nucleoprotein accumulation (Fig. 5E) and reduced virus titers (3.3-fold) (Fig. 5F).

Altogether, these data indicate that ISGylation, rather than free ISG15, is responsible for the anti-RSV activity of this molecule.

In addition to its role in protein ISGylation, it has been reported that ISG15 and USP18 act together to counteract IFN-α/β signaling (54, 55). Hence, the expression of IFIT1 and RIG-I (two ISGs) was measured in cells silenced for ISG15, UbE1L or USP18 that were previously treated with IFN-β and then infected with RSV. As expected, a slight increase in the expression of IFIT1 and RIG-I mRNA was observed in cells knocked down for ISG15 or USP18, but not for UbE1L (Fig. 6). Despite having similar effect on IFIT1 and RIG-I expression, ISG15 and USP18 silencing had opposite effect on RSV growth (Fig. 2A and 5F), indicating that, with regard to RSV inhibition, the effect on protein ISGylation predominates over the effect on ISGs expression. It cannot be excluded, however,

370 that the overexpression of ISGs may contribute somewhat to reduce RSV titer in
371 USP18 knocked down cells.

372 **The ISG15 anti-RSV activity affects a post-entry stage of infection before**
373 **virus release.**

374 Since it has been shown that ISG15 may affect virus entry (56) or release
375 (34, 37), two experiments were carried out to gain information about the role of
376 ISG15 in those steps of RSV replication.

377 First, A549 cells were transfected with a plasmid expressing ISG15 or a
378 control plasmid before being infected with RSV under single infectious cycle
379 conditions. Then, the accumulation of RSV nucleoprotein RNA was quantified by
380 qRT-PCR at several hpi. The results showed a significant RNA reduction in the
381 ISG15 overexpressing cells compared to control cells starting between 8-15 hpi
382 (Fig. 7A). This result shows that ISG15 restricts RSV growth at a post-entry stage
383 of infection.

384 In addition, the virus released to the supernatant and the virus associated to
385 cells was quantified in ISG15- or control-transfected cells. As expected from
386 previous experiments a more than three-time reduction in virus titer was observed
387 in the supernatant of ISG15-transfected cells with respect to control-transfected
388 cells (Fig. 7B, supernatant). Similarly, a significant decrease of more than two-fold
389 was also observed in virus titers from the cell-associated fraction of ISG15-
390 transfected cells when compared to the same fraction of control cells (Fig. 7B, cell-

associated). This indicates that an ISG15-mediated restriction on RSV infection occurs before virus release.

ISG15 expression correlates with RSV infection in pseudo-stratified respiratory epithelia and in nasopharyngeal washes from infants.

As a preliminary step to investigate the role of ISG15 in the anti-RSV response *in vivo*, its expression was analyzed in more relevant models of infection. Firstly, an *in vitro* model of differentiated pseudo-stratified columnar respiratory epithelium with ciliated and mucus producing cells that resembles *in vivo* conditions was used for RSV infection (52). Differentiated cultures from six donors were infected and viral yield and ISG15 expression were quantified by qRT-PCR at different dpi. ISG15 levels of infected samples correlated with RSV nucleoprotein expression in every sample and time post-infection tested. A high positive correlation (R^2=0.77, P<0.0001) between viral infection and the ISG15 expression level was observed (Fig. 8A).

Secondly, nasopharyngeal washes from 19 young infants infected with RSV were obtained at admission and discharge (38 samples in total). After RNA extraction, RSV nucleoprotein and ISG15 RNA levels were determined by qRT-PCR. Similarly to the pseudo-stratified epithelia, a high positive correlation (R^2=0.63, P=0.0004) between RSV nucleoprotein and ISG15 expression was observed (Fig. 8B). These data demonstrate that ISG15 is induced by RSV infection *in vivo* and suggest that ISG15 may play an antiviral role after natural infection.

DISCUSSION

In this study, the antiviral activity of ISG15 against RSV was investigated. RSV infection of A549 cells induced high levels of both free and conjugated ISG15. Furthermore, overexpression of ISG15, or pretreatment with IFN-β of cells that were ISG15-silenced or ISG15$^{-/-}$, demonstrated that ISG15 played an important role as an anti-RSV molecule. The ISG15 antiviral activity required protein ISGylation as was evidenced by the lack of effect of a non-conjugative form of ISG15, the inhibition of ISGylation by UbE1L silencing or the increase of ISGylation by USP18 knockdown. Moreover, a high correlation between RSV infection and ISG15 expression was established both in a relevant model of infection, such as human respiratory pseudo-stratified epithelia, and in nasopharyngeal washes from infected children.

Although the antiviral activity of ISG15 has been widely described (57, 58), the mechanisms through which ISG15 exerts its effects have only been hinted in some cases. The antiviral activity of ISG15 has been described to occur in the late stages of the viral cycle of HIV and in Ebola virus infections, where ISG15 inhibits virus release (34, 37). In contrast, it has been recently described that ISG15 inhibits early steps, such as entry and/or uncoating, of the Murine norovirus life cycle, although no specific target proteins responsible for these effects have been identified (56). ISG15 has been claimed to exert its antiviral activity by conjugation to either viral or cellular proteins. For instance, ISGylation of the NS1 protein of Influenza A virus reduced its capacity to antagonize the host antiviral response (59,

60) and ISG15 conjugation to IRF3 during Sendai virus infection inhibited its proteasome-mediated degradation, boosting the host antiviral response (61).

Our results show that, in order to exert its anti-RSV activity, ISG15 has to accumulate to high amounts before virus infection. They also indicate that ISG15 carry out its anti-RSV action after virus entry. In addition, ISG15 seems to affect a stage in the RSV cycle before virus release, since RSV titers decreased in both released and cell-associated virus following ISG15 overexpression. Given that ISGylation is ordinarily a co-translational modification (31), it is possible that the ISG15 anti-RSV activity is mediated by direct ISGylation of viral and/or cellular proteins essential for virus replication. In this situation, in the first RSV infected cells, ISG15 would not have an antiviral effect because most viral proteins have already accumulated to high levels before the ISGylation machinery is triggered. In cells that acquire an antiviral state before virus infection, such as those stimulated by interferon, the ISGylation machinery is ready to operate as soon as the virus enters into the cell. In this case, ISG15 may interfere with RSV replication by ISGylation of viral or cellular proteins required for RNA replication/transcription, being P, L, M2-1 and M2-2 obvious protein viral targets which are now under study. Additionally, or alternatively, binding of certain RSV protein(s) to ISG15 may be required to antagonize its antiviral effect, as occurs with vaccinia virus E3 or influenza B NS1 proteins (24, 45). In this case, when ISG15 levels increase in the first RSV infected cells, the amount of viral proteins has already reached levels capable of neutralizing ISG15 activity. In contrast, when ISG15 is expressed at high levels before virus infection, as in cells previously stimulated by IFN-β, no viral

458 proteins are present to counteract the ISG15 antiviral effect. The same reasoning
459 would apply if RSV protein(s) antagonize any of the enzymes involved in
460 ISGylation, instead of ISG15 directly.

