

## Original Article

# Interferon-induced IFIT5 promotes epithelial-to-mesenchymal transition leading to renal cancer invasion

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**Abstract:** Interferon is known as a pleiotropic factor in innate immunity, cancer immunity and therapy. Despite an objective short-term response of interferon (IFN) therapy in renal cell carcinoma (RCC) patients, the potential adverse effect of IFN on RCC cells is not fully understood. In this study, we demonstrate that IFNs can enhance RCC invasion via a new mechanism of IFIT5-mediated tumor suppressor microRNA (miRNA) degradation resulted in the elevation of Slug and ZEB1 and epithelial-to-mesenchymal transition (EMT). Clinically, a significant upregulation of IFN $\gamma$  signaling pathway (such as IFNGR1, IFNGR2, STAT1 and STAT2) is observed in RCC patients with metastatic disease. Overall, this study provides a new mechanism of action of IFN-elicited canonical pathway in regulating suppressor miRNAs. Most importantly, it highlights the potential pro-metastatic effect of IFNs, which could undermine the clinical applicability of IFNs for treating RCC patients.

**Keywords:** Interferon (IFN), interferon-induced tetratricopeptide repeat 5 (IFIT5), epithelial-to-mesenchymal transition (EMT)

### Introduction

Renal cell carcinoma (RCC) is by far the most lethal urologic malignancy of cancer-specific mortality of 40% compared with 25% of overall mortality of prostate and bladder cancers because it is resistant to chemotherapeutics and radiotherapy [1]. Pathologically, RCC is a heterogeneous disease consisting three major types: clear cell RCC (ccRCC) [2], papillary RCC [3] and chromophobe RCC [4]. As for the therapeutic strategy for RCC patients, usually localized primary tumor can be managed successfully with radical nephrectomy. On the contrary, despite the prognosis of metastatic RCC (mRCC) patients has improved with targeted therapies, 20-25% of the patients are refractory to chemotherapy at the first response assessment and acquire drug resistance during the treatment [5].

Accumulating studies have demonstrated that tumor-associated immune cells play a critical role in cancer development. The presence of tumor-associated macrophage (TAM) is able to increase tumor cell proliferation and dissemination in different cancer types [6-12]. Action of these tumor-infiltrating lymphocytes (TILs) is mainly mediated through secretion of cytokines such as interleukins (IL-1, IL-6, IL-8 and IL-10), tumor necrosis factors (TNF), and interferons (IFNs). Many studies have shown that these cytokines could stimulate tumor cell proliferation, protect tumor cells from apoptosis, or promote angiogenesis and metastasis. Interleukin-6 (IL-6) is known to act as pro-tumorigenic cytokine by facilitating cell growth and anti-apoptosis in multiple myeloma [13, 14]. High concentration of interleukin-1 (IL-1) is associated with more malignant tumor phenotype [15-18]. Both IL-1 $\alpha$  and IL-1 $\beta$  are implied to aggra-

vate tumor angiogenesis and invasiveness via induction of vascular endothelial cell growth factor (VEGF) and tumor necrosis factor (TNF) [18]. On the other hand, the role of IFNs in cancer development remains controversial. For example, both IFN and IFN $\gamma$  can exhibit anti-tumor activities [19-25]. IFN $\gamma$  is also responsible for antigen-specific tumor immunity [26-29]. In contrast, IFN $\gamma$  was reported to facilitate lung metastasis in melanoma [30] and peritoneal dissemination of ovarian cancer [31]. In prostate cancer, our recent study also indicates that IFN $\gamma$  can promote epithelial-to-mesenchymal transition (EMT) via a new mechanism of IFIT5 in specific microRNA (miRNA) turnover [32].

Clinically, a significant elevation of several key effectors (such as IFN $\gamma$  receptor 1 and 2 [IFNGR1, IFNGR2], STAT1, STAT2) in IFN $\gamma$  signaling pathway is associated with metastatic RCC tumor [33]. Nevertheless, the role of IFNs in RCC development is not fully characterized. In this study, we demonstrate that IFN-elicited RCC invasion is mediated by IFIT5-XRN1 complex responsible for specific microRNA (such as miR-363) turnover; loss of miR-363 expression has been detected in RCC specimens [34]. Taken together, IFN is a potent tumor promoter that is mediated by a new mechanism of action of IFIT5 in degrading tumor suppressor miRNA.

### Materials and methods

#### Cell lines

ACHN, 7680 and 769P cell lines were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS). 293 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% FBS. Stable IFIT5-shRNA knockdown (shIFIT5) and control (shCon) RCC cell lines were established from ACHN, 7680 and 769P cell lines using pLKO.1-shCon or pLKO.1-shIFIT5 plasmids provided from Academia Sinica, Taiwan. All RCC cell lines were authenticated using the short tandem repeat (STR) profiling by Genomic Core in UT Southwestern Medical Center (UTSW). Mycoplasma testing was performed using MycoAlert® kit (Lonza Walkersville, Inc. Walkersville, MD) every quarterly to ensure Mycoplasma-free condition.

#### Invasion assay

RCC cells cultured in the serum-free RPMI-1640 medium for 18 hrs were plated onto the

upper chamber of Transwell (8- $\mu$ m (i.e., 8-micrometer) pore size, Corning) pre-coated with 2.5% Matrigel, and bottom chamber contained RPMI-1640 medium with 10% FBS. After 24 hrs, cells from the bottom side of chamber were fixed by 4% paraformaldehyde, stained with 0.4% Crystal Violet and observed under microscope (Keyence). The crystal-violet-stained cells from each field were quantified using BZ-X Analyzer software. Relative invaded cells were normalized to control group of each experiment. Each experiment was performed in triplicates.

#### Construction of SSMut and DSMut pre-miRNA-expression plasmid

miR-363 and miR-128 expression plasmid was initially purchased from Origene and GeneCopoeia, respectively. Native miR-363 or miR-128 expression plasmid was engineered to generate mutant pre-miR-363 or mutant miR-128 with 5'-end six nucleotides single stranded overhang (pre-SS<sup>6</sup>Mut-miR-363 or pre-SS<sup>6</sup>Mut-miR-128) or double-stranded blunt end (pre-DSMut-miR-363 or pre-DSMut-miR-128) constructs using QuickChange II site-directed mutagenesis kit (Agilent Technologies) and the mutated sequence was confirmed by DNA sequencing from Genomic Core in UTSW.

