Spring 3-17-2023

Influence of Canonical and Non-Canonical IFNLR1 Isoform Expression on Interferon Lambda Signaling

John Evans
Medical University of South Carolina

Influence of Canonical and Non-Canonical IFNLR1 Isoform Expression on Interferon Signaling

J. Grayson Evans

A dissertation submitted to the faculty of the Medical University of South Carolina in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Graduate Studies

Department of Molecular and Cell Biology

Approved by:

_____ (Chair)

Dr. Eric Meissner

_____ (Co-Chair)

Dr. Stephen Duncan

_____

Dr. Betty Tsao

_____

Dr. Scott Eblen

_____

Dr. Melissa Cunningham

_____

Dr. Robin Muise-Helmericks

TABLE OF CONTENTS

LIST OF FIGURES

Supplemental Figure S2: Endogenous IL10RB and IFNLR1 isoform expression in HEK293T stable lines.

Supplemental Figure S3: Representative flow cytometry plots used for quantification in Figures 3, 4, 6, and 9.

Supplemental Figure S4: Effect of pre-incubation with anti-IFNLR1 antibody on the IFNL3 response in FLAG-Iso1 and FLAG-Iso2 lines.

Supplemental Figure S5: Dox-titratable FLAG-Isoform 2 expression in independent HEK293T stable lines.

Supplemental Figure S6: Differentially expressed genes among HEK293T cells line.

Supplemental Figure S7: *IFNAR1* and *IFNAR2* RNA expression levels are unchanged among cell lines and conditions.

Supplemental Figure S8: Western blot analysis of pSTAT1 in HEK293T stable lines.

LIST OF ABBREVIATIONS

Cas9 – CRISPR Associated Protein 9

CRISPR – Clustered Regularly Interspaced Short Palindromic Repeats

GAS – Gamma Activated Sequence

HA – Hemagglutinin

HBV – Hepatitis B Virus

HCV – Hepatitis C Virus

HIV – Human Immunodeficiency Virus

IAV – Influenza A Virus

IFN – Interferon

IFNA – Interferon Alpha

IFNAR1 – Interferon Alpha Receptor 1

IFNAR2 – Interferon Alpha Receptor 2

IFNB – Interferon Beta

IFNL – Interferon Lambda (also known as type-III interferon)

IFNLR1 – Interferon Lambda Receptor 1

IL – Interleukin

IL10RB — Interleukin 10 Receptor Beta (also known as IL10R2)

iPSC – Induced Pluripotent Stem Cell

IRF1 – Interferon Regulatory Factor 1

IRF9 – Interferon Regulatory Factor 9

ISG – Interferon Stimulated Gene

ISGF3 –Interferon Stimulated Gene Factor 3

ISRE – Interferon Stimulated Response Element

JAK – Just Another Kinase or Janus Associated Kinase

KD – Knock Down

KO – Knock Out

PAMP – Pathogen Associated Molecular Pattern

PBMC – Peripheral Blood Mononuclear Cell

PBS – Phosphate Buffered Saline

PRR – Pattern Recognition Receptor

siRNA – Small Interfering RNA

SOCS — Suppressor of Cytokine Signaling

STAT1/STAT2 – Signal transducer and activator of transcription 1 and 2

TLR – Toll Like Receptor

USP18 — Ubiquitin Specific Peptidase

WNV – West Nile Virus

YFV — Yellow Fever Virus

ABSTRACT

Interferon lambdas (IFNLs) are innate immune cytokines that induce antiviral cellular responses by signaling through a heterodimer composed of IL10RB and the interferon lambda receptor 1 (IFNLR1). Multiple IFNLR1 transcriptional variants are expressed *in vivo* and are predicted to encode distinct protein isoforms whose function is not fully established. IFNLR1 isoform 1 has the highest relative transcriptional expression and encodes the full-length functional form that supports canonical IFNL signaling. IFNLR1 isoforms 2 and 3 have lower relative expression and are predicted to encode signaling defective proteins. To gain insight into IFNLR1 function and regulation, we explored how altering relative expression of IFNLR1 isoforms influenced the cellular response to IFNLs. To achieve this, we generated and functionally characterized stable HEK293T clones expressing doxycycline-inducible FLAG-tagged IFNLR1 isoforms. Minimal FLAG-IFNLR1 isoform 1 overexpression markedly increased IFNL3-dependent expression of antiviral and pro-inflammatory genes, a phenotype that could not be further augmented by expressing higher levels of FLAG-IFNLR1 isoform 1. Expression of low levels of FLAG-IFNLR1 isoform 2 led to partial induction of antiviral genes, but not pro-inflammatory genes, after IFNL3 treatment, a phenotype that was largely abrogated at higher FLAG-IFNLR1 isoform 2 expression levels. Expression of FLAG-IFNLR1 isoform 3 partially augmented antiviral gene expression after IFNL3 treatment. In addition, FLAG-IFNLR1 isoform 1 significantly reduced cellular sensitivity to the type-I IFN IFNA2 when overexpressed. These results identify a unique influence of canonical and non-canonical IFNLR1 isoforms on mediating the cellular response to interferons and provide insight into possible pathway regulation *in vivo*.

CHAPTER 1: INTRODUCTION

The Innate Immune Response

The human immune system protects the host against the multitude of pathogenic threats present within the environment [1]. The first line of host defense is comprised of physical and anatomical barriers, including the skin and mucosal membrane-lined gastrointestinal, respiratory, and reproductive tracts [2]. While each barrier has unique physiological properties, they share common features that are essential towards preventing pathogenic entry, including organization of epithelial cells into layers linked together through tight junctions to hinder pathogenic entry, regulation of temperature and pH to promote an inhospitable environment, and production of anti-microbial peptides (lysozyme, defensins, cathelicidin, etc.) to actively neutralize pathogenic threats [1, 2]. Despite these robust physical and anatomical barriers, whether through traumatic injury or underlying pathology, pathogenic infections do occur. Once a pathogen overcomes barriers and establishes infection, the innate immune system initiates concurrent biological processes to hinder spread, clear infection, and engage the adaptive immune response [3].

Infected cells and tissues have the capacity to detect and recognize a broad range of pathogenic features in order to promote a robust innate immune response [4]. Cells constitutively express specialized proteins known as pattern recognition receptors (PRRs) which have evolved over time to recognize common pathogenic features, referred to as pathogen associated molecular patterns (PAMPs) [4]. PRRs can be categorized based on the chemical composition of PAMP recognized (carbohydrate, lipid, or nucleic acid) and location of recognition (extracellular vs. intracellular vs. organelle specific), which, in turn, influences the type of pathogen recognized [5]. For example, some PRRs (e.g. TLR3, RIGI) are specific for detection of elements produced by viral pathogens [6].

Once a PRR is activated, a response is propagated through a series of signaling cascades that culminates in a cornerstone of the innate immune response, inflammation [7]. A classic manifestation of the observable effects of inflammation in the skin upon injury or infection include prominent vasodilation (redness, *rubor*), localized edema (swelling, *tumor*), increased temperature (warmth, *calor*) and pain (*dolor)* [8]. These macroscopic findings are mediated through synthesis and release of immunologically active proteins known as cytokines [9, 10]. In addition to serving pro-inflammatory roles, additional cytokines directly promote anti-pathogenic mechanisms and interfere with pathogen replication within infected cells. One family of cytokines, known as interferons (IFNs), have been the intense focus of study since their discovery [11]. There are three major classes of IFNs: type-I (alpha/beta), type-II (gamma), and type-III (lambda) [12]. Type-I IFNs and type-III IFNs (henceforth referred to as interferon lambdas, IFNLs) are the primary IFNs induced during the initial innate immune response to viral infection [13].

Principle of Viral Interference and Discovery of "The Interferon"

By the end of the 1800s and early 1900s, the fields of microbiology and immunology and their applicability to public health was indisputable. Along with a growing understanding of the microbiological world came a deep appreciation for the role of the host immune response and how it could be modulated or augmented to prevent or neutralize infectious diseases. Laboratories from around the world turned their focus on how cells and tissues defend themselves against the seemingly infinite number of pathogenic threats.

Several investigators reported observing an interesting phenomenon characterized by acquired cellular resistance to viral infection when preceded by earlier viral infection, later termed the principle of viral interference [14]. The history of these investigations is described by

Dr. M. R. Hilleman in a review published in 1963 [15], noting that one of the earliest insights into the principle of viral interference was made in 1935, when Hoskins et al. demonstrated that concurrent exposure of monkeys to attenuated and live yellow fever virus led to increased survival [16]. From these findings, the logical hypothesis at the time was that co-injection of the attenuated virus resulted in production of beneficial "immune bodies." This hypothesis was tested and ruled out by Findlay and MacCallum in 1937, who showed that the effect of viral interference was still observed when two antigenically unrelated viruses were used [17]. Lennette and Koprowski showed in 1946 that fluid derived from chicken embryo tissue infected with a strain of the yellow fever virus was able to inhibit infection of fresh tissue with West Nile virus [18].

The seminal studies that firmly established the phenotype of viral interference, and first proposed the term “interferon,” were published in a series of papers beginning in 1957 by Isaacs and Lindenmann [19] and Isaacs, Lindenmann, and Valentine [20]. In the first paper, the authors demonstrated that incubation of chorioallantoic membrane derived from chicken embryos with a heat or ultra violet (UV) light neutralized influenza A virus reduced the tissue’s capacity to serve as a host to a test virus [19]. These data suggested that either 1) a byproduct of the neutralized virus hindered replication of the test virus, or, that 2) the tissue itself underwent changes that increased its resiliency to infection. In either case, the term “interferon” was adopted to describe the theoretical factor at play.

In their second paper, the authors set out to describe the properties of the interferon [20]. They did so through a series of biochemical experiments using crude isolation of interferon from the supernatant of virus-infected chicken embryo membranes. First, they demonstrated a dose-response relationship between the amount of interferon used and its antiviral effect. Second, they showed that interferon could be inactivated at 60 degrees Celsius. Critically, this

heat susceptibility contrasted with the heat stability of ribonuclease, an enzyme that had previously been shown to exhibit viral interference properties and was a leading candidate for the true identity of interferon. Further, viral interference was lost when the crude isolates were incubated with proteinases. These data suggested the interferon particle was a protein. In addition to these biochemical properties, the authors provide evidence that the effect of interferon occurs through the cellular membrane, as fresh embryo tissue treated with crude interferon preparations and thoroughly washed was still resistant to challenge with a novel virus [20].

Purification of interferon from crude isolates would be achieved a few years later in Dr. Hilleman's laboratory and by others [21, 22]. Subsequently, multiple experimental strategies were employed to identify the gene encoding the interferon, which led to the discovery of genes encoding distinct interferon proteins, ultimately leading to the establishment of the type-I IFN class of proteins, comprised primarily of interferon alpha (IFNA) and beta (IFNB) ligands [23, 24].

Mechanisms Underlying Viral Interference: The JAK-STAT Pathway

Through investigating the means by which type-I IFNs produce antiviral interference, fundamental discoveries were made in the understanding of cytokine receptor biology, signal transduction and intracellular signaling, and ultimately, the means by which an extracellular signal can affect cellular transcriptional programming.

Multiple laboratories observed that IFN-treated cells upregulate novel protein synthesis [25]. These genes induced by IFN treatment were classified as interferon stimulated genes (ISGs) [26, 27]. In a simple yet insightful study, Dr. Joyce Taylor-Papadimitriou observed that administration of transcriptional inhibitor actinomycin D prior to treatment with type-I IFNs

abolished the antiviral effect, suggesting that IFNs require *de novo* expression of ISGs [28]. However, the mechanisms by which IFN induced these antiviral genes was unknown.

Several features of IFN-induced gene expression led researchers to hypothesize that a novel signaling pathway was at play. For instance, the changes in gene expression and protein synthesis stimulated by treatment with type-I IFNs occurred very quickly and did not rely on *de novo* protein synthesis [26], IFN was still able to induce an antiviral state when denied entry into the cell [29], and did not require cyclic-adenosine monophosphate (cAMP) as a second messenger [30].

Dr. J. E. Darnell Jr.'s group hypothesized that IFNs induced ISGs through a common promoter sequence [31]. His group went on to clone a gene reliably induced by IFN treatment, *ISG54K*, and identified a region within its promoter sequence that, when taken away, abolished its IFN-responsiveness [32]. This sequence was further characterized and termed interferon-stimulated response element (ISRE) [33].

The next step in understanding this novel gene expression pathway was to identify the protein and/or protein complex responsible for binding to the ISRE to promote gene expression. This was demonstrated in a series of studies using gel-shift assays, whereupon labeled ISRE sequences were incubated with cellular lysates from both non-treated and IFN-treated Hela cells [34, 35]. A notable shift was observed in ISRE migration upon incubation with IFN-treated Hela cells, suggesting that IFN led to protein association with the ISRE sequence. This protein complex, termed interferon-stimulated factor 3 (ISGF3), was later purified and separated into its component parts, revealing three distinct proteins of varying molecular weights [36]. Through biochemical analyses, the identities of each protein were characterized as: interferon regulatory factor 9 (IRF9), signal transducer and activator of transcription 1 (STAT1), and signal transducer

and activator of transcription 2 (STAT2) [37]. Furthermore, it was firmly established that activation of STAT proteins relied on a phosphorylation event occurring on tyrosine 701 (Y701) [38].

The discovery of STAT molecules was a major advancement in not only IFN research, but also for receptor biology and cell signaling as a whole. However, the mechanism(s) by which IFNs induced the phosphorylation event necessary to activate STAT1/STAT2 was still unknown. This question was answered by Velazquez et al. through a process known as back-selection [39]. Briefly, the investigators derived an IFN-unresponsive mutant cell line (2fTGH 11.1) containing an IFN-driven gene (guanine phosphoribosyl transferase, *GPT)* conferring resistance to growth in media supplemented with hypoxanthine-aminopterin-thymidine (HAT). They transfected whole wild-type (WT) genomes into the mutant cells and selected for surviving cell colonies in HAT media after continuous treatment with IFN. The resulting clones were then characterized to identify the gene that complemented the IFN-dependent phenotype, identifying *TYK2*, a previously reported tyrosine kinase [40]. Further investigation would identify multiple tyrosine kinases, later termed as JAK molecules, which, depending on the source, stands for “Just another kinase” [31].

Structure and Function of the Type-I Interferon Receptor

With the identification of the fundamental mechanisms by which this novel signaling pathway functions, it was then necessary to identify the means by which an extracellular ligand could initiate phosphorylation of STAT molecules, formation of ISGF3, and expression of ISGs through activation of ISRE promoter regions.