461 It has been shown that USP18 regulates negatively the IFN-α/β signaling
462 independently of its ISG15 isopeptidase activity (54) (Fig. 9).This regulation
463 required ISG15 to stabilize USP18, a process that was ISGylation-independent
464 since it was mediated by non-conjugating ISG15 and it was not affected by UbE1L
465 silencing (55). According to this, the lack of ISG15 would destabilize USP18
466 leading to an increased response to IFN type I and viral resistance (55) (Fig. 9). In
467 line with this, we have observed a modest increase in the expression of the
468 interferon stimulated genes IFIT1 and RIG-I in both ISG15 and USP18 silenced
469 cells treated with IFN-β and infected with RSV (Fig. 6). By contrast, UbE1L
470 silencing reduced the expression of those genes (Fig. 6), perhaps by increasing
471 free ISG15 levels due to impaired ISGylation. Our results, however, demonstrated
472 that the anti-RSV activity of ISG15 in A549 cells was ISGylation-dependent and
473 largely independent of its effect on USP18 stabilization because: i) non-conjugating
474 ISG15 is able to stabilize USP18 (55), but it does not have any effect on RSV
475 replication (Fig. 5A and 5B); ii) UbE1L silencing had no effect on USP18
476 stabilization (55), but it increased virus titers in IFN-β treated cells (Fig 5D); iii)
477 while ISG15 and UbE1L silencing had opposite effects on the expression of IFIT1
478 and RIG-I (Fig. 6), both increased RSV replication (Fig. 2A and 5D); and iv) ISG15
479 or USP18 silencing in IFN-β treated cells had opposite effects on RSV titers (Fig.
480 2A and 5F), consistent with the ISG15 isopeptidase activity of USP18 but contrary

481 to the results expected from a joint action of ISG15 and USP18, such as that
482 observed in the IFN response regulation (Fig. 9). Our results agree with the
483 recently reported observations showing that selective inactivation of the USP18
484 isopeptidase activity in knock-in mice enhanced protein ISGylation and resistance
485 against vaccinia and influenza B viruses without inducing any obvious changes in
486 the IFN signaling pathway (62).

487 The positive correlation observed in this study between RSV infection and
488 ISG15 expression in human pseudo-stratified epithelia and in nasopharyngeal
489 washes from infected children point to a role in RSV infections *in vivo*. Virus
490 replication in infected cells generates products that act as pathogen associated
491 molecular patterns (PAMPs), which directly trigger intracellular innate pathways
492 leading to the expression of ISG15 and other antiviral and proinflammatory genes
493 that initiate the immune response (7, 8). In this setting, uninfected respiratory
494 epithelial cells might acquire an antiviral status, which includes high levels of
495 ISG15, through stimulation by IFN secreted from neighboring infected cells and/or
496 immune cells attracted to the site of infection in the respiratory tract (63, 64).
497 Subsequent infection of those cells by RSV would be impaired by the previously
498 acquired antiviral state. In this scenario, ISG15 would play an important role to
499 hinder RSV dissemination. In addition, overexpression of ISG15 during RSV
500 infection may contribute to control the excessive inflammation by stabilizing USP18
501 (55). The role of ISG15 in viral infections *in vivo* requires, however, further
502 investigation since it seems to be complex and multifaceted, as demonstrated by
503 conflicting results from ISG15-deficient human and mice (55, 57), as well as by the

identification of a novel ISG15 conjugation-dependent mechanism by which mice are protected against influenza A and Sendai virus infection without obvious effect on virus replication and immune response (65), which contrast with the observation that protein ISGylation restricted virus replication and enhanced resistance to vaccinia virus and influenza B virus in mice (62).

The close correlation between RSV load and ISG15 expression in infants and the fact that ISG15 is secreted to human fluids, where it can be quantified, raises the possibility to use this molecule to monitor RSV-induced inflammation (66). Related to this, it is important to stress that ISG15 levels decreased between admission and discharge, for every single infant, reflecting the reduction in virus load and inflammation (data not shown).

In conclusion, we have described for the first time that ISG15 has a conjugation-dependent antiviral activity against RSV. In addition, data from nasopharyngeal washes from infants infected with RSV suggest that ISG15 may play an important role in RSV infection *in vivo*. Therefore, although further research is required to elucidate the mechanisms of ISG15 interference with RSV, this study enhances our understanding of the innate immune response against RSV and identifies ISG15 as a potential target for virus control.

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712 **FIGURE LEGENDS**

713 **Figure 1**. **RSV infection of A549 cells enhances ISG15 expression and protein**
714 **ISGylation.** (A) A549 cells were infected with RSV at a MOI of 3 and harvested at
715 the indicated hpi. RSV nucleoprotein and ISG15 RNAs were quantified by qRT-
716 PCR. Data represent the fold increase of ISG15 RNA in infected cells compared
717 with mock infected cells, and the fold increase of RSV nucleoprotein RNA with
718 respect to zero hpi. (B) Linear regression plot of RSV nucleoprotein and ISG15
719 RNA expression data from graphic A. (C) A549 cells were infected with RSV and
720 harvested at the indicated hpi. Protein accumulation was analyzed by western blot
721 using anti ISG15 and anti RSV nucleoprotein specific antibodies. Normalization
722 was carried out using an anti β-actin antibody. (D) Western blot comparing protein
723 ISGylation patterns induced by interferon-β (500 U/ml) or RSV infection after 48
724 hours. Arrows show treatment-specific ISGylated proteins.

725 **Figure 2. ISG15 downregulation in IFN-β stimulated cells increases viral titer**
726 **and viral protein and RNA levels.** (A) A549 cells were transfected with either
727 control siRNAs or ISG15 siRNAs and infected twenty four hours later at a MOI of 3.
728 In the case of IFN-β treatment, culture medium was replaced four hours after
729 transfection with fresh medium containing 500 U/ml of IFN-β and maintained during
730 the whole infection period. Cell supernatants were harvested at 48 hpi and virus
731 titer was determined by plaque assays. (B) Protein extracts from IFN-β-treated
732 RSV-infected cells were collected 24 or 48 hpi and analyzed by western blot using
733 anti ISG15 and anti RSV nucleoprotein specific antibodies. Proteins were

734 quantified and normalization was carried out using an anti β-actin antibody. (C)
735 IFN-β treated cells were either transfected with control siRNAs or ISG15 siRNAs
736 and then infected with RSV. RSV nucleoprotein RNA was quantified by qRT-PCR
737 at the indicated hpi in ISG15 silenced cells and represented as a percentage of the
738 nucleoprotein RNA expressed in cells transfected with a control siRNA (100%).
739 Data from (A) and (C) represent the mean and standard deviation from at least
740 three independent experiments. Comparisons between conditions were done using
741 the t test. * P< 0.05; ** P< 0.01.