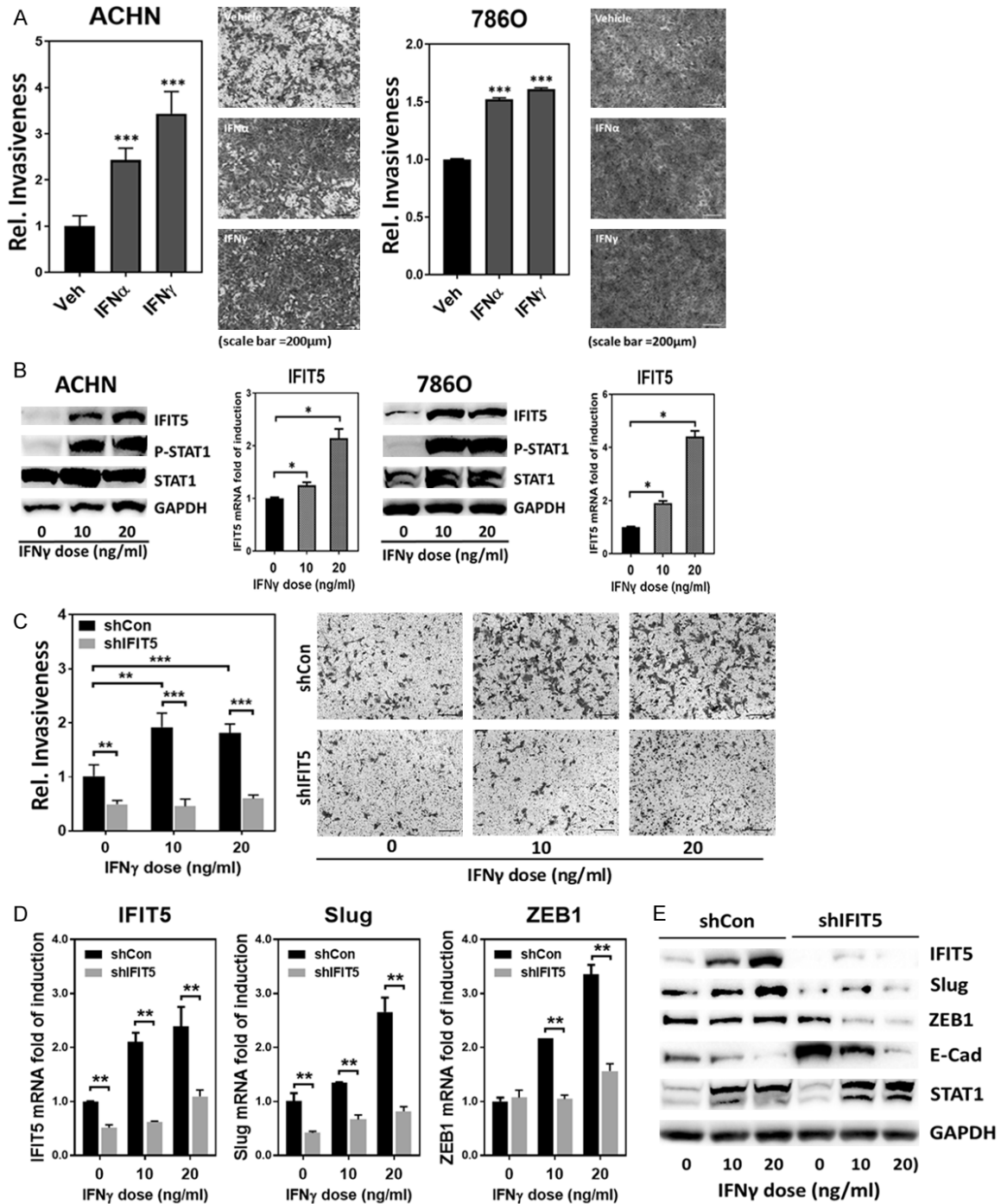
#### RNA purification

RCC cells were pelleted by centrifuge at 5000 rpm for 2 mins then snap-frozen in liquid nitrogen before RNA extraction. Chilled 1-Thioglycerol/Homogenization Solution (200  $\mu$ l) was added to re-suspend the pellet, followed by additional of 200  $\mu$ l Lysis Buffer with 15  $\mu$ l Proteinase K solution. Samples were vortexed and incubated at room temperature for 10 mins before loading into Maxwell® cartridge. RNA sample was purified in the Maxwell® RSC Instrument using the microRNA Tissue Kit and eluted in 40-60  $\mu$ l Nuclease-Free Water.

#### Quantitative real-time RT-PCR (qRT-PCR)

For the quantification of miRNA expression level, total RNA 2.5  $\mu$ g (i.e., 2.5 microgram) was subjected to miScript II RT kit (QIAGEN) then 2.0  $\mu$ l cDNA was applied to a 25- $\mu$ L reaction volume using miScript SYBR® Green PCR kit (QIAGEN) in CFX96 Touch Real-Time PCR detection system (BioRad). Primer assays for each miRNA species were purchased from QIAGEN.

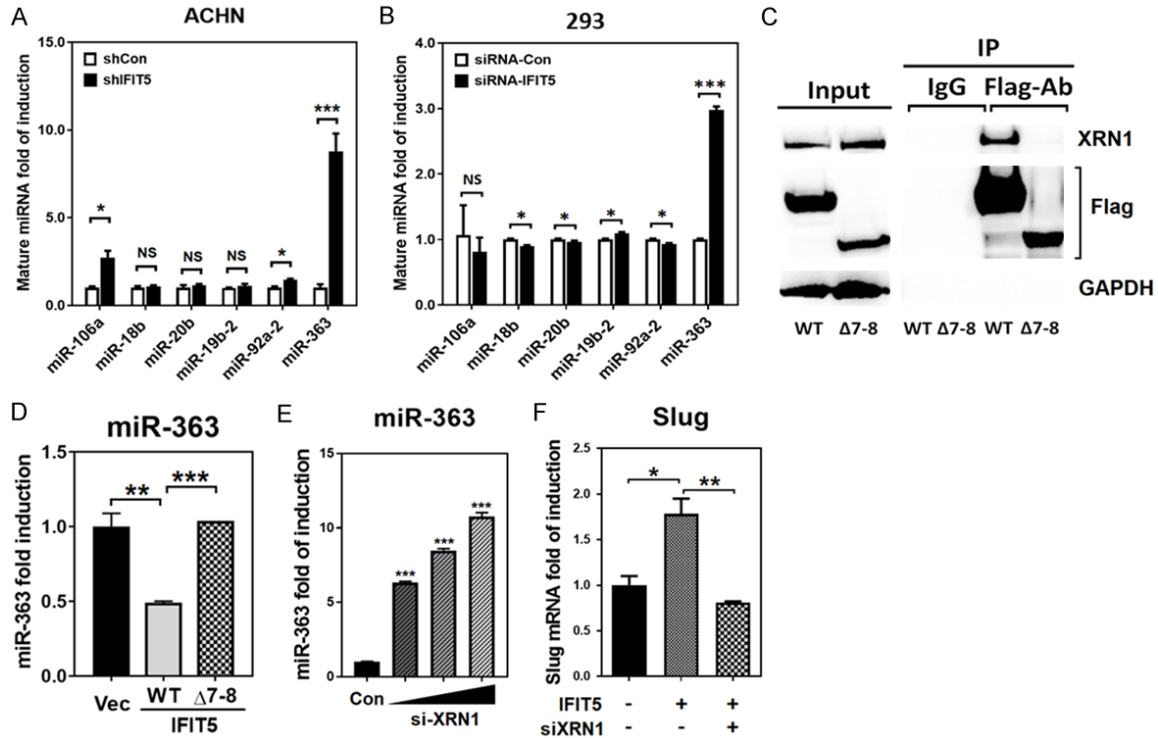
## The role of IFIT5 in RCC cell invasion