In its initial discovery, Isaac and Lindenmann predicted that the interferon worked through the cell membrane [19, 20]. However, it took several additional years of research to

identify the receptor through which type-I IFNs exert their cellular effects. Initial studies identified that type-I IFNs acted through a membrane receptor, distinct from the type-II IFN class [41]. In a landmark advancement, researchers were able to clone the human type-I IFN receptor and show that transfection into mouse cells enhanced their sensitivity to human type-I IFNs [42]. Two distinct protein subunits were subsequently identified as essential to type-I IFN signal transduction and were named interferon alpha receptor 1 (IFNAR1) and interferon alpha receptor 2 (IFNAR2) [43].

IFNAR1 and IFNAR2 were demonstrated to be expressed in almost all human cell types [44]. Several models suggested that type-I IFN ligands first bind to the high-affinity IFNAR2 subunit [45], followed by the recruitment of the IFNAR1 subunit to form a heterodimer complex [46]. By itself, the type-I ligand-receptor complex is incapable of signal transduction, as it has no intrinsic enzyme activity; instead, it serves as a messenger or "cellular bridge," connecting the events of the extracellular space to intracellular machinery, comprised of JAKs [37]. There are four members of the JAK family: JAK1, JAK2, JAK3, and TYK2, which differ based on their affinity and association to different cytokine receptors [47, 48]. IFNAR1 utilizes TYK2, while IFNAR2 has been demonstrated to transduce signal through JAK1 [49]. Binding of the IFNAR1/IFNAR2 subunits to its ligand results in deactivation of the JAK inhibitory domain, allowing the kinase domain to function. The JAK proteins associated with each subunit cross-phosphorylate each other, and subsequently phosphorylate their own membrane receptors. The phosphorylated tyrosine residues on IFNAR1/IFNAR2 can then bind to proteins with SH2 (Src homology 2) domains. As described previously, activated IFNAR1/IFNAR2 recruit members of the STAT family of proteins, specifically, STAT1 and STAT2 [50]. Once bound, tyrosine residues within STAT1 and STAT2 are phosphorylated by JAKs, causing a conformational change, prompting them to dissociate from the receptor. The recently freed SH2 domain of each STAT

molecule can bind to the exposed phosphor-tyrosine on another, allowing them to dimerize in the cytosol. The STAT1:STAT2 heterodimer may associate with interferon regulatory factor 9 [51], forming the transcription factor complex ISGF3 [36, 52]. This complex translocates into the nucleus, where it binds ISREs and promotes expression of ISGs (**Figure 1.1**) [53, 54].

A more comprehensive understanding of IFNAR1/IFNAR2 signaling was gained through identification of how cytokine receptors control association with JAK proteins and regulate their activity. The relationship between receptor subunit and its JAK is largely controlled by two unique amino acid motifs present in the intracellular domain, known as Box1 and Box2 [49]. Box1 and Box2 do not serve universal functions on all receptors and require independent analysis for each specific cytokine receptor that harbors these domains. Targeted receptor mutations in Box1 and Box2 of IFNAR1 showed these domains influence Tyk2's ability to bind IFNAR1 and to cross-phosphorylate JAK1 [55]. Specifically, loss of Box1 in IFNAR1 led to a reduction of phosphorylated JAK1 of receptor-JAK interaction but did not affect TYK2 binding. Loss of Box2 in IFNAR1, however, abrogated TYK2 binding. In contrast, both Box1 and Box2 of IFNAR2 are important for JAK1 binding and activity [56]. Notably, mutation of Box1 and Box2 on either IFNAR1 or IFNAR2 resulted in direct changes in receptor phosphorylation, STAT activation, and downstream gene expression [55]. Consequently, the biochemical interactions between cytokine receptor and its associated JAK are significant to evaluate when attempting to understand the complexities of interferon signaling.

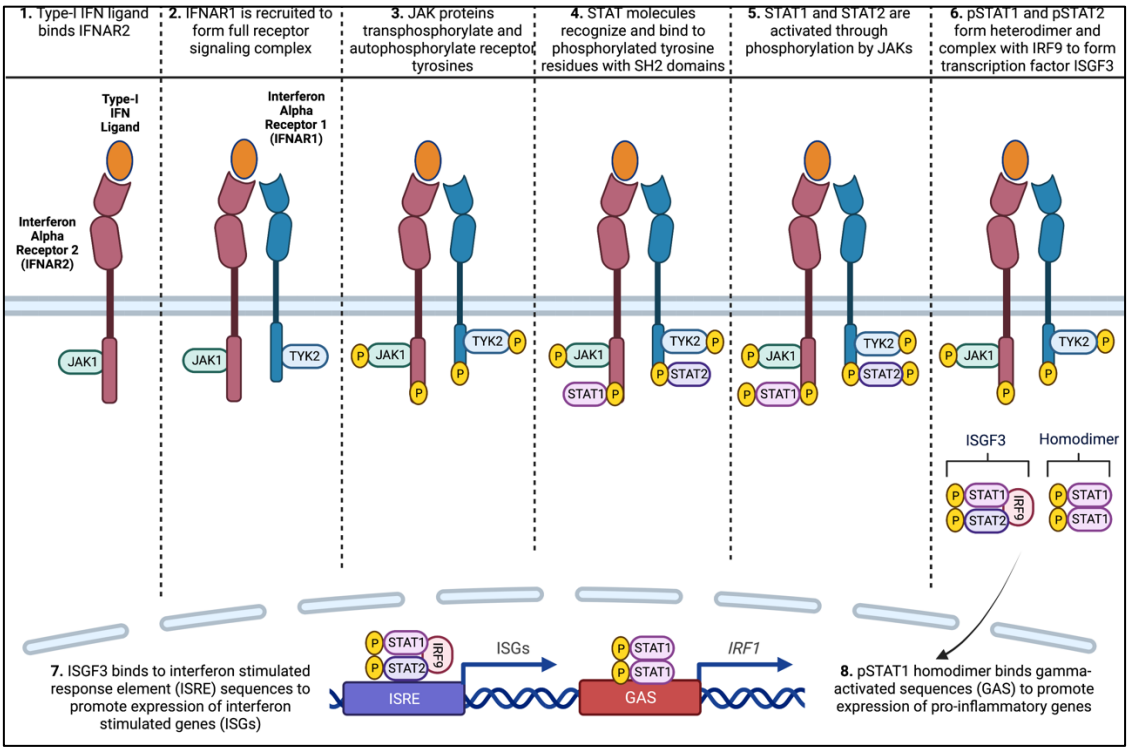


Figure 1.1: Type-I Interferon Signaling. 1) The high affinity subunit IFNAR2 is first to bind type-I IFN ligand. **2)** Upon ligand binding, the lower affinity subunit IFNAR1 is recruited to form the full receptor signaling complex. **3)** Each subunit's respective JAK protein trans-phosphorylates the other, leading to further activation of kinase activity and autophosphorylation of receptor tyrosine residues. **4)** Phosphorylated tyrosine residues in the intracellular domain serve as docking sites to molecules with SH2-domains, such as STAT1 and STAT2. **5)** Upon binding, STAT1 and STAT2 are phosphorylated by JAK1 and TYK2, activating them and facilitating their heterodimerization. **6)** pSTAT1:pSTAT2 complex with IRF9 to form the transcriptional activator ISGF3. Alternatively, pSTAT1 can homodimerize. **7)** ISGF3 translocates to the nucleus and binds to interferon stimulated response elements (ISREs) found within promoter regions of interferon stimulated genes (ISGs). **8)** Alternatively, pSTAT1 homodimer translocates to the nucleus and binds to gamma activated sequences (GAS) within promoter regions of certain pro-inflammatory genes, such as *IRF1*. Figure created with Biorender.com.

Regulation of the Type-I IFN Response through Differential Expression of Non-Canonical IFNAR2 Isoforms

Via alternative splicing, exon skipping, and modulation of polyadenylation sites [57], multiple transcriptional variants of *IFNAR2* are expressed, resulting in the translation of three distinct protein isoforms [58]. IFNAR2c is the long, transmembrane fully functional form of the receptor, which has been shown to restore sensitivity to type-I IFNs in a IFNAR2 deficient cell line (U5A) [58]. IFNAR2b is missing key cytosolic domains, rendering it incapable of signaling [58, 59]. Furthermore, IFNAR2b may act as a negative regulator of type-I IFN signaling, as it still contains its ligand binding and transmembrane domains [60]. IFNAR2a (also known as sIFNAR2a, with "s" standing for "soluble" or "secreted") is missing significant portions of the transmembrane and cytosolic domains, preventing it from being able to insert into the plasma membrane. Consequently, it is secreted into the extracellular space, where it can bind to free ligand [61-64]. Interestingly, the ratio of sIFNAR2a to IFNAR2c varies by tissue type, with sIFNAR2a being the predominant transcript found in the liver, and equal amounts being observed in bone-marrow macrophages [62]. When sIFNAR2a was pre-incubated with IFNA1 or IFNB for 1 hour, followed by treatment of L929 cells for 7 hours, the authors observed a marked reduction in the type-I IFN response [62]. The authors sought to test whether sIFNAR2a exerted effects on cells without endogenous IFNAR2c present by treating IFNAR2c knock-out lines with sIFNAR2a in the presence of IFNA and IFNB. They found that addition of sIFNAR2a restored the ability of cells to respond to type-I IFNs in a sIFNAR2a concentration dependent manner, presumably through interaction with the endogenous IFNAR1 subunit [62]. These data led to the postulation of a dynamic model of type-I IFN regulation via sIFNAR2a, whereby both abundance of soluble receptor concentration and membrane receptor isoforms contribute to the ultimate

augmentation or inhibiting effect. As demonstrated in these studies, it is clear that the type-I IFN response can be modulated via relative expression of IFNAR2 isoforms [43, 62].

Use and Limitation of Type-I IFNs as Antiviral Therapeutic

Type-I IFNs have been used clinically for the treatment of chronic viral hepatitis caused by hepatitis C virus (HCV) and hepatitis B virus (HBV). When efficacious, it is thought that over-stimulation of innate immune signaling pathways imparts an antiviral phenotype when endogenous signaling has proven inadequate to promote viral clearance (HCV) or silencing of viral genome (HBV). However, the use of type-I IFNs has been tempered by suboptimal rates of clinical success in HBV infection and availability of superior alternatives for HCV. In addition, patients can experience significant untoward effects associated with systemic immune activation and inflammation. This systemic inflammation may be molecularly explained by the capacity of type-I IFNs to induce STAT1:STAT1 homodimers, which translocate to the nucleus and bind to gamma activated sequence (GAS) regions, which can result in the transcription of IRF1, a well-characterized pro-inflammatory transcription factor [65]. Consequently, while type-I IFN stimulation has demonstrated powerful antiviral ability, it can result in the production of pro-inflammatory cytokines that result in systemic, and potentially deleterious, inflammation [66].

Discovery of Interferon Lambdas

Type-III interferons (referred to here as interferon lambdas, IFNLs) were discovered in the early 2000s by two separate research groups [67-69]. Both groups aimed to identify novel members of the type-II cytokine family, as they play a critical role in host innate immunity and modulation of the adaptive immune response. These groups employed different approaches: Sheppard et al. used computational methods to identify novel helical cytokine ligands, using

sequence homology to the IL10 family [68], while Kotenko et al. used similar methods to identify receptors bearing structural features of class-II cytokine receptors [67]. Both investigations led to the discovery of the same family of novel IFN ligands and receptor, which were named using distinct nomenclatures, listed here with Sheppard et al. first and Kotenko et al. in parentheses: IL-29 (IFNL-1), IL-28A (IFNL-2), IL-28B (IFNL-3), and IL-28R (IFNLR1). As the field has adopted the nomenclature presented in Kotenko et al. study, it will be used exclusively in this thesis.

The *IFNLR1* gene was sequenced and mapped against the human genome to reveal a structure containing 7 distinct exons: Exon 1 encoding the signal peptide for membrane localization, exons 2-5 and partial 6 encoding the extracellular domains, and exons 6 and 7 encoding the transmembrane and intracellular domains [67]. The researchers postulated that IFNLR1 represented the R1 subunit of the traditional class II cytokine receptor complex, due to the length of its intracellular domain, which suggested it could bind JAK protein and serve as a substrate for tyrosine phosphorylation [67]. Consequently, they sought to determine the identity of the R2 subunit. IL10RB had previously been characterized as a receptor subunit with wide cell type expression and ability to serve as the R2 subunit to facilitate signal transduction in other cytokine systems [70]. To test the hypothesis that IL10RB was necessary to support IFNLR1 signaling, they expressed IFNLR1 either alone or in tandem with IL10RB in multiple hamster cell lines and treated with conditioned media obtained from cells transfected with IFNL ligand constructs [67]. The researchers did not observe any STAT phosphorylation or upregulation of MHC class-I when either subunit was expressed alone, but observed marked STAT1 phosphorylation and MHC class-I upregulation when the subunits were co-expressed [67]. Furthermore, the requirement of IL10RB for signal transduction was confirmed by observing loss of transduction with IFNL treatment after pre-incubation with a neutralizing anti-IL10RB antibody [67].

These initial studies also demonstrated that binding of IFNLs to IFNLR1 resulted in phosphorylation of STAT1/2 and upregulation of ISG expression (**Figure 1.2**) [67, 68]. Thus, from a signaling perspective, the IFNL response has a reliance on the JAK-STAT pathway for signal transduction similar to the type-I IFN system [71, 72]. However, investigation of IFNLR1 expression in tissues and cell types throughout the host (mouse, human) identified low and restricted expression in cell types of epithelial origin, such as those found in the skin, liver, respiratory, gastrointestinal, reproductive tracts, and some immune cells [12, 69, 73], which is in stark contrast to the near ubiquitous expression of the type-I IFN receptor. Consequently, it was hypothesized that the IFNL system was a major driver of innate immunity in tissues and cells that comprise anatomical barriers [74]. Subsequent work identified that the IFNL system is not simply a redundant system active in tissues that face more regular exposure to pathogens in the environment; rather, it displays distinct kinetics of activation, can exert significant regulatory influence on the type-I IFN response, and in addition plays an active role in modulating the adaptive immune response during chronic viral infection [75, 76].

Structure and Function of the Interferon Lambda Receptor

The basic biochemical and structural nature of the interferon lambda receptor 1 (IFNLR1) protein have been established since its discovery [65, 69, 77-79]. Consistent with other class II helical cytokine receptors, IFNLR1 has an N-terminal extracellular region, followed by transmembrane and C-terminal intracellular domains [77]. The N-terminal region contains the ligand binding domain, which provides specificity to the receptor complex to transduce signal initiated by receptor complexation with IFNL ligands (IFNL1, IFNL2, IFNL3, or IFNL4) [80]. Notably, IFNL ligands display distinct affinities for IFNLR1 (IFNL3 > IFNL1 > IFNL2 = IFNL4) [65]. The transmembrane domain of IFNLR1 contains amino acids necessary for association with the co-receptor, IL10RB [77, 78, 81]. It is important to note that although IL10RB is required for IFNL

signaling [82], several studies have shown that loss of TYK2 does not compromise the IFNL response [83]. Consequently, the role of TYK2 in the IFNL-IFNLR1 signaling axis is hypothesized to be modulatory, but not obligatory [84]. The intracellular domain of IFNLR1 consists of regions essential for signal transduction. Specifically, as described above for IFNAR1 and IFNAR2, IFNLR1 contains Box1 and Box2 sequence domains that have been demonstrated to be important for JAK1 binding, with Box1 being necessary for JAK1 binding, and Box2 increasing the stability of JAK1 interaction [77, 85]. Slightly downstream of the Box1/2 regions lie the tyrosine residues essential for STAT2 binding and activation, specifically Tyrosine (Y) residues Y343 and Y517 [13, 86].