742 **Figure 3**. **ISG15 knockout in IFN-β stimulated cells leads to an increase in**
743 **viral titer.** (A) Two ISG15-knockout A549 cell lines ($ISG15^{-/-}$) (lanes 4 and 5) were
744 generated by using TALENs nucleases, and ISG15 expression was checked by
745 western blot using specific antibodies. Uncloned wild type cells (lane 1) and two
746 wild type cell clones (lanes 2 and 3) were included as controls. (B) ISG15 wild type
747 cells and $ISG15^{-/-}$ cells were either left untreated or treated with IFN-β prior to RSV
748 infection at MOI of 3. (C) Similar experiments were carried out at MOI of 0.3 and 30
749 in clones #3 (WT) and #5 ($ISG15^{-/-}$). Cell supernatants were harvested 48 hpi (MOI
750 of 3 and 30) or 72 hpi (MOI of 0.3) and virus titers determined by plaque assays.
751 Data represent the mean and standard deviation from three independent
752 experiments. Comparisons between groups were done by the t test. ** P< 0.01.
753 Lanes: 1, uncloned wild type cells; 2 and 3, wild type cell clones; 4 and 5, $ISG15^{-/-}$
754 cell clones.

755 **Figure 4**. **ISG15 overexpression leads to a decrease in viral titer, proteins and**
756 **RNA.** A549 cells were either transfected with an ISG15 overexpressing plasmid or

757 a control plasmid and infected at a MOI of 3 with the RSV 24 hours later. (A) Cell
758 supernatants were collected and virus titer determined by plaque assay at 48 hpi.
759 (B) Protein extracts from a representative experiment were collected at 24 and 48
760 hpi and analyzed by western blot using antibodies against ISG15, and the following
761 RSV proteins: glycoprotein (G), phosphoprotein (P), fusion protein (F) and
762 nucleoprotein (N). Proteins were quantified and normalization was carried out
763 using an anti β-actin antibody. 1, Cells transfected with a control plasmid and 2,
764 cells transfected with the ISG15 plasmid. (C) RSV nucleoprotein RNA was
765 quantified by qRT-PCR at 24 and 48 hpi. Data represent the percentage of
766 expression of RSV nucleoprotein RNA in ISG15-transfected cells compared with
767 cells transfected with a control plasmid (100%). Data from (A) and (C) represent
768 the mean and standard deviation from four independent experiments. Comparisons
769 between groups were done by the t test. * $P< 0.05$; ** $P< 0.01$.

770 **Figure 5. Antiviral activity of ISG15 against RSV is due to protein ISGylation.**
771 (A) A549 cells were transfected with either a control plasmid, a plasmid
772 overexpressing ISG15 or a plasmid overexpressing non-conjugative ISG15
773 (ISG15-LRAA), and infected 24 hours later with RSV at a MOI of 3. Cell
774 supernatants were collected at 48 hpi and viral titers determined by plaque assay.
775 (B) $ISG15^{-/-}$ A549 cells were transfected, infected and virus titer determined as in
776 panel A. (C) IFN-β treated A549 cells were transfected with either control siRNA or
777 UbE1L siRNAs and infected 24 hours later with RSV at a MOI of 3. Protein extracts
778 were collected at 24 and 48 hpi and analyzed by western blot using anti ISG15 and
779 anti RSV nucleoprotein specific antibodies. Proteins were quantified and

780 normalization was done using an anti β-actin antibody. (D) Untreated and IFN-β
781 treated A549 cells were either transfected with control siRNAs or UbE1L siRNAs
782 prior to RSV infection. Cell supernatants were collected 48 hpi and virus titers
783 determined by plaque assays. (E) and (F) A549 cells were treated as in panels C
784 and D but USP18 siRNA, rather than UbE1L siRNA, was used for gene silencing.
785 In the case of IFN-β treatment (C, D, E and F), culture medium was replaced four
786 hours after transfection with fresh medium containing 500 U/ml of IFN-β and
787 maintained during the whole infection period. Data represent the mean and
788 standard deviation from at least three independent experiments. Comparisons
789 between groups were done by the t test. * P< 0.05.

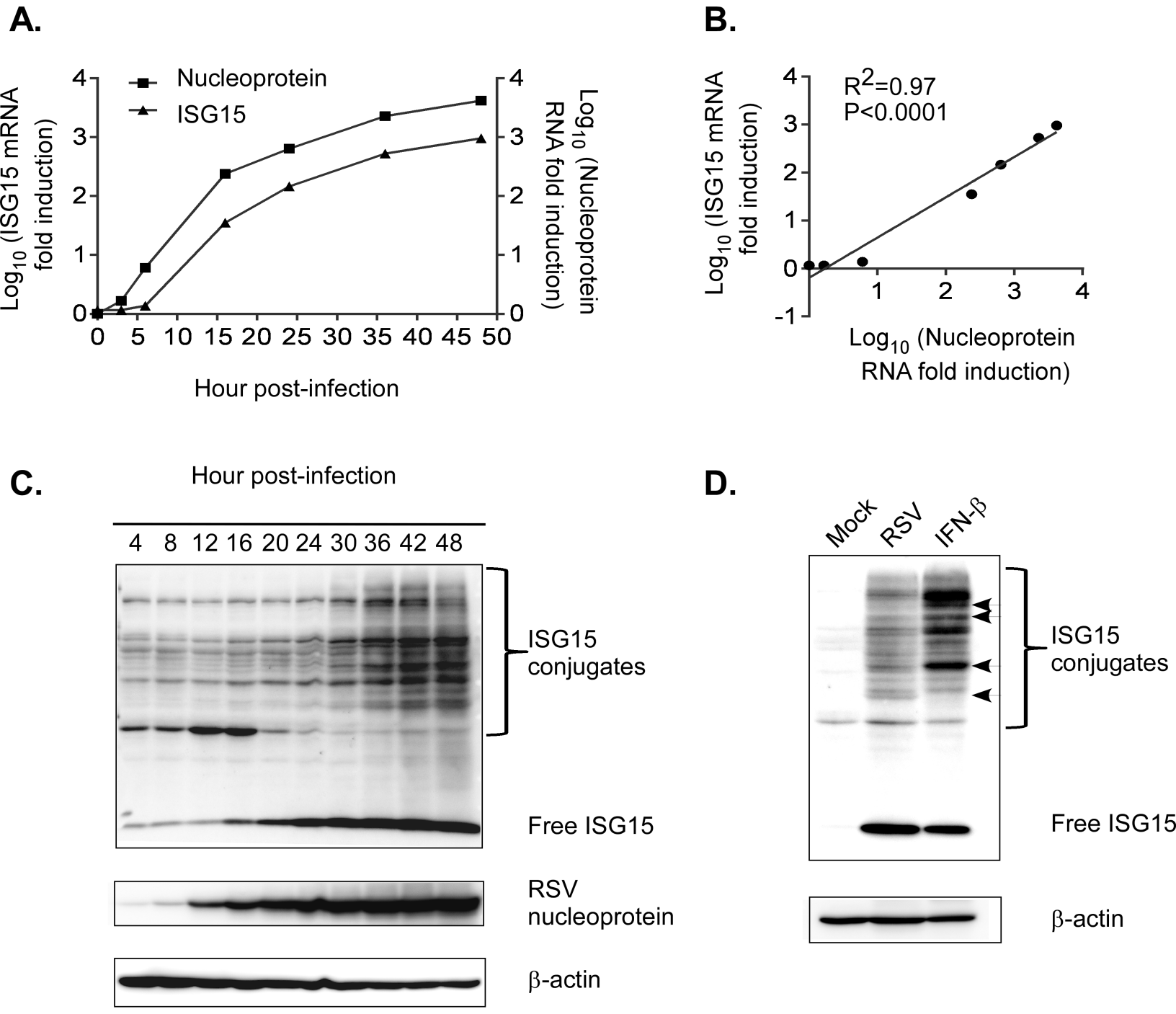
790 **Figure 6. ISGs expression in ISG15, UbE1L and USP18 silenced cells.** IFN-β
791 treated cells were either transfected with control siRNAs or ISG15 (A), UbE1L (B)
792 or USP18 siRNAs (C) and then infected with RSV. IFIT1 and RIG-I mRNA was
793 quantified by qRT-PCR at the indicated hpi in silenced cells and represented as a
794 percentage of the mRNA expressed in cells transfected with a control siRNA
795 (100%). Data from (A), (B) and (C) represent the mean and standard deviation
796 from three independent experiments. Comparisons between conditions were done
797 using the t test. * P< 0.05.