**Figure 1.** IFN $\gamma$  promotes renal cancer invasion via induction of IFIT5. **A.** Increased invasiveness of ACHN or 786O cells after IFN $\alpha$  or IFN $\gamma$  treatment (20 ng/ml) for 48 hrs, compared to vehicle control. (\*\*\* $P$ <0.00001). **B.** Dose-dependent elevation of IFIT5 protein and mRNA level in renal cancer cell lines (ACHN and 786O) treated with IFN $\gamma$  for 48 hrs, compared to vehicle control. (\* $P$ <0.05). **C.** The impact of IFIT5 loss (shIFIT5) on the IFN $\gamma$ -enhanced aggravation of invasiveness in ACHN cells, compared to shCon. (\*\* $P$ <0.001, \*\*\* $P$ <0.00001). **D.** The impact of IFIT5 loss (shIFIT5) on the IFN $\gamma$ -induced elevation of Slug and ZEB1 mRNA level in ACHN cells, compared to shCon (\* $P$ <0.001). **E.** The impact of IFIT5 loss (shIFIT5) on the IFN $\gamma$ -induced alteration of Slug, ZEB1 and E-Cadherin (E-Cad) protein level in ACHN cells, compared to shCon.

The relative expression levels of matured miRNAs from each sample were determined by nor-

malizing to SNORD95 small RNA. For the quantification of mRNA expression level, total RNA



**Figure 2.** Recruitment of XRN1 is required for the machinery of IFIT5-mediated miR-363 degradation. A, B. The impact of IFIT5 shRNA knockdown (shIFIT5) on the expression level of miRNAs derived from the miR-106a-363 cluster (miR-106a, miR-18b, miR-20b, miR-19b-2, miR-92a-2 and miR-363) in ACHN and 293T cells, compared to control shRNA (shCon). (\* $P < 0.05$ , \*\*\* $P < 0.00001$ ). C. Co-Immunoprecipitation using flag antibody to pulled down flag-tagged WT or mutant ( $\Delta 7-8$  TPR deletion) IFIT5 protein overexpressed in 293 cells, and immunoblotted with or and flag antibody. D. Expression level of miR-363 in cells overexpressed with WT or mutant ( $\Delta 7-8$  TPR deletion) IFIT5, compared to vector control (\*\* $P < 0.001$ , \*\*\* $P < 0.0001$ ). E. Dose-dependent expression level of miR-363 in IFIT5-positive 293 cells transfected with siRNA-knockdown of XRN1 (\*\*\* $P < 0.0001$ ). F. Expression level of Slug in IFIT5-overexpressed 7860 cells transfected with siRNA-XRN1, compared to vector control (\* $P < 0.05$ , \*\* $P < 0.001$ ).

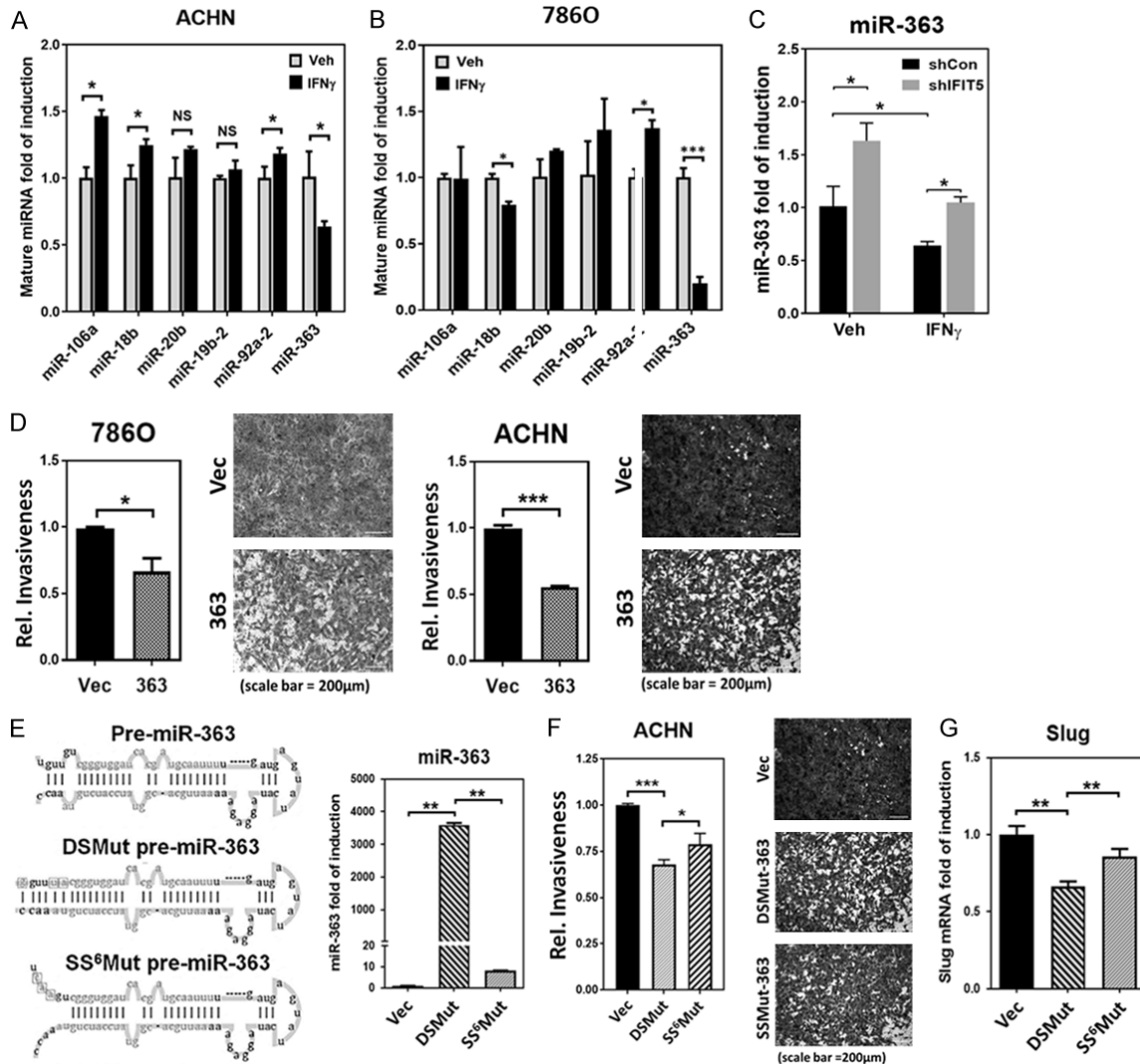
(2 g) was subjected to iScript advanced cDNA synthesis kit (BioRad) then 2.0  $\mu$ l cDNA was applied to 25- $\mu$ l qRT-PCR reaction volume using iTaq Universal SYBR® Green supermix (BioRad) in CFX384 Touch Real-Time PCR detection system (BioRad). The relative expression levels of IFIT5, Slug, ZEB1, E-cadherin, and Vimentin mRNA from each sample were determined by normalizing to 18S mRNA. All quantitative data were analyzed using  $\Delta$ Ct (Ct value normalized to internal SNORD95 miRNA or 18S RNA) and the fold change ( $\Delta\Delta$ Ct) was obtained after normalizing with the control group.