Although the molecular details are not as well characterized as the type-I IFN complex, the prevailing understanding of IFNLR1 receptor activation is as follows: Upon ligand binding to the high affinity IFNLR1 subunit, IL10RB is recruited to form the complex IFNL receptor complex [67, 68]. JAK1 (associated with IFNLR1) and TYK2 (associated with IL10RB) phosphorylate both their receptor substrates and each other, revealing sites for STAT binding and subsequent activation [13]. STAT1 and STAT2 molecules form a heterodimer with IRF9 to form the signaling complex ISGF3, which translocates to the nucleus to bind and activate genes under the transcriptional control of ISREs [71]. Of note, activation of the IFNL system does not typically induce formation of STAT1:STAT1 homodimers (**Figure 1.2**).

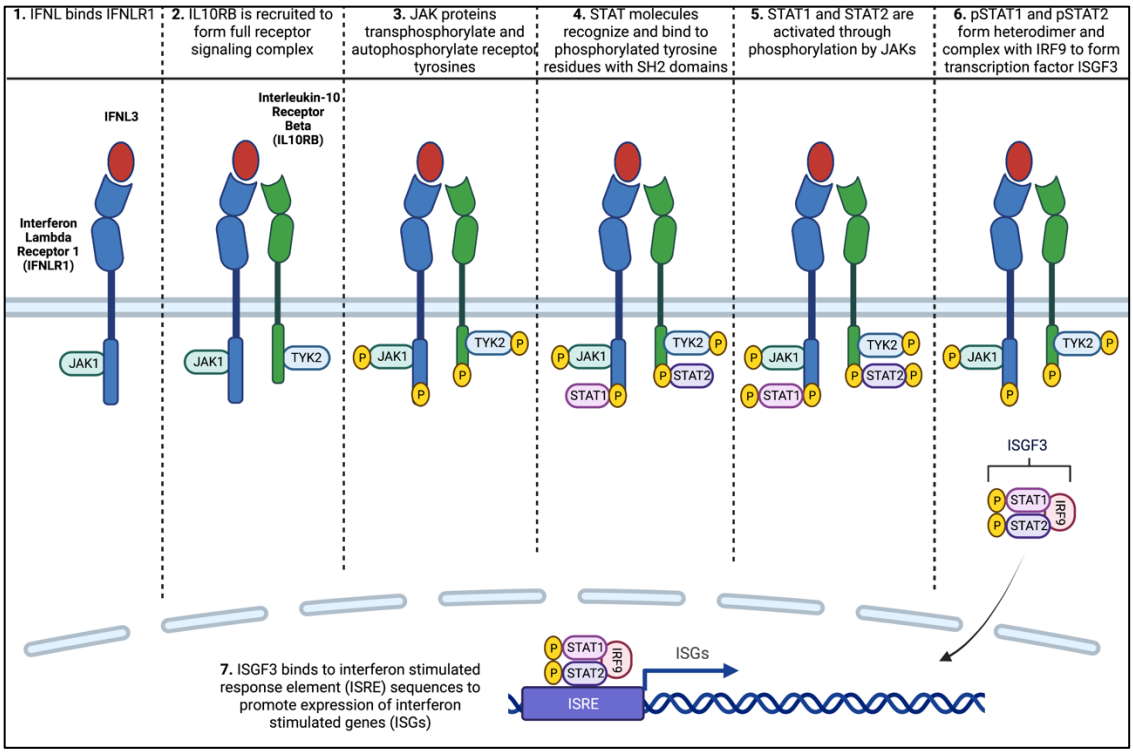


Figure 1.2: Interferon Lambda Signaling. 1) The high affinity subunit IFNLR1 (isoform 1) is first to bind IFNL ligand. **2)** Upon ligand binding, the lower affinity subunit IL10RB is recruited to form the full receptor signaling complex. **3)** Each subunit's respective JAK protein trans-phosphorylates the other, leading to further activation of kinase activity and autophosphorylation of receptor tyrosine residues. **4)** Phosphorylated tyrosine residues in the intracellular domain serve as docking sites to molecules with SH2-domains, such as STAT1 and STAT2. **5)** Upon binding, STAT1 and STAT2 are phosphorylated by JAK1 and TYK2, activating them and facilitating their heterodimerization. **6)** pSTAT1:pSTAT2 complex with IRF9 to form the transcriptional activator ISGF3. **7)** ISGF3 translocates to the nucleus and binds to interferon stimulated response elements (ISREs) found within promoter regions of interferon stimulated genes (ISGs). Figure created with Biorender.com.

Unique Characteristics of the Interferon Lambda Antiviral Response

While utilization of JAK-STAT signaling is conserved between the type-I and IFNL systems, there are notable and significant differences that impact the biology of cells. In contrast to the type-I IFN response, which is initiated through either formation of STAT1:STAT2 heterodimers (leading to the formation of ISGF3 and upregulation of ISRE-driven genes) or homodimerization of STAT1 (leading to GAS-driven upregulation of pro-inflammatory transcription factor IRF1), the canonical IFNL response is driven primarily by ISGF3 formation and upregulation of ISRE-dependent antiviral genes [87, 88]. Of note, even stimulation with supraphysiological concentrations of IFNLs does not generate appreciable levels of STAT1:STAT1 homodimers or upregulation of IRF1. Consequently, the transcriptional profile associated with IFNL-stimulation is less inflammatory than that induced by type-I IFNs [66, 87-89].

It was initially thought that the IFNL response exhibited no unique signaling pathway activation relative to type-I IFNs. However, a recent study by Pervoloraki et al. in 2017 demonstrated that the IFNL system uniquely utilizes the mitogen-activated protein kinase pathway (MAPK) [90]. Specifically, the authors showed in mini-gut organoids and T84 (colon adenocarcinoma) cells exhibit protection against mammalian reovirus (MRV) when pre-treated with either type-I IFN or IFNL and that genetic ablation of either IFNAR1/IFNAR2 or IFNLR1 in T84 cells abrogated their ability to respond to their respective ligand (IFNB for IFNAR1/IFNA2 KO and IFNL2/3 for IFNLR1 KO), with both KO lines showed robust antiviral capacity when stimulated with the proper ligand (IFNAR1/IFNAR2 KO stimulated with IFNL2/3 or IFNLR1 KO stimulated with IFNB), suggesting that the interferon systems are capable of working independently [90]. In addition, although treatment with both type-I or IFNL ligands led to phosphorylation of MAPK, use of small molecule inhibitors of the MAPK pathway only affected the antiviral capacity in cells pre-treated with IFNLs, suggesting that the IFNL system relies on

the MAPK pathway to confer antiviral protection [90]. While these results have yet to be further characterized and the significance of MAPK activation elucidated, this study established that the IFNL system is not simply a redundant form of the type-I IFN response, but rather may play a critical role in shaping multiple fundamental processes of cell biology, including cell cycle and growth, differentiation, and cell death [13].

As the type-I IFN response can cause undue inflammation, subsequent investigations have examined how the type-I IFN response is regulated in the hopes that insight would allow more efficient targeting of type-I IFN pathways while minimizing untoward inflammatory side effects. It had been observed that the ISG response after treatment with type-I IFNs peaked ~24 hours, rapidly diminished after, and was refractory to treatment with type-I IFN ligands. These data suggested that the type-I IFN response is actively negatively regulated [91]. Further studies identified multiple proteins that negative regulate type-I IFN signaling including SOCS1, SOCS3, and USP18 [91-93]. Highlighting the importance of these proteins, loss of these negative regulators of the type-I IFN response resulted in autoimmunity [94, 95]. In contrast, the IFNL response exhibits delayed ISG expression, peaking after 24 hours and lasting for several days. The durability of the IFNL response is largely attributed to lack of negative regulation, although recent studies have shown that SOCS1 can negatively regulate the IFNL response under certain conditions [92]. The mechanisms by which IFNL signaling is regulated are incompletely understood.

Clinical Use of Interferon Lambdas

As IFNLR1 exhibits restricted expression to cells of epithelial origin comprising tissues along anatomical barriers, the influence of IFNL on viral infection was evaluated in respiratory, gastrointestinal, and reproductive tracts, in addition to the liver [96]. Specifically, the IFNL response has been shown to engage in host defense against multiple viruses, such as: SARS-CoV-2, influenza A [97], zika virus [98], hepatitis B and C viruses [99], norovirus [100], West Nile virus [101], and yellow fever virus [102]. IFNLs have also been evaluated for potential therapeutic use [103], with IFNL1 evaluated for treatment of chronic hepatitis C and B viruses [104-107], and more recently, for SARS-CoV-2 [108], with the hope that a robust antiviral effect can be obtained without off target proinflammatory side effects.

Pegylated (peg)-IFNL1 was first used in a therapeutic setting in the treatment of HCV, which showed robust antiviral effects in patients over a 4-week course of treatment [109]. However, with the marked clinical efficacy of more recently developed direct acting antiviral (DAA) agents, further investigation of IFNLs for treatment of HCV was abandoned [110]. For chronic HBV infection, PEG-IFNL1 was tested head-to-head with PEG-IFNA2 (a type-I IFN) in a clinical trial for patients with chronic hepatitis B viral infection [107]. This study demonstrated that peg-IFNL1 treatment reduced HBV infection and had fewer untoward effects than peg-IFNA2 treatment [107]. However, patients who received peg-IFNL1 exhibited greater rebound of viral markers when taken off drug, compared to those on peg-IFNA2 [107].

A recent study published in the New England Journal of Medicine tested the efficacy of a single-dose of peg-IFNL1 in SARS-Cov-2 positive patients presenting with acute symptoms no longer than 7-days after symptom development [108]. Patients who received peg-IFNL1 treatment exhibited a 51% lower rate of hospitalization or visit to an emergency room due to

COVID-19 related issues, and a 47% decrease in hospitalization [108]. These data support that there is clinical potential for the use of IFNLs and that further investigation into how the IFNL system can be modulated to optimize its use is warranted.

Genetics of the IFNL Response

When considering the therapeutic potential of IFNL, it is important to note that there is a strong genetic influence on both host response to viral infection and efficacy of IFN treatment. This concept is best illustrated in the context of HCV infection, where the rate of spontaneous clearance and sustained viral response (SVR) after treatment with peg-IFNA was correlated with several factors, including race, suggesting a genetic influence [111-113]. Specifically, it was shown that African American patients were less likely to achieve SVR than Caucasian patients after administration of a peg-IFNA therapeutic regimen [113].

To help explain these differences, several genome wide association studies (GWAS) were conducted to determine whether these clinical observations were associated with a particular genotype [114-118]. Multiple single nucleotide polymorphisms (SNPs) were identified that correlated with spontaneous viral clearance and SVR, including rs12979860 and rs8099917, on chromosome 19q13.13 [114-116, 118]. Notably, these SNPs are in close proximity to the *IFNL* genes, which at the time of investigation, included *IFNL1*, *IFNL2*, and *IFNL3*. In an attempt to understand the functional impact of these SNPs, Prokunina-Olsson et al. performed RNA sequencing in primary human hepatocytes after treatment with polyinosinic:polycytidylic (polyI:C), a synthetic double stranded RNA reagent commonly used to stimulate the endogenous IFN response [119]. Their data revealed upregulation of all known IFNL ligand genes (*IFNL1*, *IFNL2*, and *IFNL3*), in addition to transcription of an uncharacterized region located upstream of *IFNL3* [119]. Through genomic mapping, the group identified the transcriptional and

translational start sites, which allowed them to detect a novel dinucleotide variant within exon 1 (rs368234815), termed *IFNL4*-ΔG/TT [119]. The *IFNL4*-ΔG allele led to a frameshift mutation that gave rise to a novel 179 amino acid protein that exhibited sequence homology to IFNL3 (29.1% amino acid identify), and was consequently classified as a fourth IFNL ligand (IFNL4) [119]. In contrast, the *IFNL4*-TT allele resulted in the production of a truncated transcript and abrogated expression of IFNL4 [119, 120]. While this dinucleotide variant exhibited high linkage disequilibrium with SNPs rs12979860 and rs8099917, it proved to be a more accurate predictor of treatment response, as evidenced by association of the *IFNL4*-ΔG allele with poor decline in HCV RNA in African American patients treated with a combination peg-IFNA/ribavirin therapeutic strategy [119]. Interestingly, the *IFNL4* genotype has also been associated with rate of viral decay and drug efficacy in HCV-infected patients treated with Sofosbuvir, a direct acting antiviral agent (DAA), and ribavirin [121]. The authors suggest that prior knowledge of the *IFNL4* genotype could inform the treatment decision-making process (i.e. treatment duration/intensity), thereby allowing for more personalized therapeutic strategies [121].

The functionality of IFNL4 was tested in various cell lines, showing that it results in upregulation of ISGs through IFNLR1 and establishment of an antiviral state, similar to previously discovered IFNL ligands [119, 122]. Consequently, this presents an apparent contradiction, as the capacity to produce IFNL4 *in vivo* is associated with poor host response to HCV, while it demonstrates antiviral properties *in vitro* [123, 124]. Treatment of HepG2 cells with IFNL4 exhibited increased levels of negative regulators of the IFN response, specifically USP18, compared to IFNL3-treatment, leading to the hypothesis that production of IFNL4 *in vivo* may alter cell responsiveness to type-I IFNs [125]. However, additional studies are needed to thoroughly understand the role of IFNL4 in influencing the host response to endogenous and exogenous IFNs.

IFNLR1 Regulation, Transcriptional Variants, and Receptor Isoforms

A more complete understanding of IFNL regulation could help identify new ways to optimize and improve the use of IFNL as a therapeutic agent. Due to low IFNLR1 expression *in vivo* and lack of effective commercially available reagents to detect endogenous IFNLR1, detailed evaluation of IFNLR1 has been challenging [13, 65]. Interestingly, distinct transcriptional splice variants predicted to encode distinct IFNLR1 protein isoforms were identified by RNA-Seq in multiple cell lines and tissues (**Figure 1.3)** [67, 68, 126]. Canonical IFNLR1 isoform 1, produced from transcriptional variant isoform 1, is signaling-competent and supports JAK-STAT signaling induced by IFNLs [67, 68]. In contrast, non-canonical IFNLR1 isoform 2 and isoform 3 are missing exons that encode key signaling and trans-membrane domains, respectively, and are predicted to encode signaling-defective proteins [126]. The presence of these isoforms suggests that they may play a potential role in modulating the IFNL response and could be of importance in understanding how the IFNL system can be manipulated for therapeutic purpose.