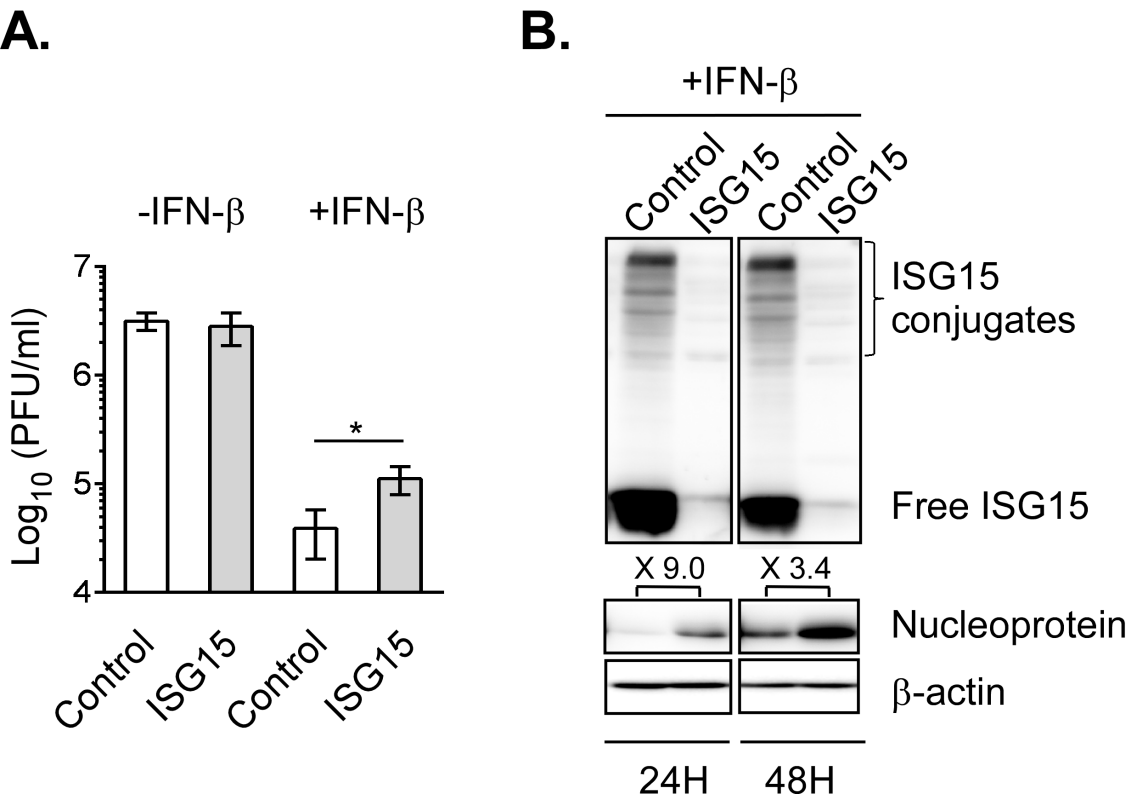
798 **Figure 7. The anti-RSV activity of ISG15 affects a post-entry stage of infection**
799 **before virus release.** A549 cells were either transfected with an ISG15
800 overexpressing plasmid or a control plasmid and infected at a MOI of 3 with the
801 RSV 24 hours later. (A) RSV nucleoprotein RNA was quantified by qRT-PCR at
802 different hpi. Data represent the percentage of expression of RSV nucleoprotein

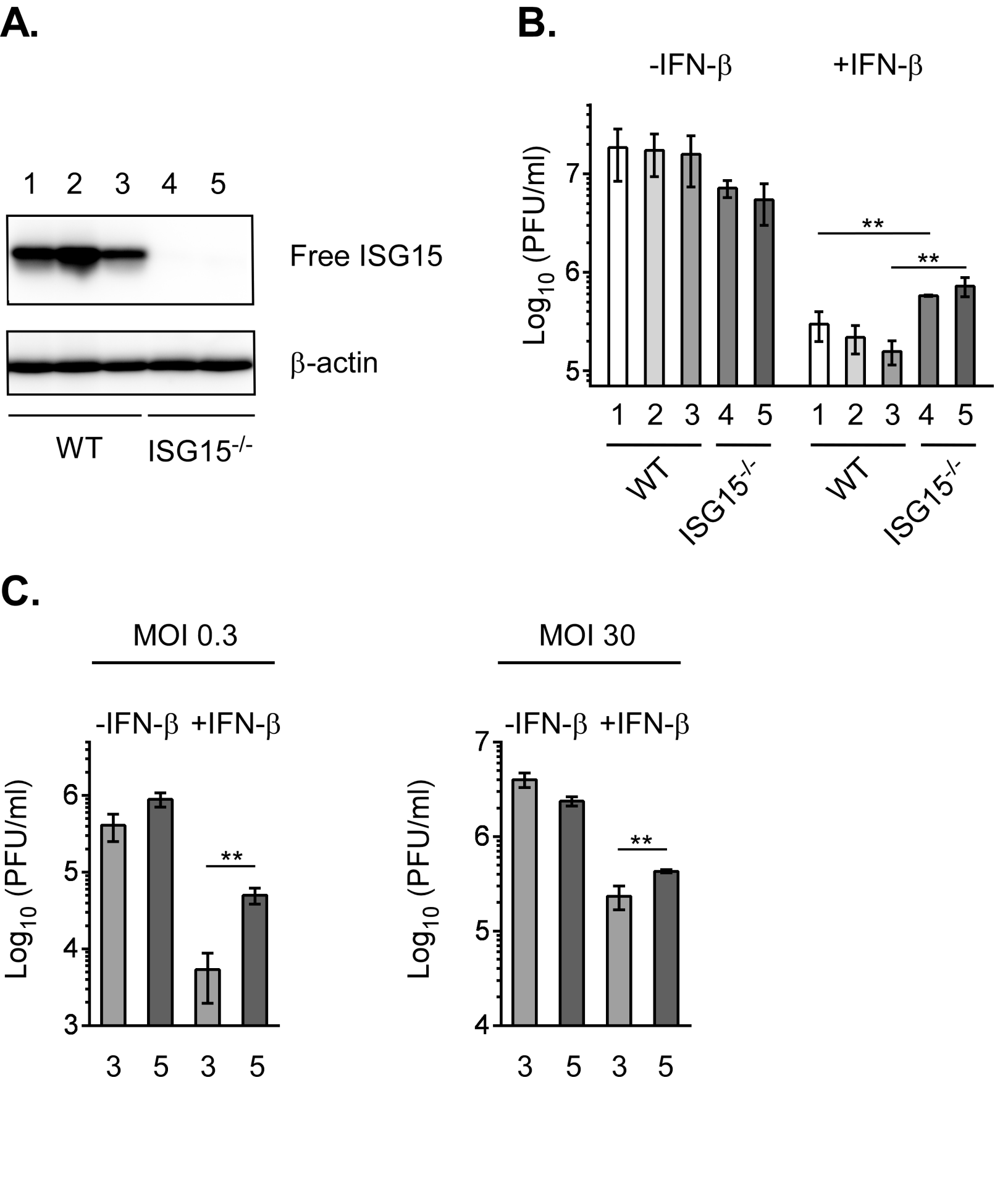
RNA in ISG15-transfected cells compared with cells transfected with a control plasmid (100%). (B) RSV titer in the culture supernatant or associated with cells was determined by plaque assay at 48 hpi. For cell-associated virus, cells were washed with DMEM2, scrapped off in fresh DMEM2, disaggregated by thoroughly pipetting and brief sonication in an ultrasonic bath, and virus titrated in the clarified supernatant. Data from (A) and (B) represent the mean and standard deviation from three independent experiments. Comparisons between groups were done by the t test. * P< 0.05; ** P< 0.01.