#### Western blot analysis

RCC Cells were lysed in lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Triton X-100, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM sodium pyrophosphate, 10 mg/mL, aprotinin, 10 mg/mL leupeptin, 2

mM phenylmethylsulfonyl fluoride, and 1 mM EDTA] for 30 mins on ice. Cell lysates were spin down at 20,000 $\times$ g for 20 mins at 4°C. Protein extracts were subjected to SDS-PAGE using Bolt 4-12% Bis-Tris Plus gel (Invitrogen), and transferred to nitrocellulose membrane using Trans-Blot Turbo Transfer system (BIORAD). Membranes were incubated with primary antibodies against IFIT5 (ProteinTech), Slug (Cell Signaling Technology), ZEB1 (Cell Signaling Technology), E-Cadherin (BD Transduction Laboratory), Vimentin (Sigma-Aldrich), STAT1 (Santa Cruz Biotechnology), GAPDH (Santa Cruz Biotechnology) or HRP-conjugated Flag (Sigma-Aldrich) antibodies at 4°C for 16-18 hrs, followed by incubation with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1.5 hrs. Results were visualized with ECL chemiluminescent detection system (Thermo Scientific Pierce) using AlphaImager instrument. The relative protein ex-

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**Figure 3.** IFIT5 regulates miR-363 turnover via recognition of precursor miRNA 5' end. A, B. The impact of IFN $\gamma$  on the expression level of miRNAs derived from the miR-106a-363 cluster (miR-106a, miR-18b, miR-20b, miR-19b-2, miR-92a-2 and miR-363) in ACHN and 7860 cells, compared to vehicle control (\* $P < 0.05$ , \*\*\* $P < 0.0001$ ). C. The impact of IFIT5 loss (shIFIT5) on the IFN $\gamma$ -induced downregulation of miR-363 level in ACHN cells. (\* $P < 0.05$ ). D. Attenuated cancer invasiveness by miR-363 overexpression in 7860 or ACHN cells, compared to vector control (\* $P < 0.05$ , \*\*\* $P < 0.00001$ ). E. Left: Mutation of nucleotides (box) for generating 5'-end 6 nucleotides single stranded pre-miR-363 (SS<sup>6</sup>Mut pre-miR-363) and blunt 5'-end double stranded pre-miR-363 (DSMut pre-miR-363). Both mature miR-363 and miR-363\* sequence are shown in lighter gray. Right: Expression level of mature miR-363 in 293 cells transfected with plasmids carrying mutant SS<sup>6</sup>Mut or DSMut pre-miR-363 sequence. (\*\* $P < 0.001$ ). F. The impact of mutant SS<sup>6</sup>Mut or DSMut pre-miR-363 on suppression of cell invasion in ACHN cells (\* $P < 0.05$ , \*\*\* $P < 0.00001$ ). G. Expression level of Slug mRNA in 293 cells transfected with plasmids carrying mutant SS<sup>6</sup>Mut or DSMut pre-miR-363 sequence. (\*\* $P < 0.001$ ).

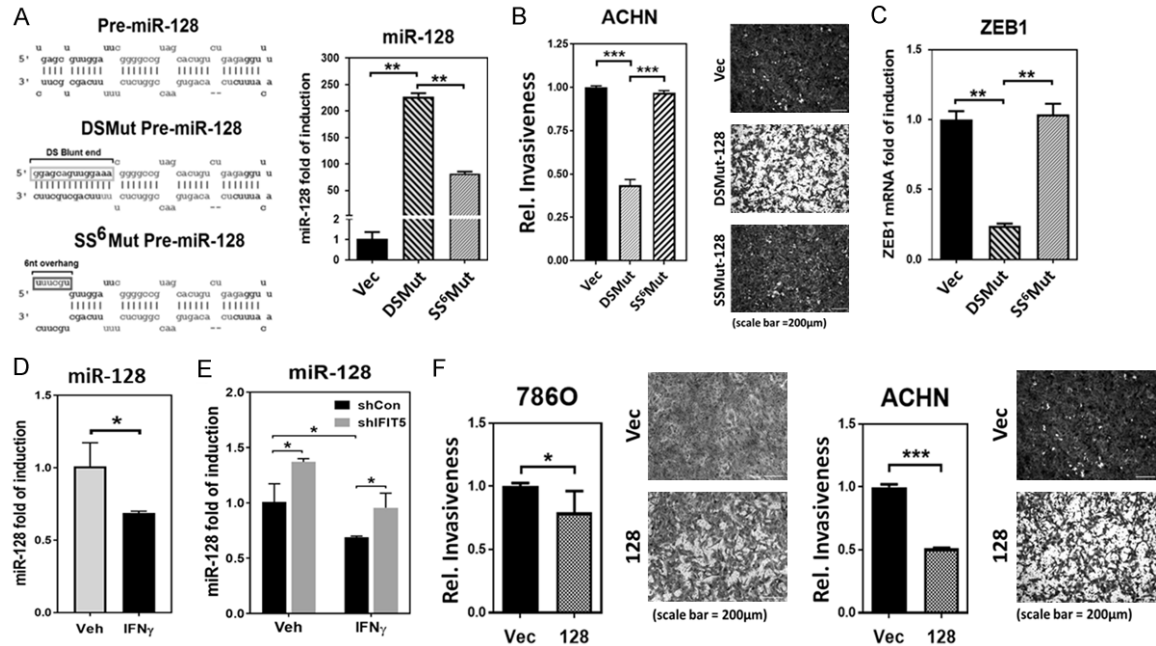
pression level in each sample was normalized by GAPDH levels.