A

Feature	Function	IFNLR1-Isoform 1 (Q8IU57-1)	IFNLR1-Isoform 2 (Q8IU57-2)	IFNLR1-Isoform 3 (Q8IU57-4)
Signal Sequence (1-21)	Protein localization to membrane	✓	✓	✓
Extracellular Domain (21-228)	Ligand binding domain (90-205)	✓	✓	✓
Transmembrane Domain (229-249)	Insertion into membrane	✓	✓	✗
Box 1 (252-269)	JAK1 binding	✓	Partial	✗
Box 2 (270-297)	JAK1 stabilization	✓	✗	✗
Tyrosines: Y343 and Y517	Necessary for STAT2	✓	✓	✗
Total Amino Acids		520 amino acids	491 amino acids	244 amino acids

B

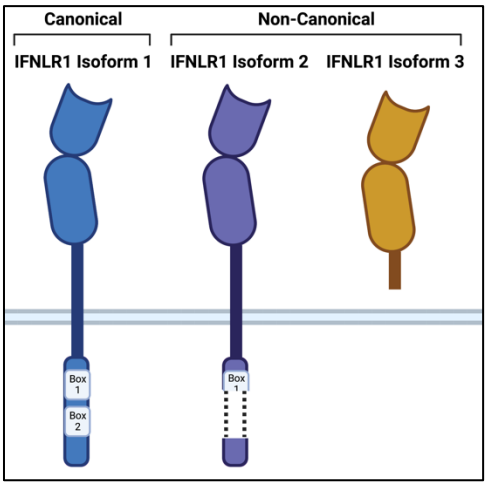


Figure 1.3: Structural features of IFNLR1 receptor isoforms. (A) Key biological features of IFNLR1 isoforms. **(B)** Graphical representation of IFNLR1 isoforms. Figure created with Biorender.com.

Prior studies that evaluated IFNLR1 isoform 1 showed that its overexpression augments the magnitude of IFNL-induced ISG expression (summarized in **Figure 1.4**) [87, 126, 127]. This phenomenon was best demonstrated in a study conducted by Forero et al. in 2019, which showed that constitutive overexpression of IFNLR1 isoform 1 in an immortalized liver cell line (Huh7) resulted in augmented expression of the antiviral gene *ISG15*, increased pSTAT1 content, and *de novo* expression of pro-inflammatory cytokine *CXCL10* after IFNL3 treatment compared to cells transfected with vector-only [87]. In addition, overexpression of IFNLR1 isoform 1 increased the relative gene expression of both antiviral gene *MX1* and pro-inflammatory cytokine *CXCL10*, when compared to cells treated with type-I IFN (IFNB) [87]. The researchers showed this effect was mediated through upregulation of STAT1:STAT1 homodimers, which led to the expression of pro-inflammatory protein IRF1, a transcription traditionally associated with type-I but not IFNL signaling [87, 88].

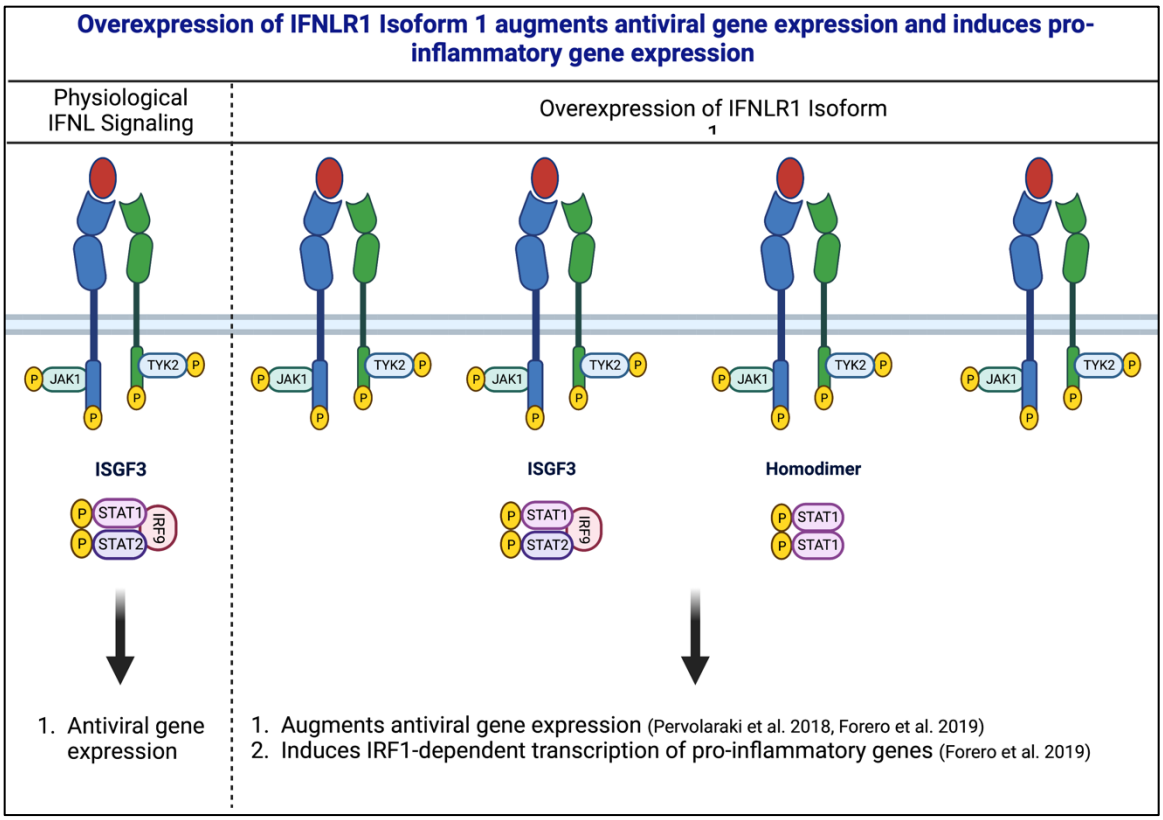


Figure 1.4: Current model of IFNLR1 Isoform 1 impact on IFNL signaling. Figure created with Biorender.com.

Much less is known about how and whether non-canonical IFNLR1 isoforms influence pathway regulation. IFNLR1 isoform 2 exhibits a partial loss of Box1 and complete loss of Box2 in its intracellular domain which play a fundamental role in JAK1 binding and stability [77]. Only one study to date by Lauber et al. in 2015 has explored its potential functionality, showing that transient transfection of IFNLR1 isoform 2 under a constitutively active promoter in HEK293 cells does not enhance or reduce ISRE activity after IFNL3 treatment (summarized in **Figure 1.5**) [126]. Two studies have evaluated the role of IFNLR1 isoform 3 on the IFN response [128, 129]. In the first study, Witte et al. in 2009 demonstrated that IFNLR1 isoform 3 is secreted, can bind to ligand IFNL1, and when co-treated in excess with IFNL1, can reduce the IFNL-dependent upregulation of MHC class-I in HepG2 cells [129]. Santer et al. in 2020 characterized the functional effects of IFNLR1 isoform 3 and showed that IFNLR1 isoform 3 was present in greater abundance in immune cell types in contrast to epithelial cell types, even though IFNLR1 isoform 1 was the predominant transcriptional variant in both cell types [128]. Further, they showed that IFNLR1 isoform 3 binds the surface of peripheral blood mononuclear cells (PBMCs) in the presence of ligand, and when co-treated in excess with IFNL3, led to a robust inhibition of the IFNL response [128]. Taken together, these data suggest that IFNLR1 isoform 3 may modulate cellular sensitivity to IFNLs and/or titrate the relative concentration of free ligand available for signal transduction in the extracellular space (summarized in **Figure 1.6**).

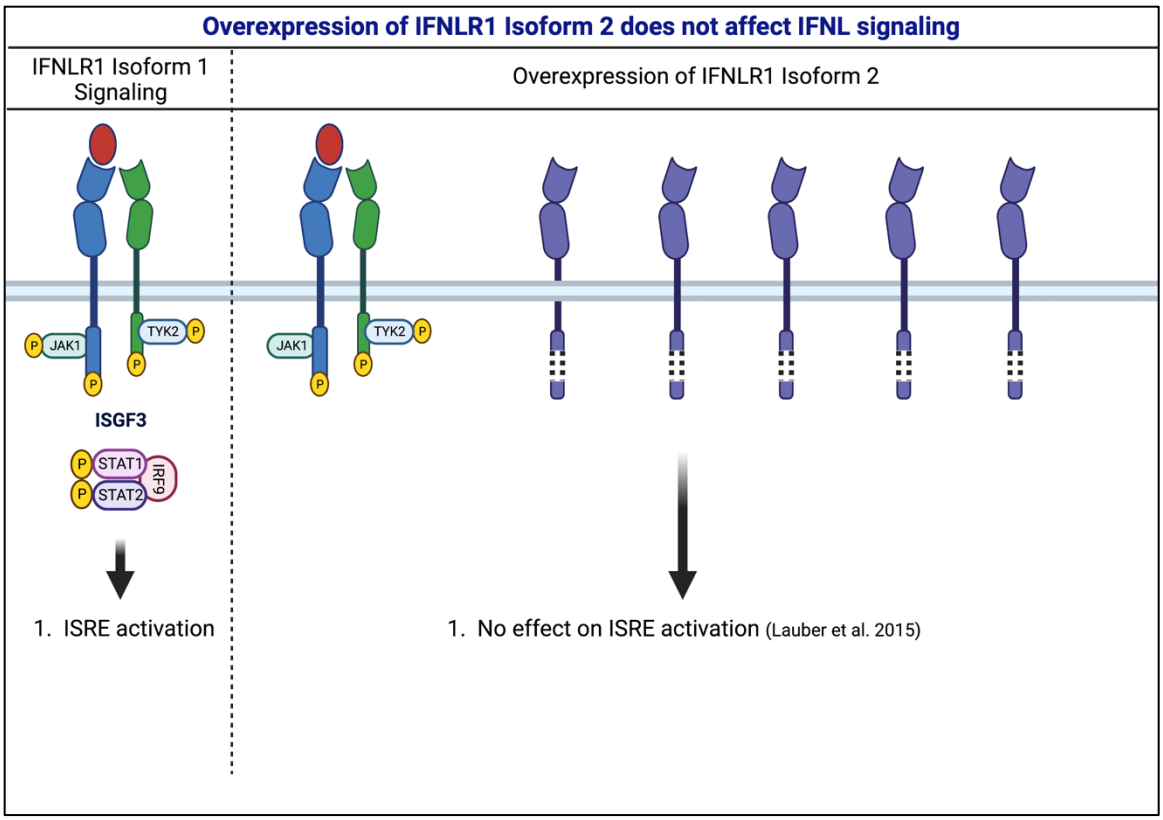


Figure 1.5: Current model of IFNLR1 Isoform 2 impact on IFNL signaling.

Overexpression of IFNLR1 isoform 2 did not augment or reduce ISRE activity after treatment with IFNL3 compared to cell controls. Consequently, it was concluded that IFNLR1 isoform 2 does not affect IFNL signaling. Figure created with Biorender.com.

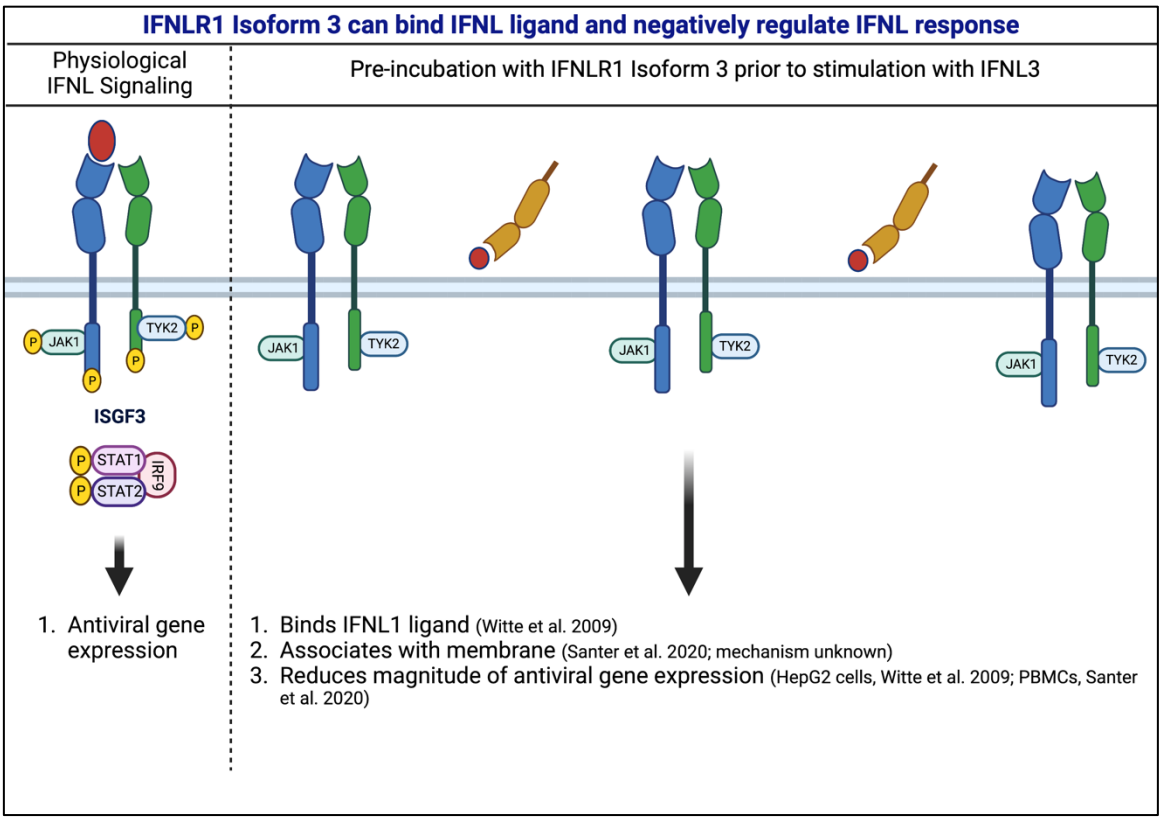


Figure 1.6: Current model of IFNLR1 Isoform 3 impact on IFNL signaling. Figure created with Biorender.com.

Challenges in Studying IFNLR1

As IFNLs are an essential component of the host innate immune response and represent a promising antiviral therapeutic option, this gap in the literature may reflect the complexity of biology and study of this receptor. The consensus in the field is that IFNLR1 is difficult to study for two major reasons: 1) Like many cytokine receptor systems, IFNLR1 exhibits extremely low receptor expression *in vivo* [130, 131], making it difficult to visualize and/or detect using commercially available reagents and methods and 2) Multiple studies suggest that overexpression of IFNLR1 may alter the cellular balance between survival and apoptosis in certain cell types [132, 133].