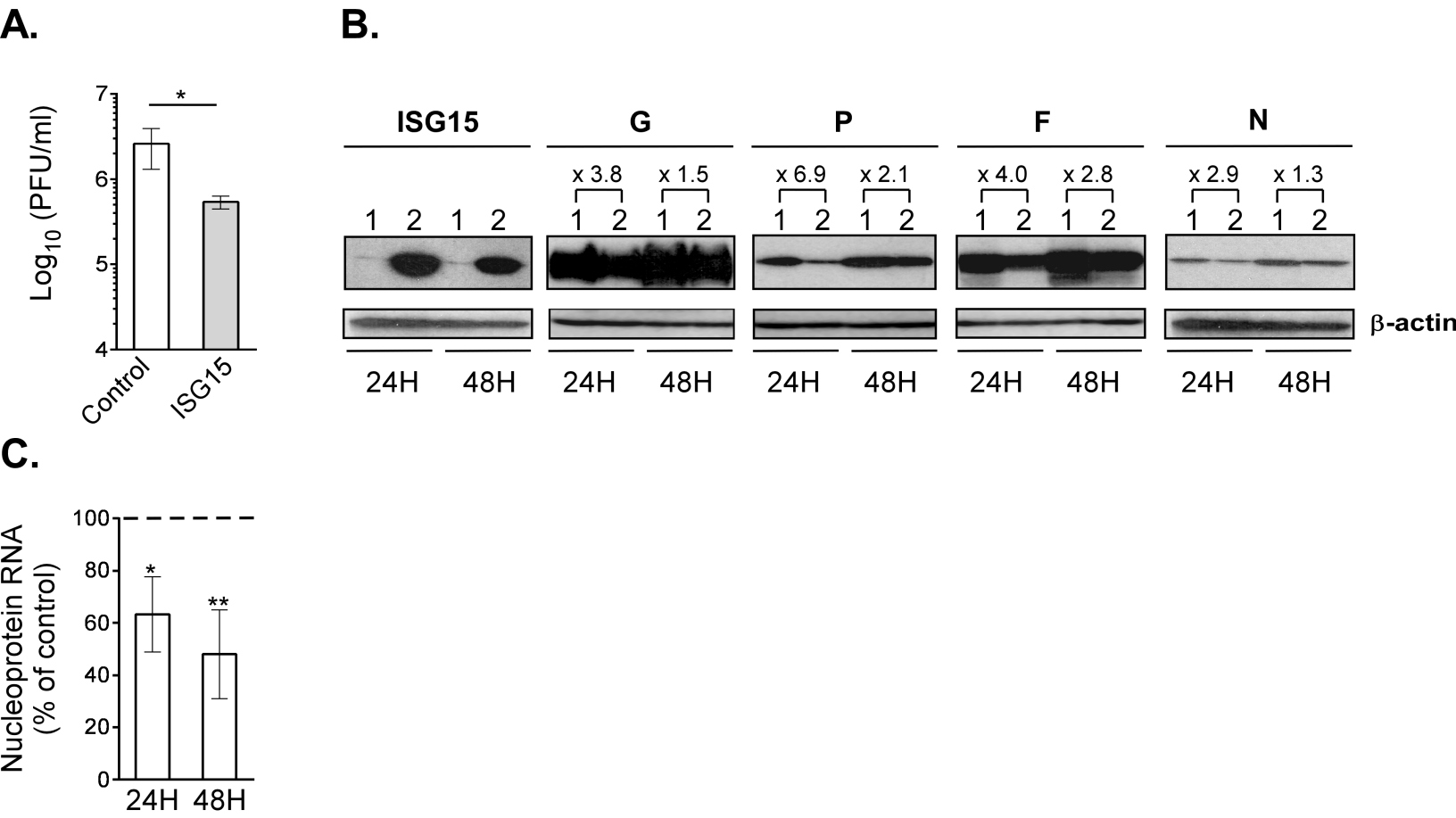
Figure 8. ISG15 expression correlates with RSV infection *in vitro* and *in vivo*. (A) Pseudo-stratified epithelia were generated *in vitro* from lung explant of six donors and either mock-infected or infected with RSV for two and four days (five individuals) or three days (one individual). RNA was extracted and ISG15 and RSV nucleoprotein RNA was quantified by qRT-PCR. A linear regression plot of RSV nucleoprotein against ISG15 RNA levels in each condition is represented. RSV nucleoprotein fold induction was obtained by comparison to the donor with the lowest value of expression, and ISG15 fold induction was calculated relative to mock-infected cells. (B) Nasopharyngeal wash samples from 19 children infected with RSV were collected at admission (open circles) and discharge (solid circles) (38 samples in total) and RNA was extracted and quantified by qRT-PCR. A linear regression plot of RSV nucleoprotein against ISG15 RNA levels in each sample is represented. RSV nucleoprotein and ISG15 fold induction were relative to an external control (a dilution of mRNA from A549 cells infected with RSV).