### Luciferase reporter assay

Established stable clones of control (shCon) or STAT1-knockdown (shSTAT1) 7860 cells ( $8 \times 10^4$ ) were seeded onto 12-well plates at 75% conflu-

ence before transfection with IFIT5 promoter-luciferase reporter plasmid. Cells were harvested and lysed with Passive Lysis buffer (Promega) at 48 hrs after transfection. Luciferase activity was measured using the Firefly luciferase reporter assay (Promega) on the Veritas Microplate Luminometer (Turner Biosystems).

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**Figure 4.** IFIT5 regulates miR-128 turnover via recognition of 5'-end of precursor miRNA. A. Left: Mutation of nucleotides (box) for generating 5'-end 6 nucleotides single stranded pre-miR-128 (SS<sup>6</sup>Mut pre-miR-128) and blunt 5'-end double stranded pre-miR-128 (DSMut pre-miR-128). Both mature miR-128 and miR-128\* sequence are shown in lighter gray. Right: Expression level of mature in 293 cells transfected with plasmids carrying mutant SS<sup>6</sup>Mut or DSMut pre-miR-128 sequence. (\*\*P<0.001). B. The impact of mutant DSMut or SS<sup>6</sup>Mut pre-miR-128 on suppression of cell invasion in ACHN cells (\*\*\*P<0.00001). C. Expression level of ZEB1 mRNA in 293 cells transfected with plasmids carrying mutant SS<sup>6</sup>Mut or DSMut pre-miR-128 sequence. (\*\*P<0.001). D. The impact of IFN $\gamma$  and IFIT5 knockdown on the expression level of miR-128, compared to vehicle and control shRNA, respectively. (\*P<0.05). E. The impact of IFIT5 loss (shIFIT5) on the IFN $\gamma$ -induced downregulation of miR-128 level in ACHN cells. (\*P<0.05). F. Attenuated cancer invasiveness by miR-128 overexpression in 7860 or ACHN cells, compared to vector control (\*P<0.05, \*\*\*P<0.00001).

Relative luciferase activity was determined by normalizing with control. Each experiment was performed in triplicates.

### Statistics analysis

Statistics analyses were performed using GraphPad Prism software. Statistical significance was evaluated using Student t-test. *P* values of *P*<0.05, *P*<0.001 and *P*<0.00001 were considered significant difference between compared groups and marked with asterisks.

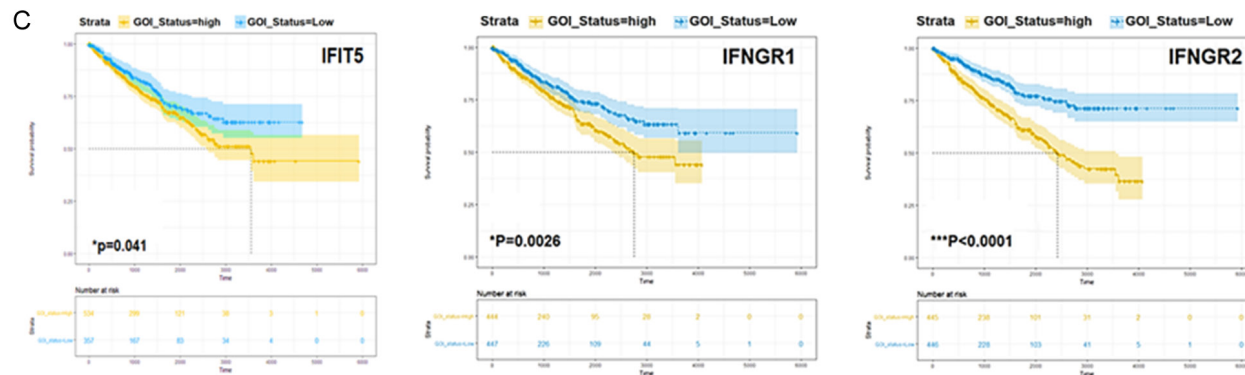
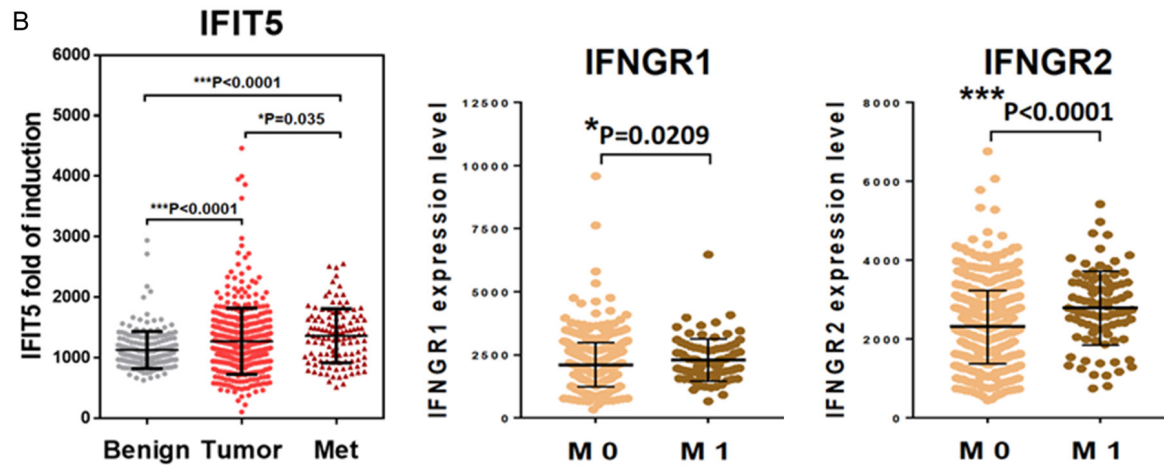
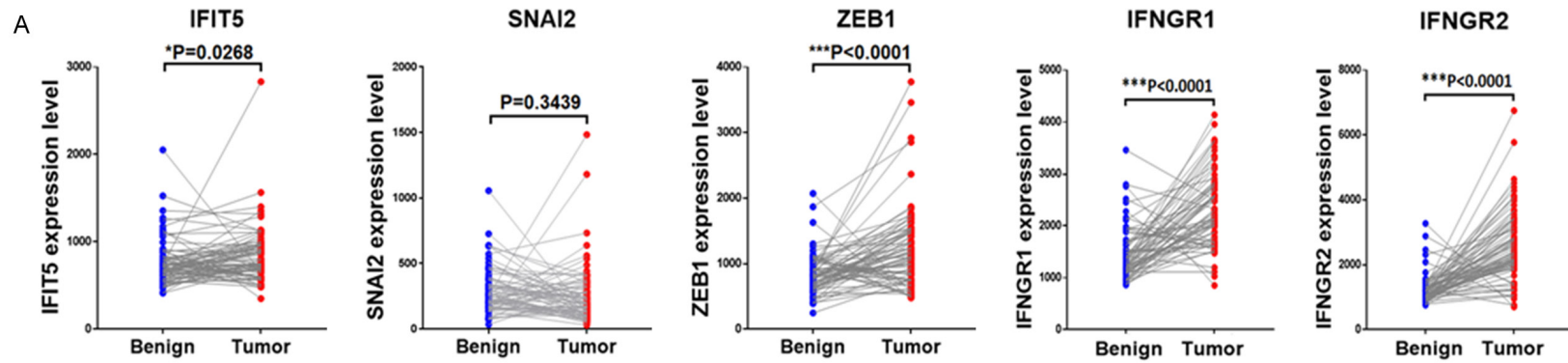
## Results