Overarching Aims and Hypothesis

Despite demonstrating antiviral activity against multiple viral pathogens including HCV, HBV, and SARS-CoV-2 [17, 18] and improved tolerability relative to type-I IFNs, IFNLs are not an approved therapy for any infection as of yet. Consequently, the overarching theme of our laboratory investigation is that an improved understanding of the regulatory mechanisms underlying the cellular response to IFNLs could provide new insight into how to effectively target this pathway for therapeutic benefit. Specifically, we hypothesize that IFNLR1 isoforms uniquely modulate IFNL signaling, and that differential expression of IFNLR1 isoforms provides a critical means by which cells can regulate their responsiveness to IFNLs.

To explore this hypothesis, we altered relative expression of IFNLR1 isoforms and evaluated the cellular transcriptional response to IFNLs *in vitro*. First, we asked whether IFNLR1 isoform 1 abundance could be titrated to allow augmentation of select antiviral genes without inducing potentially harmful pro-inflammatory cytokines. Second, we examined whether non-canonical IFNLR1 isoforms 2 and 3 act as negative regulators of the IFNL response in a

concentration-dependent manner. We utilized HEK293T cells, previously shown to be a good model for study of IFNLR1 function [21, 22], to generate stable clones with doxycycline-inducible expression of each FLAG-tagged IFNLR1 isoform to facilitate receptor visualization and allow precise control of expression.

CHAPTER 2: MATERIALS AND METHODS

Generation of FLAG-epitope Tagged IFNLR1 Expression Constructs

Thermo Fisher's GeneArt system was used to design FLAG-tagged IFNLR1 constructs. Full cDNA FASTA sequences of IFNLR1 isoforms were retrieved from the NCBI gene database, with IFNLR1 isoform 1 denoting the full length signaling-capable receptor, IFNLR1 isoform 2 missing a portion of the cytoplasmic domain, and IFNLR1 isoform 3 missing its transmembrane domain [126, 128, 129]. A Kozak sequence (ACCAUGG) was added to the extreme 5' end to promote ribosomal binding and translation in mammalian cell lines [134]. The UniProt database was used to design placement of an N-terminal 3X-FLAG tag immediately downstream of the predicted signal sequence (henceforth referred to as FLAG-Iso1, FLAG-Iso2, and FLAG-Iso3) [135]. Sequences were codon optimized to maximize expression in mammalian cells. Nucleotide and protein sequences, in addition to graphical representations of each FLAG-IFNLR1 isoform, are depicted in **Figures 3.1** and **S1.**

Cloning of FLAG-IFNLR1 Isoform Inserts into Doxycycline Inducible Vector

FLAG-IFNLR1 isoform constructs were flanked with 5' MluI and 3' EcoRV restriction enzyme sites to facilitate downstream sub-cloning into the pTRE-Tight-IP doxycycline-inducible expression vector (gift from Dr. Stephen Duncan) [136] . Plasmids were transformed into 5-alpha Competent *E. coli* (C2987H, New England BioLabs) and ampicillin selected (100 µg/mL, A5354-10ML, Sigma Aldrich) followed by purification using an endotoxin-free plasmid maxiprep Kit (12362, Qiagen). Nucleotide sequencing (Eurofin Genomics) was used to confirm construct sequence and alignment after cloning.

Generation of Stable HEK293T FLAG-IFNLR1 Isoform Cell Lines

HEK293T cells were cultured in in Dulbecco's modified Eagle's medium with F12 (DMEM/F12) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin, according to ATCC guidelines. Wild-type HEK293T cells were transfected using ViaFect (E4981, Promega) with empty vector (pTRE-Tight-IP), or FLAG-Isoform encoding pTRE-Tight-IP vectors and cultured in 3 μg puromycin/ml to generate stable cell lines (A11138-03, Gibco). Surviving colonies were picked via application of cloning rings, expanded, and continuously cultured in 1 μg puromycin/ml.

Doxycycline Induction and Interferon Treatments

To induce construct expression, cells were treated with doxycycline (dox, D9891, Sigma) for 24 hours prior to IFNL3 treatment. IFNL3 (5259-IL-025/CF, R&D Systems) and IFNA2 (10984-IF, R&D Systems) stock solutions were aliquoted and stored at -80 ^{o}C prior to thaw on ice immediately before use.

Western Blot

To evaluate construct expression, cellular protein was harvested using RIPA buffer (89900, Thermo Fisher Scientific) supplemented with protease and phosphatase inhibitors (A32959, Thermo Fisher Scientific). Protein concentration was standardized by BCA assay (23227, Thermo Fisher Scientific) prior to electrophoresis using gradient SDS/PAGE gels (Bio-Rad, Hercules, CA, USA).

To detect the FLAG epitope, blots were blocked with 5% milk (M17200-1000.0, Research Products International, Mount Prospect, IL, USA) in TBST (J77500-K8, Thermo Fisher Scientific) for 1 h at room temperature and then incubated with mouse monoclonal anti-FLAG M2

antibody (1:1000, F3165, Sigma-Aldrich) overnight at 4 °C in 1% milk/TBST. After washing, blots were incubated with goat anti-mouse IgG conjugated to HRP (NB7539, Novus Biologicals, Bio-Techne, Minneapolis, MN, USA) for 1 h at room temperature and then developed using ECL (32106, Thermo Fisher Scientific). To detect phosphorylated STAT1 protein (pSTAT1-Y701), blots were blocked in 3% bovine serum albumin (BSA) in TBST for 1 h at room temperature. Blots were then incubated with primary antibody (1:500, mouse monoclonal anti-pSTAT1 (A-2), sc-8394, Santa Cruz Biotechnology, Dallas, TX, USA) overnight at 4 °C in 1% BSA. After washing, blots were incubated with m-IgGk BP-HRP (sc-516102, Santa Cruz Biotechnology) for 1 h at room temperature and developed using ECL. GAPDH was assessed as a loading control by staining with rabbit anti-GAPDH (1:2000, BS2188R, Bioss, Woburn, MA, USA) for 1 h at room temperature, followed by incubation with donkey anti-rabbit IgG conjugated to HRP (1:2000, 406401, BioLegend, San Diego, CA, USA) for 1 h at room temperature. Imaging was performed using FluorChem R (ProteinSimple, Bio-Techne, Minneapolis, MN, USA).

To analyze protein secretion, a 4x volume of chilled acetone was added to culture supernatant, followed by brief vortexing and incubation for 1 hour at -20˚C. Samples were then centrifuged 10 minutes at 15,000 x g. Protein pellets were air dried for 30 minutes at room temperature and resuspended in an equal volume of RIPA buffer prior to analysis by western blot.

Flow Cytometry

To detect receptor expression on the cell surface, cells were fixed with fresh 4% paraformaldehyde (PFA; 15710, Electron Microscopy Sciences) for 20 minutes at 4˚C and stained with rat anti-DYKDDDK Alexa Fluor 488 (1:500, 637317, BioLegend) or rat IgG2a Alexa Fluor 488 Lambda Isotype Control (1:500, 400525, BioLegend) for 45 minutes at 4˚C. To detect

intracellular receptor expression, cells were fixed and permeabilized using BD Cytofix/Cytoperm Fixation/Permeabilization solution kit (BDB554714) prior to staining.

To detect phosphorylated STAT1 (pSTAT1), cells were harvested, fixed with 4% PFA for 20 minutes at 4˚C, and permeabilized in 100% chilled methanol (A411-4, Fisher Chemical) for 15 minutes at 4˚C. Cells were stained with mouse anti-pStat1-PE (pY701) (5µl/100µl sample, 612564, BD Phosflow) prior to flow cytometry. Non-IFNL3 treated and non-permeabilized cells were used as negative controls.

Flow cytometry was performed using a Guava HT8 Incyte System (Luminex). Live cells were identified using scatter-area (forward and side scatter) and by staining with a viability dye (1:1000, Zombie Green Fixable Viability, 423111 or 1:1000, Zombie Red Fixable Viability, 423109, BioLegend). FlowJo software was used for analysis of frequency and median fluorescence intensity (MFI).

Quantitative Reverse Transcriptase-Polymerase Chain Reaction

Expression of individual genes was measured by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). Whole cell total RNA was isolated using a Qiagen Rneasy kit (74106, Qiagen). Concentration and quality were assessed using Nanodrop and cDNA was synthesized using a high-capacity RNA-to-cDNA kit (4368814, Applied Biosystems). qRT-PCR was performed on a CFX96 Real-Time System (Bio-Rad) using TaqMan Fast Advanced Master Mix (4444557, Applied Biosystems) and commercially available TaqMan primer-probes specific for individual genes, including VIPERIN (RSAD2, Hs00369813_m1) and CXCL10 (Hs0017042_m1), relative to GAPDH (Hs02786624_g1). qRT-PCR experiments were conducted with biological and technical duplicates.

Luciferase Assay

To assess receptor function using a dual luciferase assay, cells in 96-well plates were co-transfected with a 5:1 ratio of interferon stimulated response element (ISRE)-firefly luciferase (E4141, Promega) and CMV-renilla luciferase plasmids (E2261, Promega) (300 ng total DNA). Cells were incubated 12-18 hours post-transfection, then washed and incubated with fresh media containing ± dox for 24 hours, followed by IFNL3 treatment for an additional 24 hours. Quantitation of luminescence from lysed cells was determined on a microplate reader (FilterMax F3, Molecular Devices) after applying DualGlo Stop and Glo reagent (E2920, Promega) according to manufacturer guidelines.

Immunofluorescence

To detect protein targets via immunofluorescence, cells were first fixed in 4% PFA for 15 minutes on ice, followed by extensive washing with phosphate buffered saline (PBS). Next, cells were permeabilized with 0.05% Triton X-100 for 10 minutes at room temperature. After washing with PBS, cells were blocked with 3% bovine serum albumin (BSA) solubilized in PBS for 1 hour at room temperature. After washing with PBS, cells were incubated with primary antibody (1:1000) for 1 hour at room temperature. After washing with PBS, cells were incubated with secondary antibody (1:2000) for 1 hour at room temperature. Cells were washed and treated with Fluoromount with DAPI and stored at 4˚C overnight prior to imaging.

Receptor Internalization and Co-Localization

HepG2 cells were transiently transfected with FLAG-Iso1 construct using ViaFect reagent (as described previously) and induced with ± dox (100ng/ml) overnight. Cells were incubated with M2 anti-FLAG antibody at 4˚C for 30 minutes to label surface receptor. Cells were then

washed extensively with chilled PBS and either kept at 4˚C or incubated at 37 ˚C for 30 minutes. Cells were washed with PBS, fixed with 4% PFA for 15 minutes, and permeabilized with 0.05% Triton X-100 for 10 minutes at room temperature. Cells were blocked with 3% BSA for 1 hour. After, cells were incubated with anti-EEA1 (marker of early endosome) antibody for 1 hour at room temperature. After washing with PBS, cells were incubated with secondary antibodies against both anti-FLAG and anti-EEA1 primary antibodies. Samples were washed and treated with Fluromount with DAPI and stored at 4˚C overnight prior to imaging. For co-localization experiments, imaging was performed using the Zeiss 880 confocal microscope.

NanoString Analysis

Global transcriptional profiling was performed on samples using the NanoString nCounter Human Immunology v2 Panel. Gene counts were normalized to 15 housekeeping genes using nSolver software. Gene counts below the assay's threshold of detection (20) were assigned a value of 20 to facilitate analysis of differentially expressed genes (DEGs). Normalized gene counts were compared within each cell line between -dox/+dox and no IFNL3/+ IFNL3 conditions to identify 61 DEGs exhibiting upregulation or downregulation of 2-fold, which were log transformed and standardized to lowest value of 0.

Data and Statistical Analysis

Statistical significance was evaluated in Microsoft Excel and GraphPad Prism 9 using non-parametric two-tailed student's T-tests. Data are presented as mean ± standard error of the mean (SEM).

CHAPTER 3: RESULTS

The majority of the data presented in this section can be found in the following article published in the peer-reviewed journal *Viruses* [137]:

Evans, J.G., L.A. Novotny, and E.G. Meissner, *Influence of Canonical and Non-Canonical IFNLR1 Isoform Expression on Interferon Lambda Signaling.* Viruses, 2023. **15**(3): p. 632.

Generation of doxycycline-inducible, FLAG-tagged IFNLR1 isoform expression constructs and stable lines

Endogenous expression of IFNLR1 is low and difficult to detect [65, 87]. To facilitate protein visualization and enable precise control of IFNLR1 expression, we synthesized cDNAs that incorporated a 3X-FLAG-epitope at the amino terminus of each IFNLR1 splice variant (referred to as FLAG-Iso1, FLAG-Iso2, FLAG-Iso3) (**Figure 3.1, Supplemental Figure S1**) under control of a doxycycline (dox)-inducible promoter [136].

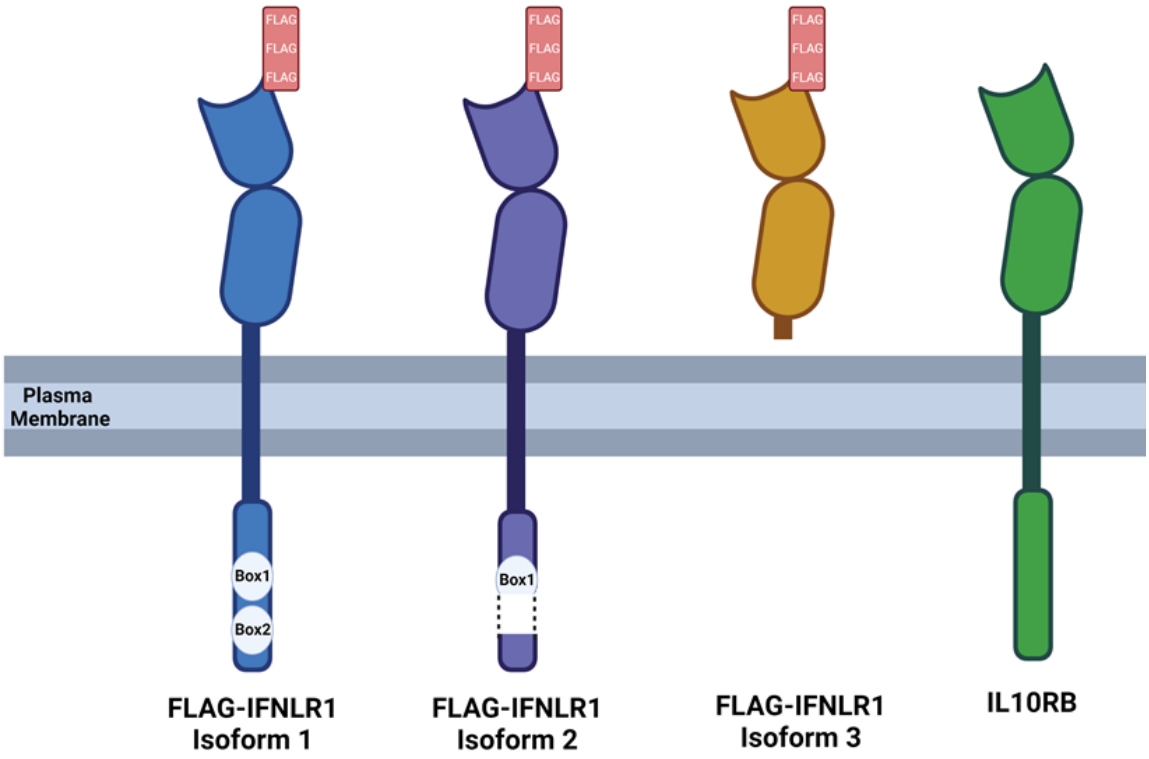


Figure 3.1: FLAG-IFNLR1 Isoforms and IL10RB. Schematic depicting the structure of each FLAG-tagged IFNLR1 isoform and IL10RB. Box1 and Box2 depict the Jak1 binding domain that is fully present in FLAG-IFNLR1 isoform 1, truncated in FLAG-IFNLR1 isoform 2, and absent in FLAG-IFNLR1 isoform 3. Image created with BioRender.com using default settings to represent a transmembrane protein.