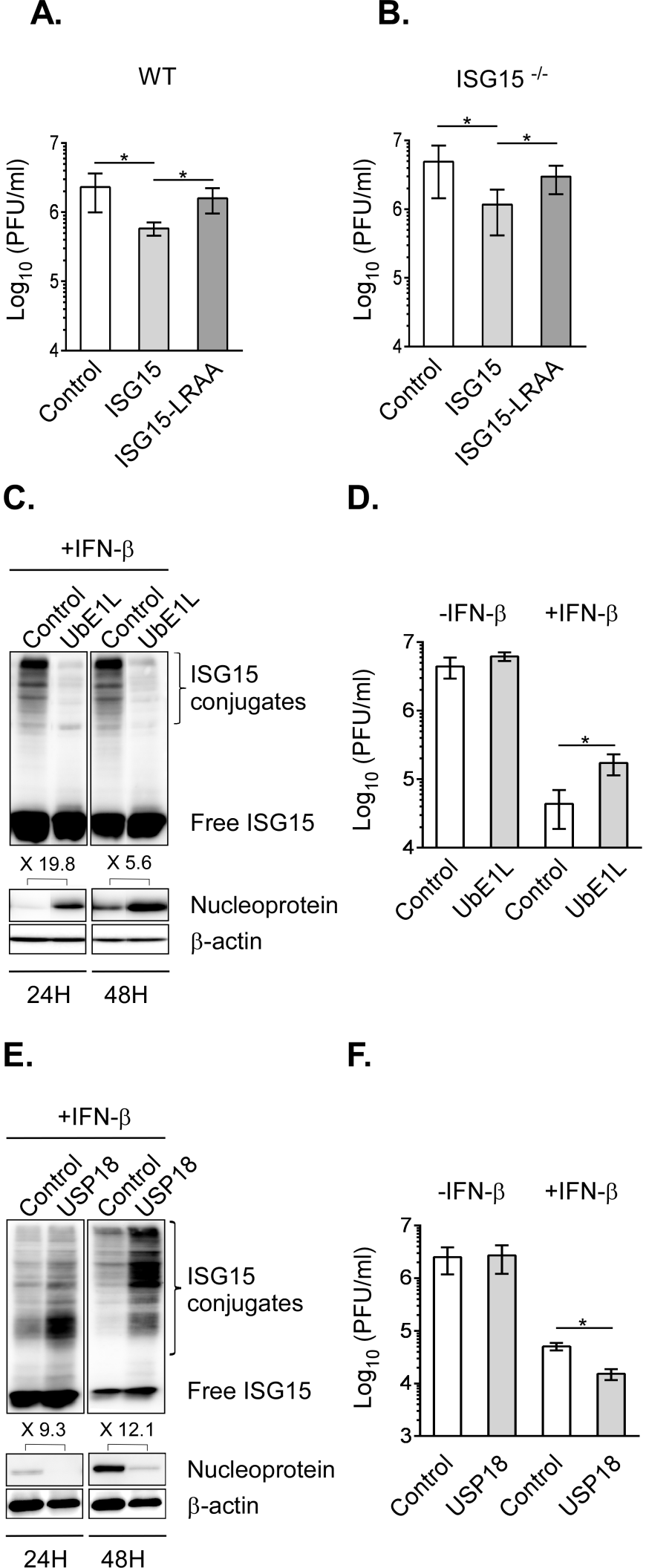
Figure 9. Intracellular ISG15 conjugation-dependent and independent mechanisms of action. IFN-α/β stimulates the expression of a wide range of genes termed interferon-stimulated genes (ISGs) involved in the antiviral response. ISG15 is one of those genes that conjugates to target proteins through a three-step enzymatic process termed ISGylation, for which the E-1 activating enzyme UbE1L is required. This process is reversed by the ubiquitin-specific isopeptidase USP18. In addition, free ISG15 stabilizes USP18 to compete with JAK1 for binding to the IFNAR2, thereby negatively regulating the IFN-α/β signaling in an isopeptidase-independent manner. The impact of these mechanisms on RSV replication is discussed in the text.


A.
Nucleoprotein
ISG15
Log10 (ISG15 mRNA fold induction)
Log10 (Nucleoprotein RNA fold induction)
Hour post-infection
B.
R2=0.97
P<0.0001
Log10 (ISG15 mRNA fold induction)
Log10 (Nucleoprotein RNA fold induction)
C.
Hour post-infection
4 8 12 16 20 24 30 36 42 48
ISG15 conjugates
Free ISG15
RSV nucleoprotein
β-actin
D.
Mock
RSV
IFN-β
ISG15 conjugates
Free ISG15
β-actin

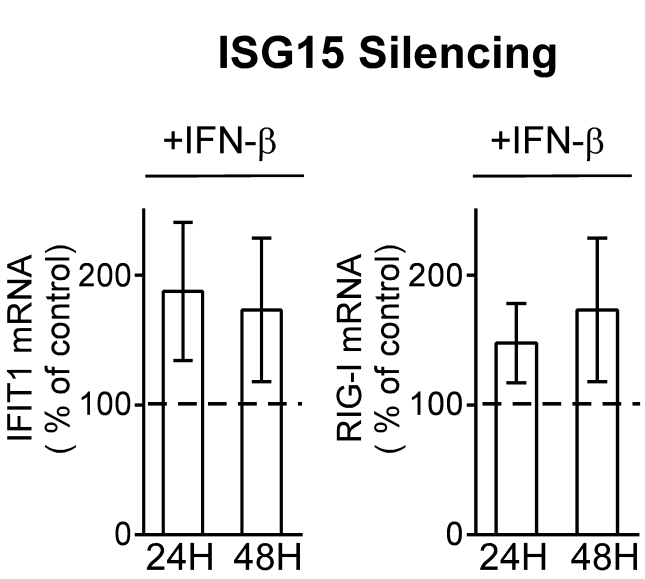

A.
-IFN-β
+IFN-β
Log10 (PFU/ml)
7
6
5
4
*
Control
ISG15
Control
ISG15
B.
+IFN-β
Control
ISG15
Control
ISG15
ISG15
conjugates
Free ISG15
X 9.0
X 3.4
Nucleoprotein
β-actin
24H
48H
C.
+IFN-β
Nucleoprotein RNA
(% of control)
200
150
100
50
0
**
*
24H
48H


A.
1 2 3 4 5
Free ISG15
β-actin
WT
ISG15-/-
B.
-IFN-β
+IFN-β
Log10 (PFU/ml)
7
6
5
**
**
1 2 3 4 5 1 2 3 4 5
WT
ISG15-/-
WT
ISG15-/-
C.
MOI 0.3
MOI 30
-IFN-β +IFN-β
-IFN-β +IFN-β
Log10 (PFU/ml)
6
5
4
3
**
**
3 5 3 5
3 5 3 5