### IFN promotes RCC invasion via induction of IFIT5

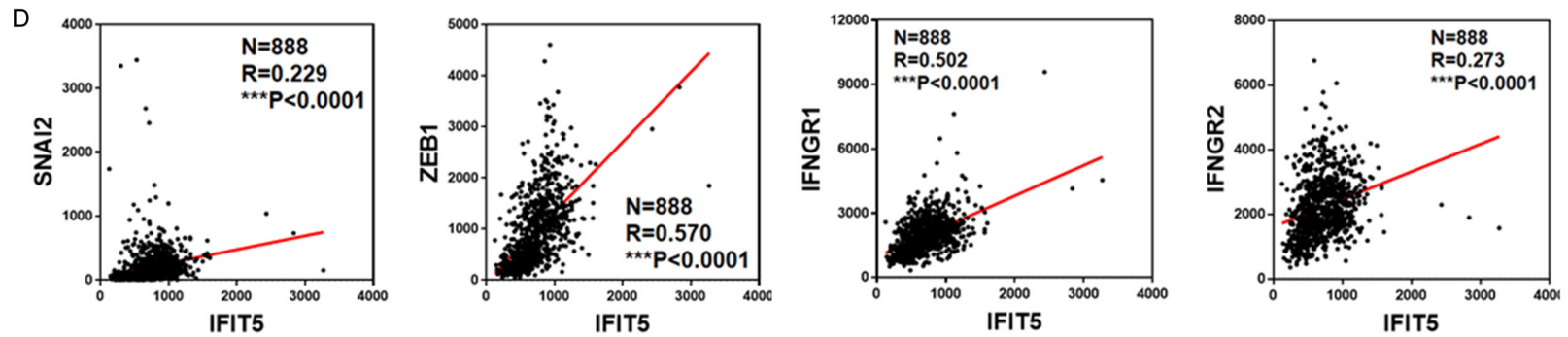
Prior to targeted therapy for RCC patients, IFN $\alpha$  has demonstrated a short-term efficacy as a single agent [35-39] and improved the overall survival by combining with other agents such as cyclooxygenase-2 inhibitor (celecoxib) [40],

interleukin-2 [41], capecitabine [42] or sora-fenib [43, 39, 44]. On the other hand, IFN $\gamma$  therapy resulted in minimal anti-tumor activity among mRCC patients [45, 46]. Thus, we decided to study potential adverse effect of IFN $\gamma$  and observed that either IFN $\alpha$  or IFN $\gamma$  was able to facilitate cell invasion of ACHN and 7860 cell lines using Transwell invasion assay (**Figure 1A**). Since IFN $\gamma$  appeared more potent than IFN $\alpha$ , we decided to focus IFN $\gamma$  to unveil its mechanism of action. Indeed, IFN $\gamma$  treatment is able to activate the canonical pathway of STAT1 phosphorylation to increase IFIT5 expression (**Figures 1B, S1A and S1B**), a bona fide IFN-induced gene [47, 48] that is capable of promoting EMT in prostate cancer [32]. To elucidate the role of IFIT5 in IFN $\gamma$ -induced RCC cell invasion, we knocked down IFIT5 in ACHN, 7860 and 769P cell lines and demonstrated that IFN $\gamma$ -induced cell invasion is diminished in IFIT5-knockdown (KD) cells (**Figures 1C, S1C and S1D**). Indeed, IFN $\gamma$  is able to increase both

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## The role of IFIT5 in RCC cell invasion



**Figure 5.** Machinery of IFIT5-mediated miRNA degradation is enhanced in the metastatic progression of RCC. A. Expression level of IFIT5, SNAI2/Slug, ZEB1, IFNGR1 and IFNGR2 in paired adjacent benign and tumor specimens derived from 75 RCC patients. B. Expression of IFIT5 in the patient-derived xenografts from normal benign (N=191), primary (N=294) and metastatic tumors (N=86) of RCC patients. Expression level of IFNGR1 and IFNGR2 genes in tumor specimens derived from RCC patients with non-metastasis (M0, N=550) and metastasis (M1, N=90) status. C. Kaplan-Meier overall survival curves estimate patients with RCC including types of clear cell RCC, papillary RCC and Chromophobe RCC, stratified in low and high expression of IFIT5, IFNGR1 or IFNGR2 genes. D. Clinical correlation between IFIT5 and SNAI2(Slug), ZEB1, IFNGR1 or IFNGR2 in TCGA dataset derived from 888 RCC patient tumor specimens.



Slug and ZEB1 gene expression leading to EMT in ACHN cells (**Figure 1D** and **1E**). However, in IFIT5-knockdown (KD) cells, IFN $\gamma$  failed to induce Slug and ZEB1 gene expression as well as EMT change based on E-Cadherin and Vimentin expression (**Figures 1D, 1E** and **S1E**). Taken together, these data support the notion that IFIT5 is the key mediator in IFN $\gamma$ -induced RCC invasion.