Several cell lines were screened prior to generation of stable cell lines for suitability for these investigations. Criteria considered were the following: 1) Ease of genetic manipulation 2) Lack of endogenous interferon responses when transiently transfected with foreign DNA and 3) Tolerance to overexpressing high levels of recombinant protein. HepG2 (hepatocellular carcinoma) and T84 (colon adenocarcinoma) lines were characterized but found to be difficult to transfect and we were unable to generate stable cell lines overexpressing FLAG-IFNLR1. Next, Hela (cervical carcinoma), A549 (lung carcinoma), and HEK293T (kidney epithelial) cells were screened for suitability to support our studies. In contrast to A549 and Hela cells, HEK293T cells showed no endogenous IFN response upon treatment with transient transfection reagent (ViaFect) or introduction of foreign DNA (**Figure 3.2**), thereby providing a suitable model to evaluate the impact of construct expression. In addition, transient transfection of HEK293T cells with FLAG-Iso1 led to augmentation of antiviral ISG *VIPERIN* after treatment with IFNL3 in both -dox and +dox conditions (**Figure 3.3**), demonstrating these cells support IFNL signaling and that our construct functioned as predicted.

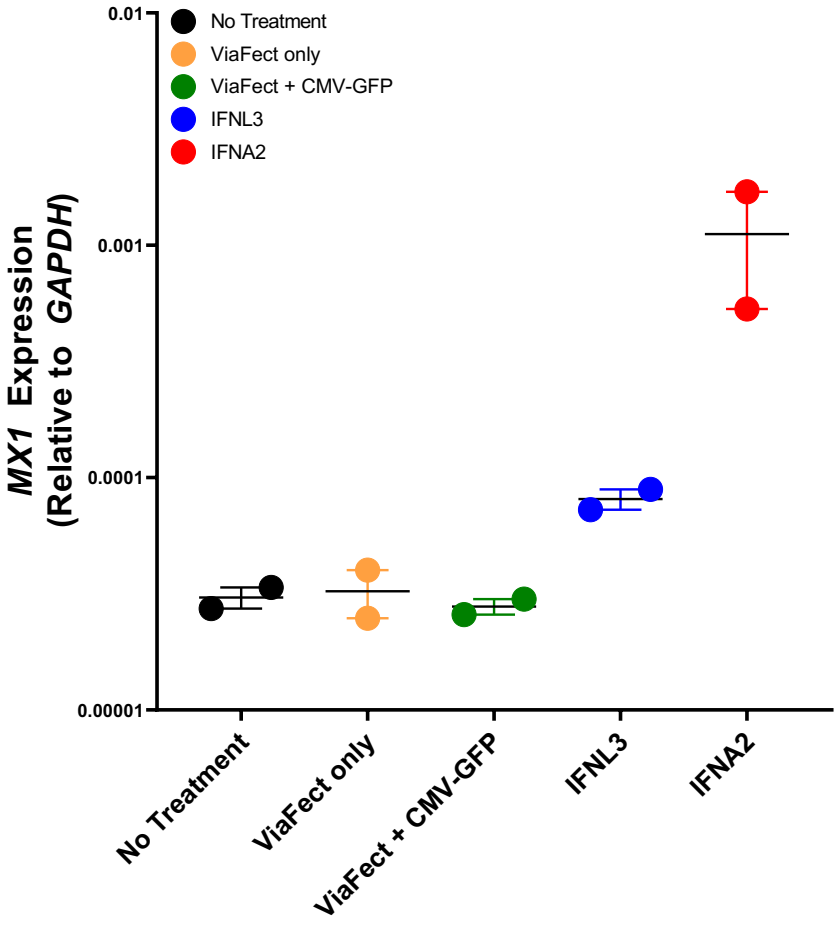


Figure 3.2: Transfection of HEK293T cells with foreign DNA does not elicit endogenous IFN response. HEK293T cells were stimulated with no treatment, ViaFect reagent only, ViaFect complexed with CMV-GFP plasmid, IFNL3 ligand (100ng/ml), or IFNA2 ligand (100ng/ml) for 24 hours prior to collection of RNA and evaluation of expression of antiviral gene *MX1*.

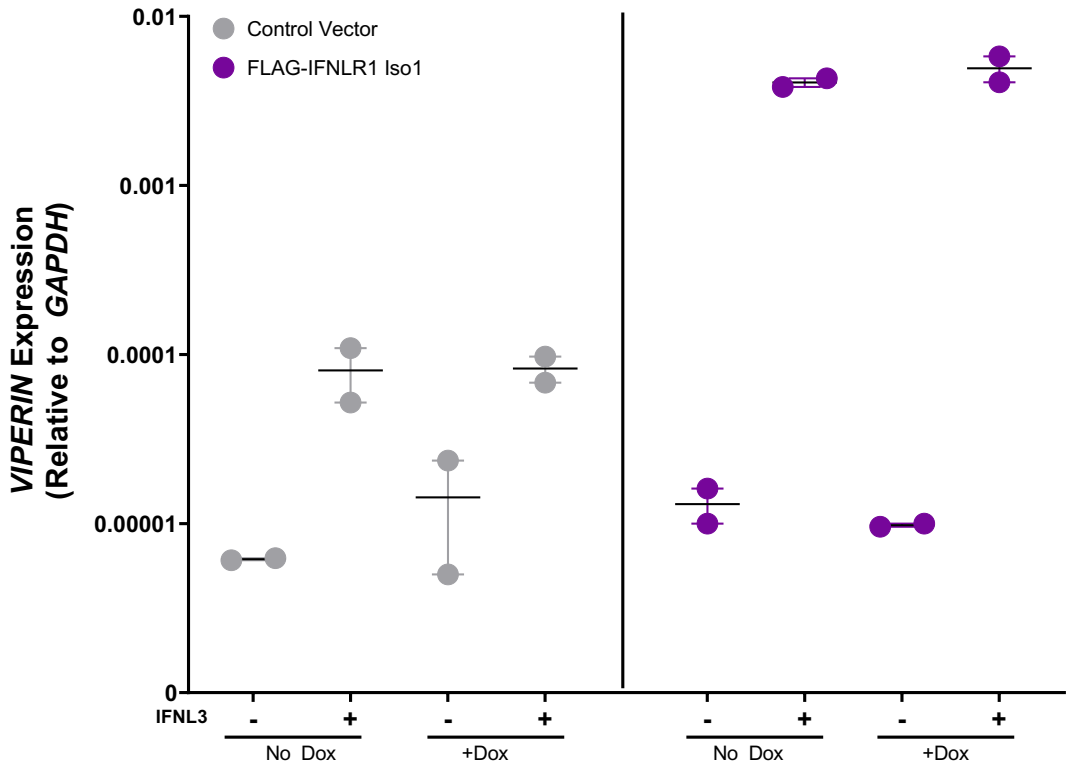


Figure 3.3: Transient transfection of HEK293T cells with FLAG-Iso1 augments response to IFNL3 regardless of doxycycline treatment. HEK293T cells were transiently transfected with FLAG-Iso1 plasmid and induced with ± dox (100ng/ml) for 24 hours prior to treatment with IFNL3 (100ng/ml). RNA was collected after 24 hours to assess expression of antiviral gene *VIPERIN*.

Having established that HEK293T cells serve as a suitable cell model and that transient expression of the FLAG-Iso1 augments cell response to IFNL3, we next generated stable HEK293T clones expressing each construct and demonstrated dox-inducible expression of 58kDa (FLAG-Iso1), 54kDa (FLAG-Iso2), and 31kDa (FLAG-Iso3) proteins whose size was consistent with the predicted molecular weight of non-glycosylated protein (**Figure 3.4A**). Slightly larger bands were consistent with the predicted molecular weight of glycosylated protein (**Figure 3.4A**). Multiple independent clones were evaluated for expression prior to selecting a representative clone for further in-depth study. Several clones had detectable construct expression in the absence of dox, most clearly observed in the FLAG-Iso2 cell line (**Figure 3.4A**), even when cells were cultured in media containing dox-free FBS (data not shown). FLAG-Iso3 had lower relative expression in cell lysates but was readily detected in cellular supernatants (**Figure 3.4B**) consistent with it being a secreted protein [129]. Further, while each FLAG-IFNLR1 isoform was detected with intracellular staining, only FLAG-Iso1 and FLAG-Iso2 were detected on the cell surface by flow cytometry (**Figure 3.4C**). We observed no impact of FLAG-IFNLR1 isoform expression on endogenous *IL10RB* or *IFNLR1* isoform 1 expression by qRT-PCR and only minimal changes in endogenous expression of *IFNLR1* isoforms 2 and 3 (**Supplemental Figure S2**). We did not detect any differences in IL10RB surface expression by flow cytometry (FAB874G, R&D Systems; data not shown) or any differences in cell viability between lines by cell counting or flow cytometry using a Zombie viability stain (data not shown).

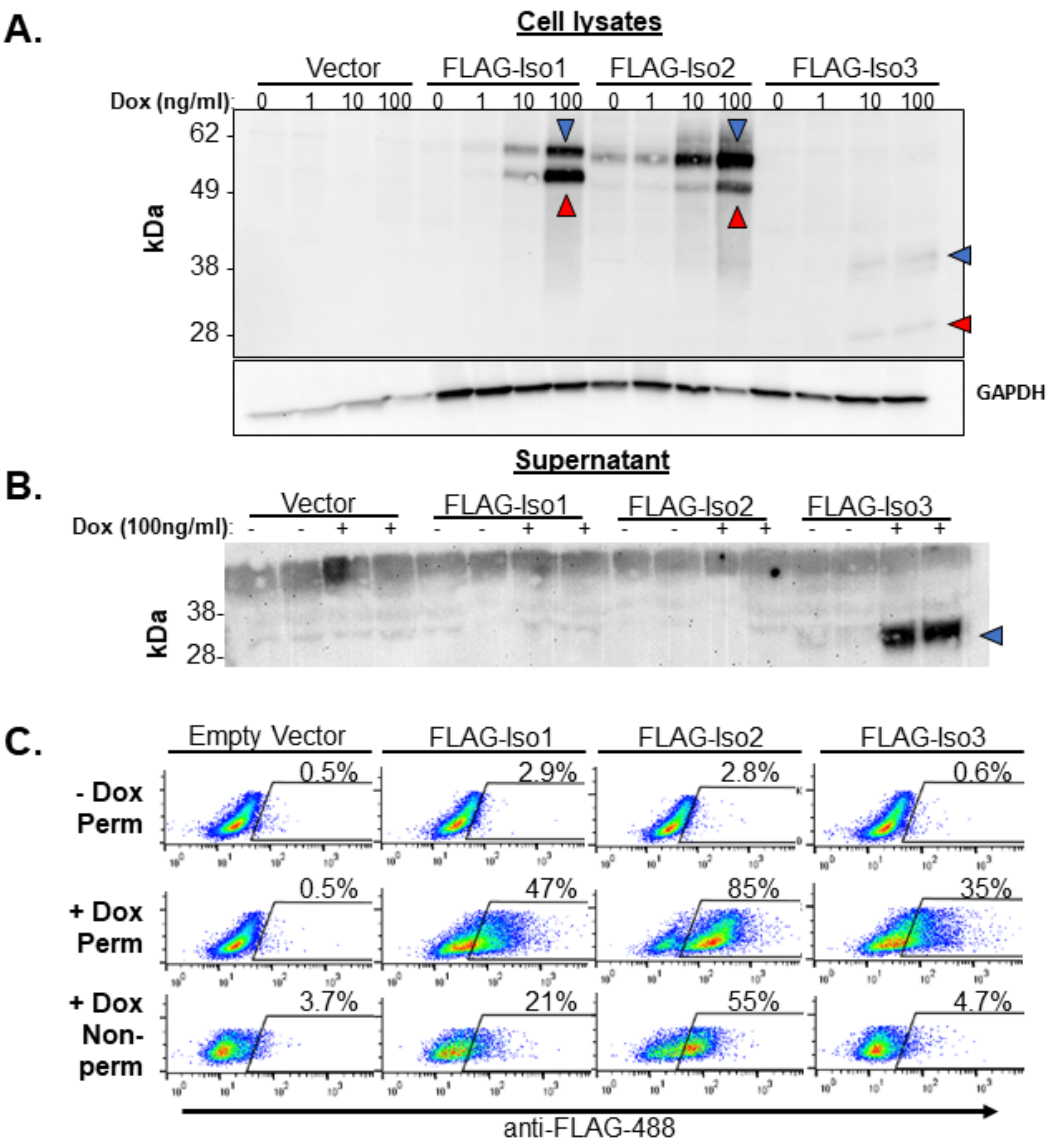


Figure 3.4: Doxycycline-inducible expression of FLAG-IFNLR1 isoforms in HEK293T cells. (A-B) Dox-inducible expression of IFNLR1 isoforms in stable HEK293T clone cell lysates **(A)** and culture supernatant **(B)** was detected by western blot using anti-FLAG antibody. Predicted non-glycosylated and glycosylated proteins are denoted by red and blue arrows, respectively. **(C)** Percent of FLAG-positive cells after 24 hrs induction ± dox (100 ng/ml) followed by permeabilization (perm) or no permeabilization (non-perm), staining with anti-FLAG antibody, and analysis by flow cytometry (representative data, 3 independent experiments).