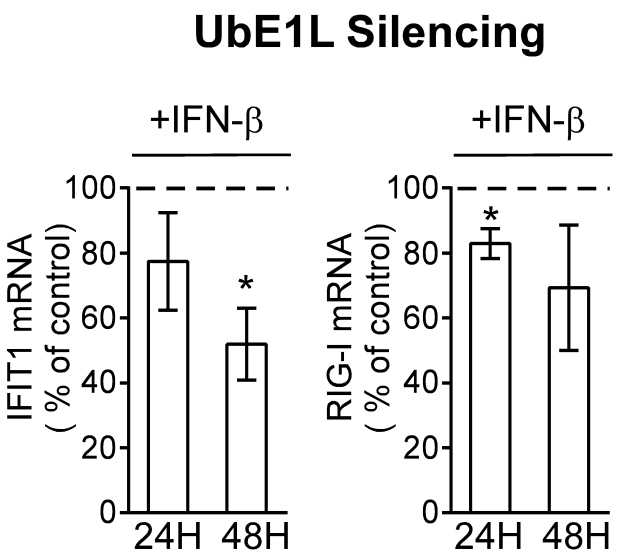

A.
B.
C.
Log10 (PFU/ml)
7
6
5
4
*
Control
ISG15
ISG15
G
P
F
N
x 3.8
x 1.5
x 6.9
x 2.1
x 4.0
x 2.8
x 2.9
x 1.3
1 2 1 2
1 2 1 2
1 2 1 2
1 2 1 2
1 2 1 2
β-actin
24H
48H
24H
48H
24H
48H
24H
48H
24H
48H
Nucleoprotein RNA
(% of control)
100
80
60
40
20
0
*
**
24H
48H


A.
WT
Log10 (PFU/ml)
7
6
5
4
*
*
Control
ISG15
ISG15-LRAA
B.
ISG15 -/-
Log10 (PFU/ml)
7
6
5
4
*
*
Control
ISG15
ISG15-LRAA
C.
+IFN-β
Control
UbE1L
Control
UbE1L
ISG15
conjugates
Free ISG15
X 19.8
X 5.6
Nucleoprotein
β-actin
24H
48H
D.
-IFN-β
+IFN-β
Log10 (PFU/ml)
7
6
5
4
*
Control
UbE1L
Control
UbE1L
E.
+IFN-β
Control
USP18
Control
USP18
ISG15
conjugates
Free ISG15
X 9.3
X 12.1
Nucleoprotein
β-actin
24H
48H
F.
-IFN-β
+IFN-β
Log10 (PFU/ml)
7
6
5
4
3
*
Control
USP18
Control
USP18

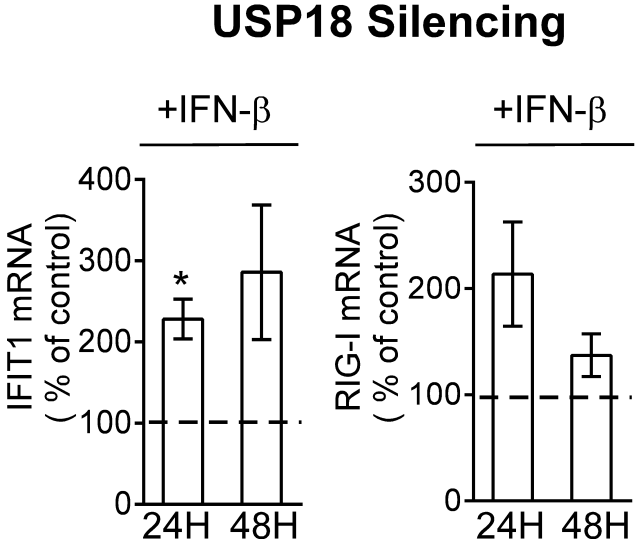
A.


ISG15 Silencing
+IFN-β
+IFN-β
IFIT1 mRNA (% of control)
RIG-I mRNA (% of control)
200
100
0
24H 48H

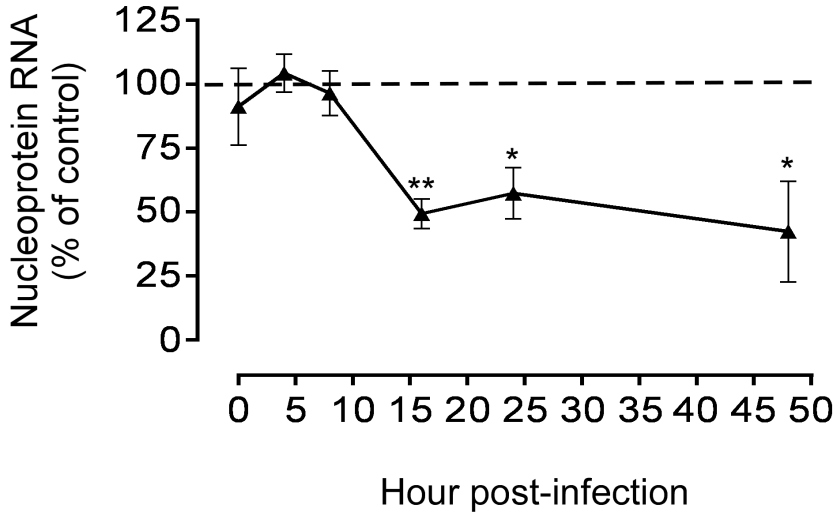
B.


UbE1L Silencing
+IFN-β
+IFN-β
IFIT1 mRNA (% of control)
RIG-I mRNA (% of control)
100
80
60
40
20
0
*
24H 48H

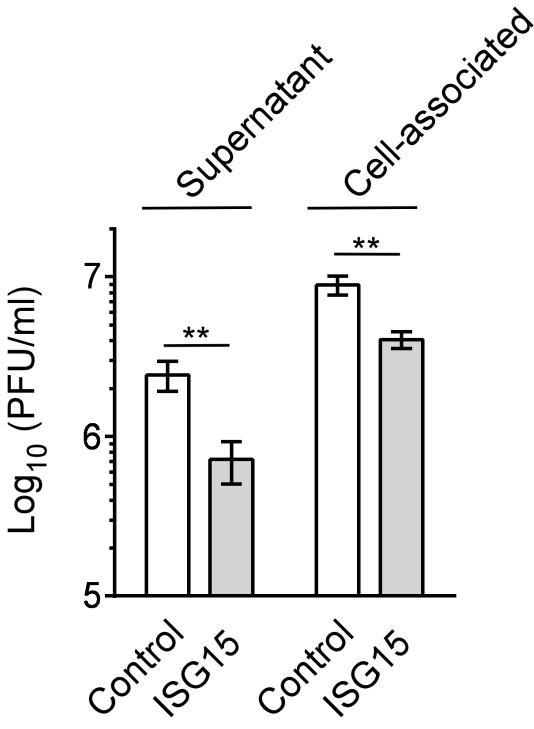
C.