### *IFIT5 complex regulates miR-363 turnover*

IFIT5 has been characterized to function as a binding protein for various RNA species (such as viral RNA, tRNA) [47, 49, 50]. Our recent data [32] demonstrate that a new function of IFIT5 is to recruit XRN1 exoribonuclease to degrade miRNA by recognizing the unique 5'-structure of precursor miRNA. In addition, loss of miR-363 is detected in RCC specimens [34]. Thus, we further validated whether IFIT5 with a similar functional role could contribute the loss of miR-363 in RCC cells. Noticeably, miR-363 belongs to the miR-106a-363 cluster containing miR-106a, miR-18b, miR-20b, miR-19b, miR-92a-2 and miR-363 [51-54]. As shown in **Figure 2A** and **2B**, only mature miR-363 but no other miRNA species from this cluster was elevated in IFIT5-KD cells.

Since XRN1 is required for the activity of IFIT5 complex, we further determine the binding domain in IFIT5 for XRN1 and found 7-8 TPR domain ( $\Delta$ 7-8) as a key binding domain (**Figure 2C**). This truncated IFIT5 was incapable of degradation of miR-363 (**Figure 2D**). Furthermore, our data demonstrate that XRN1 is required for IFIT5-mediated miRNA degradation (**Figure 2E**). Consistently, the restoration of miR-363 level by siRNA-knockdown of XRN1 resulted in the suppression of Slug mRNA expression in 7860 cells (**Figure 2F**). Taken together, we believe that the presence of IFIT5-XRN1 contributes to the loss of miR-363 in RCC cells.

### *miR-363 functions as a potent suppressor in IFN $\gamma$ -induced cell invasion*

Knowing IFIT5 as a potent regulator for miR-363 turnover (**Figure 2A** and **2B**), we further demonstrated that IFN $\gamma$  exhibited the same specific degradation of miR-363 from miR-106a-363 cluster in both ACHN and 7860 cells (**Figure 3A** and **3B**). Also, IFN $\gamma$ -induced down-regulation of miR-363 can be reversed in IFIT5

KD cells (**Figure 3C**), supporting the mechanism of IFN $\gamma$  on miRNA turnover is mediated by IFIT5 complex.

By increasing miR-363 expression in either ACHN and 7860 cells, a significant reduction of IFN $\gamma$ -elicited cell invasion was observed (**Figure 3D**). Recently, we have demonstrated that IFIT5 preferentially bind to 5'-end single-stranded overhang sequence of precursor miRNAs [32]. Thus, the blunt-end (i.e., DSMut) of pre-miR-363 appears more resistant to IFIT5-mediated degradation than open-end (i.e., SS<sup>6</sup>Mut) of pre-miR-363 (**Figure 3E**). Functionally, the DSMut of pre-miR-363 is more potent in suppressing cell invasion of several RCC cells (**Figures 3F** and **S2A**) as well as Slug gene expression (**Figure 3G**). Overall, these data provide some understanding of induction of Slug by IFN $\gamma$  in RCC cells.

### *IFIT5 regulates the turnover of miR-128 that can target ZEB1*

In addition, ZEB1 induction was detected in IFN $\gamma$ -treated RCC cells (**Figure 1E**). We hypothesized that this regulation could be mediated by the same mechanism. Thus, by searching IFIT5-binding miRNA candidates, we identified pre-miR-128 with as similar single-stranded overhang structure at its 5'-end (**Figure 4A** left panel) and targeting ZEB1. By modify its native structure, the DSMut of pre-miR-128 became more resistant than the SS<sup>6</sup>Mut of pre-miR-128 (**Figure 4A** right panel). Similar to miR-363, the DSMut of pre-miR-128 is more potent in suppressing cell invasion of several RCC cells (**Figures 4B** and **S2B**) as well as ZEB1 gene expression (**Figure 4C**).

Consistent with the down-regulation of miR-363 by IFN $\gamma$  (**Figure 3A**), we also observed the reduction of miR-128 level in IFN $\gamma$ -treated ACHN cells (**Figure 4D**). Moreover, IFN $\gamma$ -induced down-regulation of miR-128 can be rescued in the IFIT5 KD cells (**Figure 4E**). On the other hand, by increasing miR-128 expression in either ACHN and 7860 cells, a significant reduction of IFN $\gamma$ -elicited cell invasion was observed (**Figure 4F**). Taken together, we believe that decreased miR-128 regulated by IFIT5 complex underlies IFN $\gamma$ -induced ZEB1 contributing to RCC cell invasion.

### *IFN $\gamma$ -signaling pathway is correlated with RCC progression*

To strengthen our observation from in vitro model, we further analyzed the IFN $\gamma$ -signaling pathway from 75 paired adjacent benign and tumor tissues of ccRCC patients and the results (**Figure 5A**) indicated a significant elevation of IFN $\gamma$  receptor 1 or 2 (IFNGR1, IFNGR2) and IFIT5 in tumor specimens. In addition, the expression levels of IFNGR1, IFNGR2, and IFIT5 in mRCC patients are significantly higher than primary tumor or benign tissue (**Figure 5B**). Moreover, patients with higher level of elevated IFNGR1, IFNGR2, and IFIT5 predicts poor overall survival of RCC patients based on the Kaplan-Meier curves analyses (**Figure 5C**). As expected, a positive correlation between IFIT5 and Slug, ZEB1, IFNGR1 or IFNGR2 expression level is also observed after analyzing TCGA RCC dataset (**Figure 5D**). Overall, these clinical data support the pro-tumorigenic role of IFN $\gamma$ -signaling axis in RCC progression.

### **Discussion**

The incidence of RCC has been steadily rising by 2-4% each year; a 5-fold increase in the incidence and a two-fold increase in mortality compared to 1971 [55]. RCC is by far the most lethal urologic malignancy of cancer-specific mortality of 40% compared with 25% of overall mortality of prostate and bladder cancers once it becomes metastatic. The median OS of untreated metastatic disease is 5 months with 1-year survival of only 29%. Although, a recent study elegantly demonstrated the predisposition of different genetic mutation in RCC patients to various organ metastases [56, 57], the influence of tumor microenvironment and its regulatory network are not fully characterized.