Minimal expression of FLAG-IFNLR1 isoform 1 is sufficient to support a robust augmentation of the IFNL3 response

As IFNL binding to IFNLR1/IL10RB results in STAT1 phosphorylation (pSTAT1) [31, 69], we examined how relative FLAG-Iso1 expression influenced IFNL3-dependent pSTAT1 using flow cytometry. We observed robust pSTAT1 signal in both -dox and +dox IFNL3-treated FLAG-Iso1 cells relative to HEK293T-empty vector (EV) IFNL3-treated control cells (**Figure 3.5A,** representative flow plots in **Supplemental Figure S3)**. Minimal pSTAT1 was detected in mock-treated FLAG-Iso1 cells, indicating expression of FLAG-Iso1 alone did not result in pSTAT1 formation. These data suggest that minimal amounts of FLAG-Iso1 in -dox conditions, below the level that could be detected by flow cytometry or western blot (**Figure 3.4**), were sufficient to allow phosphorylation of STAT1 after IFNL3 treatment. We observed reduced pSTAT1 abundance in HEK293T-EV cells between 1 and 4 hours of IFNL3 treatment, but no significant decline in pSTAT1 between 1 and 4 hours in either -dox or +dox treated FLAG-Iso1 cells (**Figure 3.5A**). We also consistently observed lower pSTAT1 in +dox relative to -dox FLAG-Iso1 cells treated with IFNL3, most notably after 1 hr of treatment, suggesting excess FLAG-Iso1 expression could partially reduce STAT1 phosphorylation. No significant differences in pSTAT1 were observed at intermediate dox concentrations (1 and 10 ng/ml, data not shown).

During canonical IFNL signaling, pSTAT1 heterodimerizes with pSTAT2 and associates with IRF9 to form ISGF3, which translocates to the nucleus to bind interferon stimulated response elements (ISREs) to promote transcription of ISGs [69]. To evaluate the impact of FLAG-Iso1 overexpression on ISRE activity, we optimized a dual luciferase reporter assay that allows quantitation of ISRE promoter activity relative to a control CMV promoter. FLAG-Iso1 cells

demonstrated a marked increase in ISRE activity only in the presence of IFNL3 and equally in both -dox and +dox conditions (**Figure 3.5B**).

To evaluate how FLAG-Iso1 influences expression of specific ISGs, HEK293T-EV and FLAG-Iso1 cells were dox-induced and IFNL3-treated followed by quantitation of the antiviral ISG *VIPERIN* relative to *GAPDH*. In -dox conditions, FLAG-Iso1 cells exhibited a marked increase in *VIPERIN* expression relative to HEK293T-EV cells (~4,000-fold) that was not further augmented in +dox conditions (**Figure 3.5C**). Culture of FLAG-Iso1 lines in media supplemented with dox-free FBS did not reduce IFNL3-induced *VIPERIN* expression in -dox conditions (data not shown). *VIPERIN* was not induced in mock-treated FLAG-Iso1 cells and was minimally induced in HEK293T-EV cells treated with IFNL3, which indicated that *VIPERIN* induction was both IFNL3 and FLAG-Iso1 dependent. No changes in cell viability were observed as a result of dox-induction or IFNL3 treatment (data not shown).

To further verify the specificity of the observed response, FLAG-Iso1 cells were pre-incubated with anti-IFNLR1 neutralizing antibody prior to IFNL3 treatment. ISRE activity was significantly reduced in -dox conditions, but not in +dox conditions, suggesting that the IFNL response was specifically mediated through IFNLR1 and that antibody inhibition could be overcome with excess amounts of FLAG-Iso1 (**Supplemental Figure S4**).

Taken together, these data demonstrate that minimal FLAG-Iso1 was sufficient to support a marked augmentation of IFNL3 signaling which could not be further increased by greater receptor expression levels.

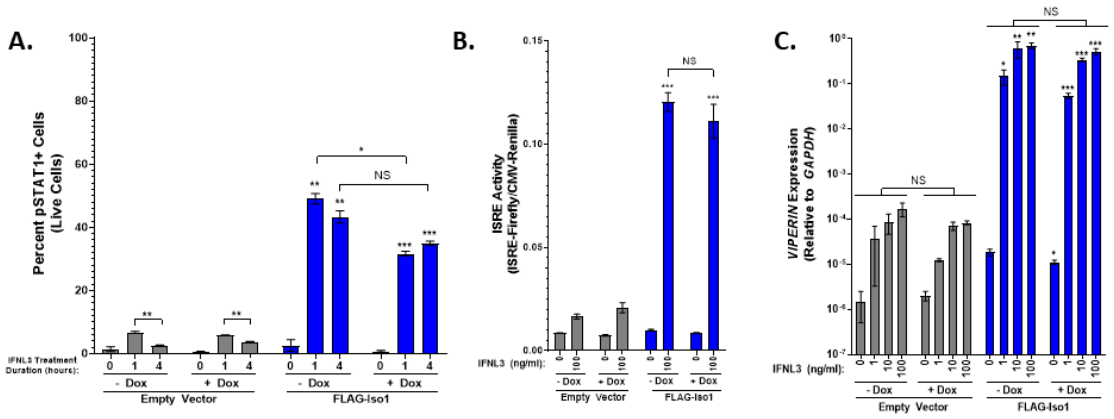


Figure 3.5: FLAG-Iso1 overexpression augments the cellular response to IFNL3. (A) Quantitation of percent pSTAT1+ in HEK293T-EV and FLAG-Iso1 cells after 0, 1, and 4 hr of IFNL3 treatment (100 ng/ml) (representative data, 2 independent experiments). **(B)** HEK293T-EV and FLAG-Iso1 cells were co-transfected with plasmids encoding Firefly Luciferase under control of an ISRE promoter and Renilla Luciferase under control of a CMV promoter. Cells were treated ± dox (100 ng/ml) for 24 hr prior to mock or IFNL3 (100 ng/ml) treatment for 24 hr and harvest for dual luciferase assay (representative data, 3 independent experiments). **(C)** HEK293T-EV and FLAG-Iso1 cells were treated ± dox (100 ng/ml) for 24 hr prior to IFNL3 treatment for 24 hr (0, 1, 10, or 100 ng/ml), then harvested for qRT-PCR analysis of *VIPERIN* and *GAPDH* (representative data, 4 independent experiments). Statistical significance represented by lone asterisks reflect comparisons between identically treated EV and FLAG-Iso1 cells. Statistical significance represented by bars and asterisks reflect comparisons between -dox and +dox conditions within each cell line. Error bars represent standard error of the mean. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. NS = not significant.

Non-canonical FLAG-IFNLR1 isoform 2 differentially modulates the cellular response to IFNL3 dependent on relative receptor expression

IFNLR1 isoform 2 is predicted to harbor a deletion in a portion of the JAK1 binding domain (**Figure 1.3 and 3.1**) [77], and a prior evaluation did not identify capacity for this isoform to support canonical IFNL signaling [126]. We hypothesized that IFNLR1 isoform 2 could function as a dominant negative receptor by binding extracellular ligand without supporting pSTAT1 formation and ISG gene expression, and thus influence the activity of canonical IFNLR1.

To our surprise, pSTAT1 was robustly induced in FLAG-Iso2 cells in -dox, but not +dox conditions, after 1 or 4 hours of IFNL3 treatment (**Figure 3.6A,** representative flow plots in **Supplemental Figure 3**)**.** Consistent with this finding, we observed augmented ISRE activity in IFNL3-treated -dox FLAG-Iso2 cells, which was markedly reduced in +dox conditions (**Figure 3.6B**). We also observed augmented *VIPERIN* expression in IFNL3-treated -dox FLAG-Iso2 cells that was reduced ~6.5-fold in +dox conditions (**Figure 3.6C**). ISRE activity was reduced by pre-incubation of cells with anti-IFNLR1 antibody prior to IFNL3 treatment, demonstrating receptor dependency of the observed phenotype (**Supplemental Figure S4**). Notably, the augmentation in *VIPERIN* expression in IFNL3-treated -dox FLAG-Iso2 cells was ~14 times lower than that observed in IFNL3-treated -dox FLAG-Iso1 cells.

To further evaluate the dose dependency of this unexpected phenotype, we titrated FLAG-Iso2 expression by varying dox concentration (0-100 ng/ml) prior to IFNL3 treatment (relative expression assessed by flow cytometry and western blot is shown in **Supplemental Figure S5**). We observed a decline in ISRE activity as dox concentration increased, demonstrating that higher FLAG-Iso2 expression levels reduced the partial augmentation observed at lower receptor expression levels (**Figure 3.7A**). We evaluated an independent FLAG-Iso2 clone and

found a similar concentration-dependent effect of FLAG-Iso2 expression on ISRE activity (**Figure 3.7B**).

Taken together, these data demonstrate that FLAG-Iso2 expression partially augments the IFNL3 response at low receptor expression levels, a phenotype that is attenuated as receptor expression levels are increased by dox-titration.

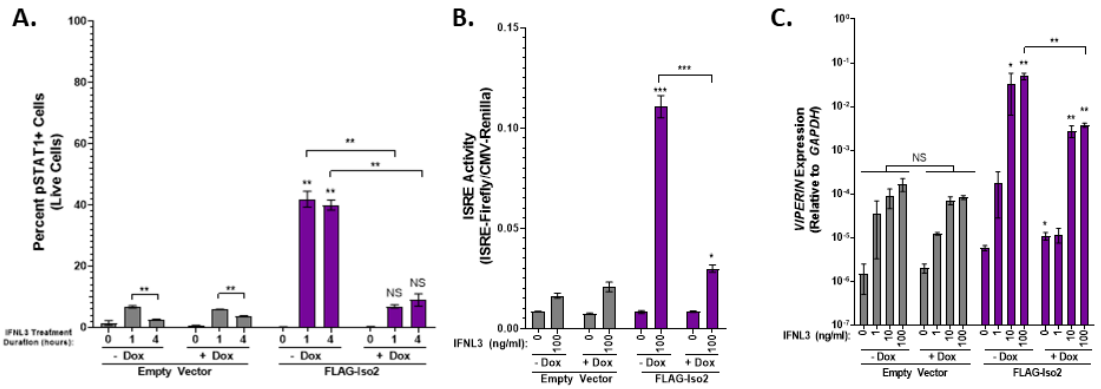


Figure 3.6: FLAG-Iso2 overexpression differentially influences the cellular response to IFNL3 based on receptor abundance. (A) pSTAT1 (2 independent experiments), **(B)** ISRE activity (5 independent experiments), and **(C)** *VIPERIN* expression (3 independent experiments) were analyzed as described in Figure 3. Statistical significance represented by lone asterisks reflect comparisons between identically treated EV and FLAG-Iso2 cells. Statistical significance represented by bars and asterisks reflect comparisons between -dox and +dox conditions within each cell line. Error bars represent standard error of the mean. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. NS = not significant.

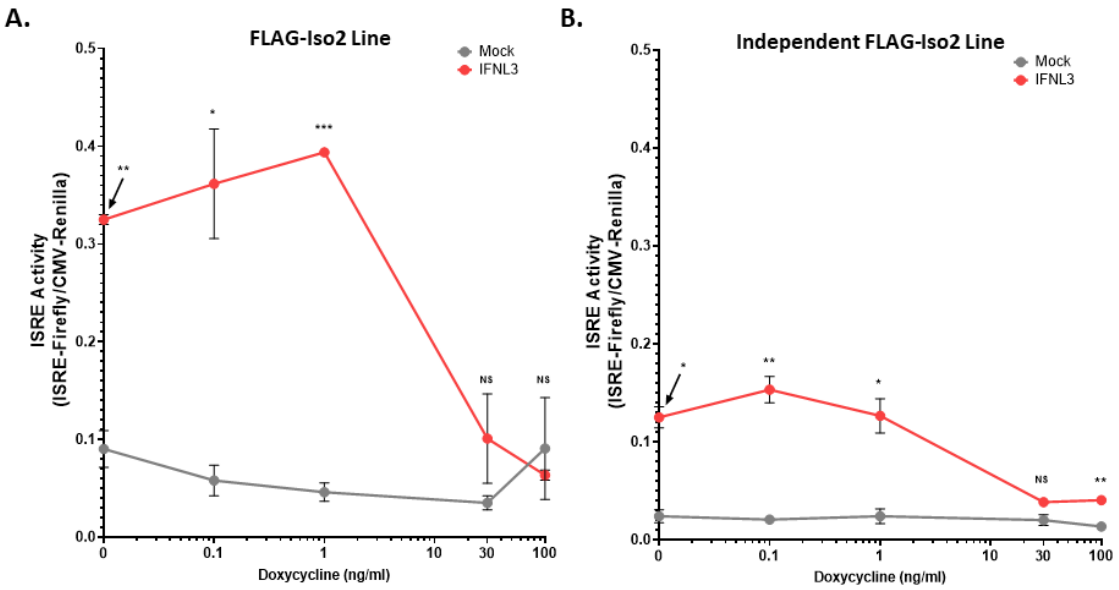


Figure 3.7: The cellular response to IFNL3 is inversely proportional to FLAG-Iso2 abundance.

Two independent FLAG-Iso2 stable lines were transfected with CMV-Renilla and ISRE-Firefly plasmids and dox-treated (dose range between 0-100 ng/ml) for 24 hr. Cells were then treated with IFNL3 (100 ng/ml) for 24 hr prior to dual luciferase assay. **(A)** FLAG-Iso2 clonal line characterized in Figure 4 and **(B)** an additional independent FLAG-Iso2 clonal line (representative data, 2 independent experiments). Statistical analysis compares IFNL3 relative to mock-treated cells at a given dox concentration. Error bars represent standard error of the mean. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. NS = not significant.

Overexpression of non-canonical IFNLR1 isoform 3 partially augments the cellular response to IFNL3

We next evaluated the effect of FLAG-Iso3 overexpression, as IFNLR1 isoform 3 has been shown to inhibit expression of ISGs in IFNL-treated HepG2 cells and PBMCs [128, 129]. Contrary to these prior reports, we observed a small but significant increase in pSTAT1 in FLAG-Iso3 cells in +dox conditions after IFNL3 treatment, but not after mock treatment (**Figure 3.8A,** representative flow plots in **Supplemental Figure S3**). FLAG-Iso3 cells also demonstrated a modest increase in ISRE activity in both -dox and +dox conditions and *VIPERIN* expression in +dox conditions after IFNL3 treatment (**Figure 3.8B-C**). Notably, the magnitude of IFNL3-induced *VIPERIN* expression (- dox: ~5-fold induction; +dox: ~29-fold induction) was substantially lower than that observed in FLAG-Iso1 and -dox FLAG-Iso2 cells.