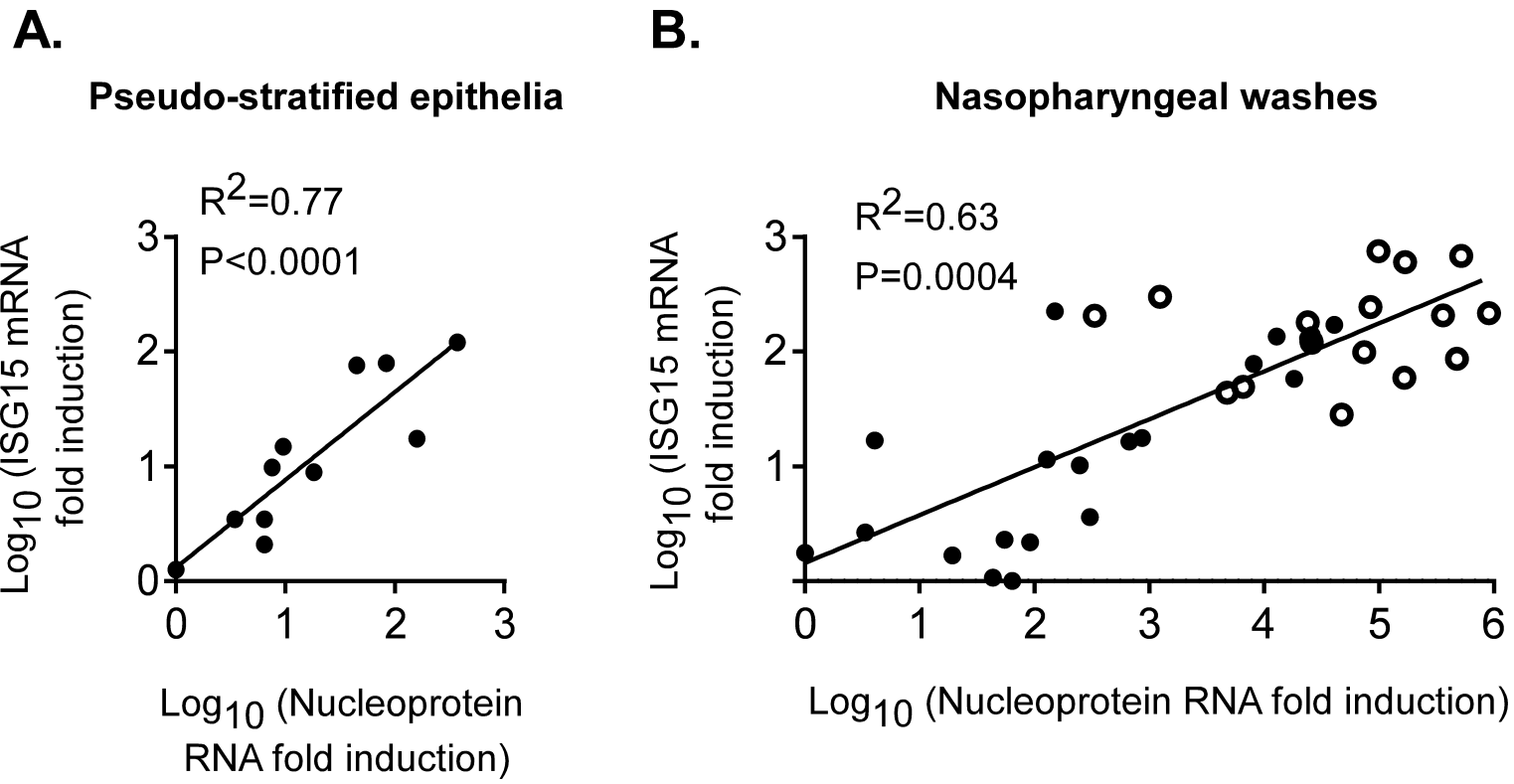

USP18 Silencing
+IFN-β
+IFN-β
IFIT1 mRNA (% of control)
RIG-I mRNA (% of control)
400
300
200
100
0
*
24H 48H

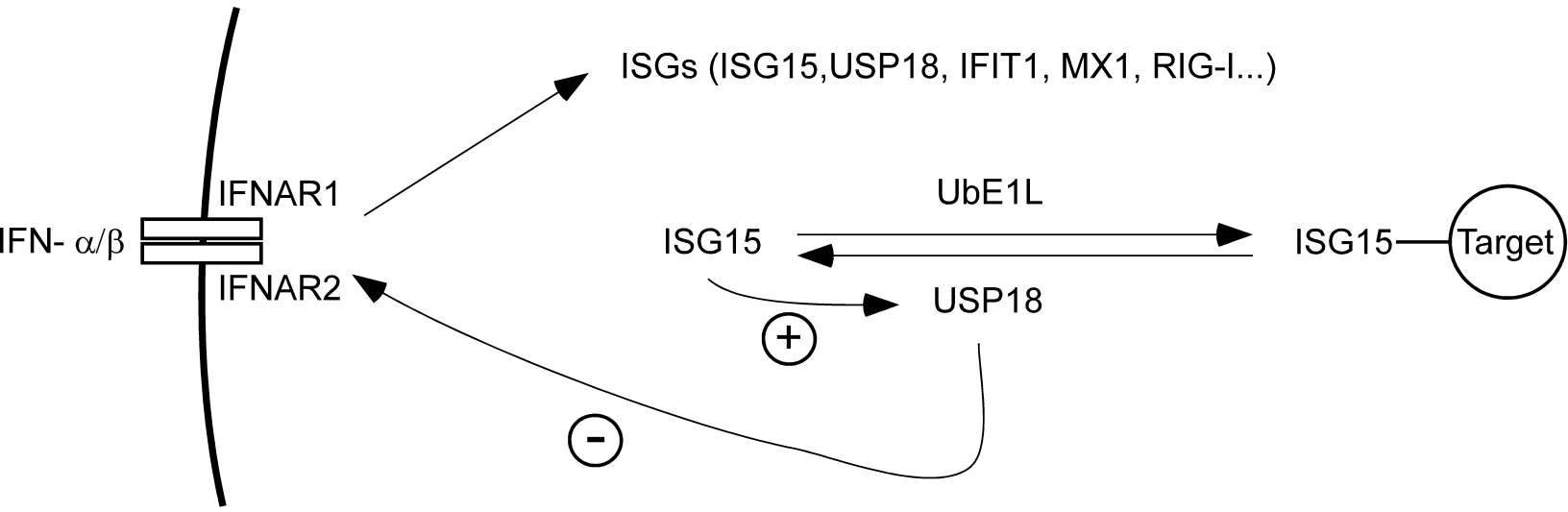
A.


Nucleoprotein RNA
(% of control)
125
100
75
50
25
0
**
*
*
0 5 10 15 20 25 30 35 40 45 50
Hour post-infection

B.


Supernatant
Cell-associated
**
**
Log10 (PFU/ml)
7
6
5
Control
ISG15
Control
ISG15


A.
Pseudo-stratified epithelia
R2=0.77
P<0.0001
Log10 (ISG15 mRNA fold induction)
Log10 (Nucleoprotein RNA fold induction)
0
1
2
3
B.
Nasopharyngeal washes
R2=0.63
P=0.0004
Log10 (ISG15 mRNA fold induction)
Log10 (Nucleoprotein RNA fold induction)
0
1
2
3
4
5
6


ISGs (ISG15,USP18, IFIT1, MX1, RIG-I...)
IFNAR1
IFN- α/β
IFNAR2
UbE1L
ISG15
ISG15
Target
USP18
+
-