At the initial stage of cancer metastasis, tumor cells could undergo a phenotypic change from epithelial type to mesenchymal type (i.e., EMT), leading to loss of cell-cell adhesion, increased cell motility and invasion into vascular circulation [58]. Loss of E-Cadherin (epithelial marker) and gain of Vimentin (mesenchymal marker) is associated with RCC metastasis [59] and the presence of a sarcomatoid component is often found in metastatic lesion of RCC and associated with high mortality. Slug (Snail2), an EMT transcription repressor of E-Cadherin, has been demonstrated to induce EMT in RCC cell lines

[60-62]. Slug expression is substantially increased in high-grade ccRCC tissues and sarcomatoid carcinoma, which predicts the worse prognosis for patients with RCC [63]. Tumor surrounding microenvironment is known to play an important role in EMT [64-67] induction by producing several cytokines such as TNF- $\alpha$  [68], TGF- $\beta$  [69] and IL-10 [67].

In RCC, a number of cytokines have shown anti-tumor activity, the most consistent results have been reported with IFN $\alpha$  [70]. IL-2 and IFN $\gamma$  have also been given to patients with metastatic RCC with limited success [70]. Although the mechanism of action of these cytokines is poorly understood, anti-tumor effects in murine models have been linked to the direct killing of tumor cells by activated T cells and natural killer cells, as well as to anti-angiogenic effects [71-73]. However, in this study, we demonstrated the pro-tumorigenic role of IFN $\gamma$  in promoting RCC cancer invasion. IFN $\gamma$  signaling pathway is highly elevated in primary as well metastatic RCC and significantly correlated with OS RCC patients, suggesting the oncogenic role of IFN $\gamma$ -IFNGR1/IFNGR2-IFIT5 signal axis in the progression of RCC. Noticeably, this study unveils a new mechanism of action of IFIT5 in regulating turnover of tumor suppressor miRNAs (miR-363 and miR-128), leading to the upregulation of EMT drivers (Slug and ZEB1). Moreover, IFN $\gamma$  promoting cancer metastasis is not only limited to RCC; it has been recently reported in other malignancies such as prostate [32] and gastric cancer [74]. In summary, we demonstrate an adverse effect of IFN $\gamma$  on RCC progression via a unique regulation of miRNA turnover. Therefore, targeting IFN $\gamma$  signaling pathway using inhibitory RNA strategy may provide a new avenue for the clinical management of RCC progression.

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### **Disclosure of conflict of interest**

None.

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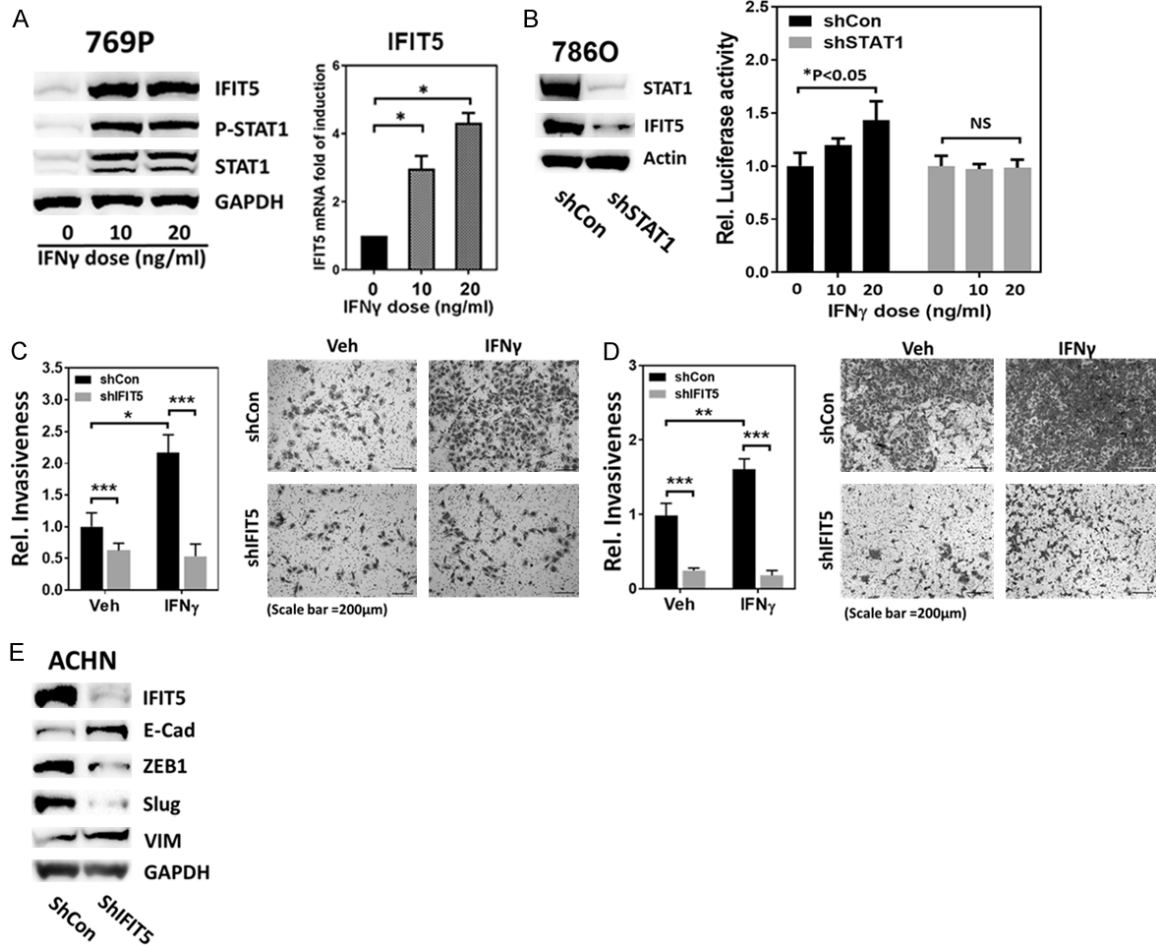
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**Figure S1.** A. Dose-dependent elevation of IFIT5 protein and mRNA level in 769P cells treated with IFN $\gamma$  for 48 hrs, compared to vehicle control. (\*P < 0.05). B. The impact of STAT1 shRNA knockdown (shSTAT1) on IFIT5 promoter activity using luciferase reporter assay. C. The impact of IFIT5 loss (shIFIT5) on the IFN $\gamma$ -enhanced aggravation of invasiveness in 786O cells, compared to shCon. (\*P < 0.05, \*\*\*P < 0.00001). D. The impact of IFIT5 loss (shIFIT5) on the IFN $\gamma$ -enhanced aggravation of invasiveness in 769P cells, compared to shCon. (\*\*P < 0.001, \*\*\*P < 0.00001). E. The impact of IFIT5 shRNA knockdown (shIFIT5) on the protein expression level of E-Cad, ZEB1, Slug and Vimentin (VIM) in ACHN cells, compared to control shRNA (shCon).



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