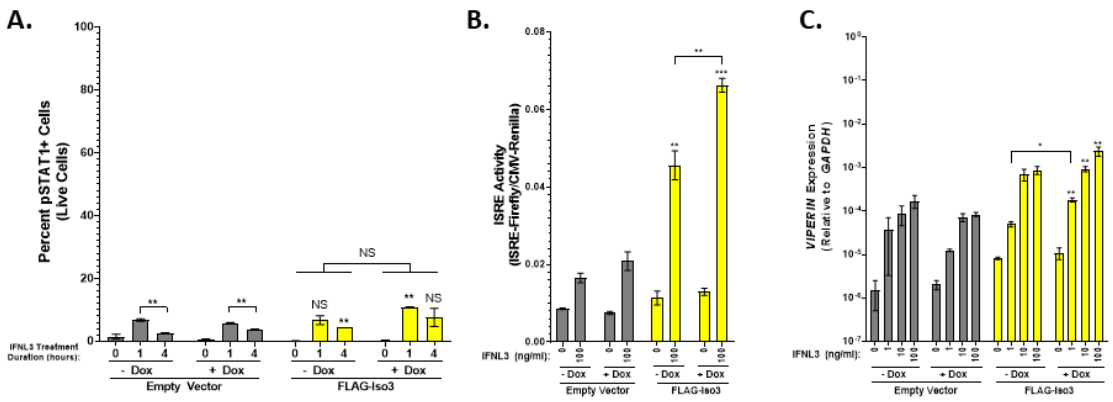


Figure 3.8: FLAG-Iso3 expression partially augments IFNL signaling. (A) pSTAT1 (2 independent experiments), **(B)** ISRE activity (3 independent experiments), and **(C)** *VIPERIN* expression (3 independent experiments) were analyzed as in Figure 3. Statistical significance represented by lone asterisks reflect comparisons between identically treated EV and FLAG-Iso3 cells. Statistical significance represented by bars and asterisks reflect comparisons between -dox and +dox conditions within each cell line. Error bars represent standard error of the mean. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. NS = not significant.

Overexpression of FLAG-Iso1 uniquely augments expression of inflammatory genes

Constitutive overexpression of IFNLR1 isoform 1 has been shown to promote *de novo* expression of the pro-inflammatory cytokine *CXLC10* after IFNL3 treatment [87]. We thus hypothesized that expression levels of FLAG-Iso1, FLAG-Iso2, and FLAG-Iso3 that supported IFNL3-dependent *VIPERIN* expression would also support capacity of cells to induce *CXCL10*. Consistent with prior studies, we observed augmented *CXCL10* expression in IFNL3-treated FLAG-Iso1 cells in both -dox and +dox conditions (**Figure 3.9**). Unexpectedly, expression of FLAG-Iso2 or FLAG-Iso3 did not result in *CXCL10* expression after 24 hours of IFNL3 treatment, suggesting non-canonical IFNLR1 isoforms may not support expression of pro-inflammatory genes.

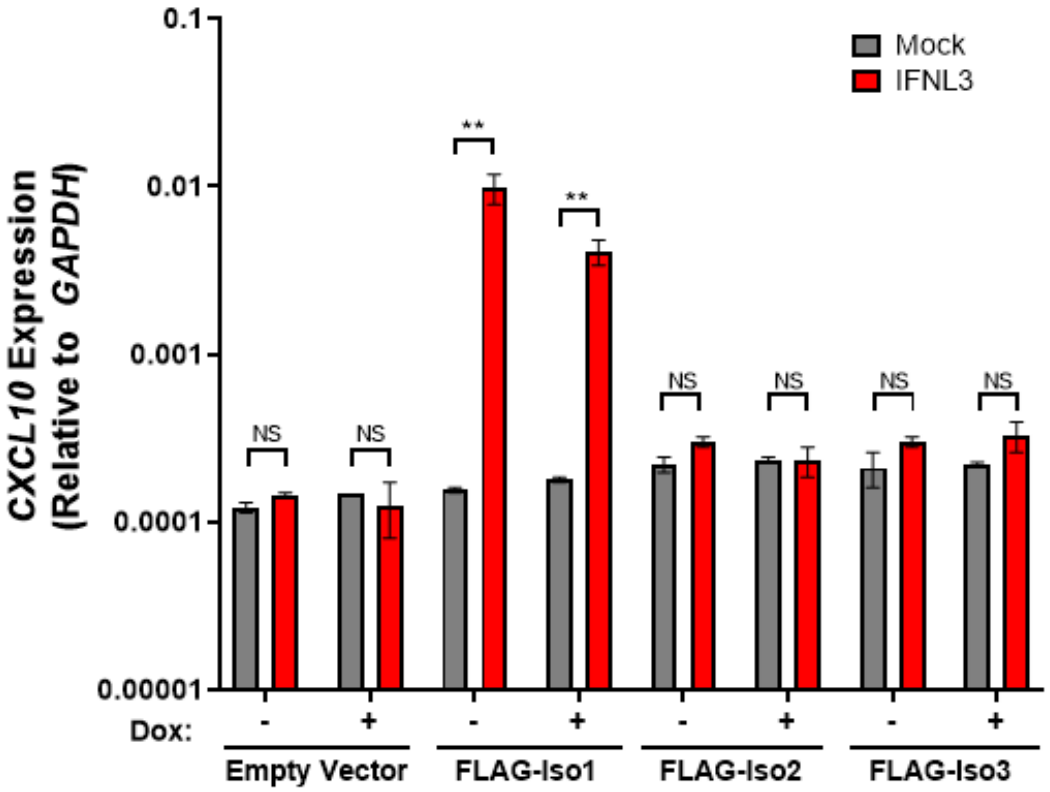


Figure 3.9: FLAG-Iso1 overexpression uniquely supports expression of the pro-inflammatory gene *CXCL10*. HEK293T stable lines were induced ± dox (100 ng/ml) for 24 hr, then mock or IFNL3 (100 ng/ml) treatment for an additional 24 hr, then evaluated by qRT-PCR for *CXCL10* expression relative to *GAPDH* (representative data, 2 independent experiments). Error bars represent standard error of the mean. ** = $p < 0.01$, NS = not significant.

To further explore differential induction of antiviral and inflammatory ISGs, we performed gene expression profiling of immune and inflammatory genes using the NanoString Immunology 2.0 panel. We identified 61 differentially expressed genes (DEGs, 2-fold change) that demonstrated varied expression upon treatment with dox or IFNL3 (**Supplemental Figure S6**). A representative subset of DEGs which include interferon stimulated genes, transcription factors, pro-inflammatory cytokines, negative regulators of IFN signaling, and antigen presentation and pathogen detection genes is displayed in **Figure 3.10**.

We identified 40 DEGs when comparing mock and IFNL3-treated FLAG-Iso1 cells, relative to only 6 and 4 DEGs affected by IFNL3 in WT and HEK293T-EV lines, respectively, demonstrating that FLAG-Iso1 expression increases the magnitude and breadth of IFNL3-dependent transcriptional activation. Consistent with our prior analysis of *VIPERIN* and *CXCL10* (**Figures 3.5** and **3.9**), IFNL3-treated FLAG-Iso1 cells exhibited upregulation of multiple antiviral ISGs (e.g. *IFITM1*, *IFIT2*, *BST2*) as well as pro-inflammatory markers and chemokines (*IRF1*, *CXCL10*, *CXCL11*), irrespective of dox-induction and consistent with prior reports [87, 88, 127]. In addition, we observed upregulation of known negative regulators of IFN signaling (*SOCS1*, *SOCS3*) [91-93], critical mediators of IFN signaling (*STAT1*, *STAT2*), and genes important for pathogen detection (*RARRES3, TLR3*) and antigen presentation (e.g. *TAP1, TAP2*).

We identified only 20 DEGs when comparing mock and IFNL3-treated FLAG-Iso2 cells, 18 of which were also identified in the FLAG-Iso1 dataset. Similar to FLAG-Iso1 cells, but to a lesser extent, we observed an IFNL3-dependent augmentation of select ISGs (e.g. *IFITM1*, *STAT1*/*STAT2*) and negative regulators of IFN signaling (*SOCS1*) in -dox conditions, an augmentation that was notably reduced by the addition of dox, consistent with **Figures 3.6 and 3.7**). In contrast to the FLAG-Iso1 dataset, we did not observe induction of pro-inflammatory genes after IFNL3 treatment in FLAG-Iso2 cells, regardless of dox induction.

We identified 11 DEGs when comparing mock and IFNL3-treated FLAG-Iso3 cells. We observed partial augmentation of select antiviral ISGs regardless of dox-induction. Similar to FLAG-Iso2, we observed slight induction of *SOCS1* but no induction of pro-inflammatory genes.

Importantly, we observed no change in expression of type-I IFN receptor subunits (*IFNAR1*, *IFNAR2*, **Supplemental Figure S7**) or apoptosis/cell stress genes (e.g. *BAX, BCL2, CASP2*, data not shown) in any condition. This is consistent with the lack of impact of construct expression and IFNL3 treatment on cellular viability we had previously observed by flow cytometry (data not shown).

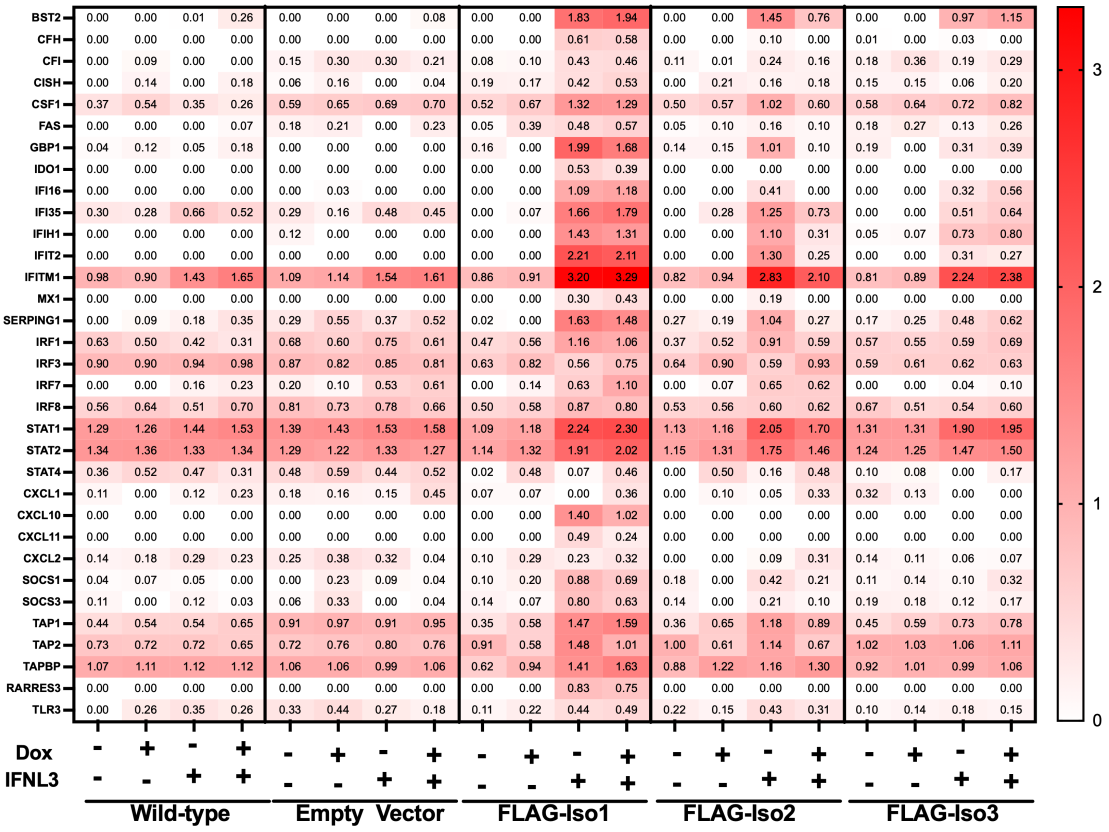


Figure 3.10: Relative expression of FLAG-IFNLR1 isoforms differentially influences IFNL3-dependent gene expression. HEK293T WT and stable lines were induced ± dox (100 ng/ml) for 24 hr prior to treatment ± IFNL3 (100 ng/ml) for an additional 24 hr. RNA was collected and gene counts were quantitated using NanoString analysis (nCounter Human Immunology v2 Panel). Data are shown as log transformed normalized counts of differentially expressed genes (>2-fold change in any condition).

Overexpression of FLAG-Iso1 reduces the impact of type-I IFN signaling

The type-I IFN response is highly regulated to temper the potentially deleterious effects of pathway overactivation [44, 91], and it is established that there is significant cross talk between type-I and IFNL signaling pathways [13]. Specifically, it has been demonstrated that stimulation of the IFNL system results in potent inhibition of a subsequent type-I IFN response, mediated in part through upregulation of negative regulators of type-I IFN signaling [93]. Consistent with this observation, our NanoString analysis identified that FLAG-IFNLR1 isoform 1 overexpression enhances IFNL3-mediated upregulation of known negative regulators of IFN signaling, including *SOCS1* and *SOCS3* (**Figure 3.10)**.

As IFNLR1/IL10RB and IFNAR1/IFNAR2 pathways both utilize several shared signaling molecules, including JAK1 and TYK2, we hypothesized that overexpression of FLAG-IFNLR1 isoforms may also affect the cellular response to type-I IFNs even in the absence of IFNL3 pre-treatment. To test this hypothesis, we evaluated pSTAT1 in HEK293T-EV and FLAG-IFNLR1 isoform lines after stimulation with the type-I IFN IFNA2 (**Figure 3.11A,** representative flow plots in **Supplemental Figure S3**). Relative to HEK293T-EV cells, each FLAG-IFNLR1 isoform line demonstrated slightly reduced IFNA2-dependent pSTAT1 signal after 1 and 4 hours of IFNA2 stimulation (**Figure 3.11A**). Surprisingly, we observed a marked reduction in IFNA2-dependent pSTAT1 signal in +dox relative to -dox FLAG-Iso1 cells after IFNA2 treatment. We observed only marginal differences in the IFNA2 response in FLAG-Iso2 and FLAG-Iso3 cells between conditions. To further evaluate this observation, we examined pSTAT1 by western blot after 1 hour of treatment with IFNL3 or IFNA2 (**Supplemental Figure S8**). Consistent with our prior flow cytometry analysis, the highest pSTAT1 induction after IFNL3 treatment was observed in FLAG-Iso1 cells, irrespective of dox treatment. In contrast, there was a notable reduction in pSTAT1 in

IFNA2-treated +dox FLAG-Iso1 cells relative to other lines. As we observed no differences in *IFNAR1* or *IFNAR2* gene expression between lines (**Supplemental Figure S7**), these data suggest that high levels of FLAG-Iso1 expression may partially inhibit IFNA2-dependent STAT1 phosphorylation.

Next, we evaluated the impact of FLAG-IFNLR1 overexpression on the capacity of IFNA2 to stimulate expression of *VIPERIN*. In HEK293T-EV, FLAG-Iso2, and FLAG-Iso3 cells, we observed robust induction of *VIPERIN* after IFNA2 treatment that was unaffected by pre-treatment with dox (**Figure 3.11B**). In contrast, dox-induction of FLAG-Iso1 prior to IFNA2 treatment resulted in a small but significant decrease in *VIPERIN* expression (**Figure 3.11B**). Taken together, these results suggest that overexpression of FLAG-Iso1 partially impairs the cellular response to IFNA2.

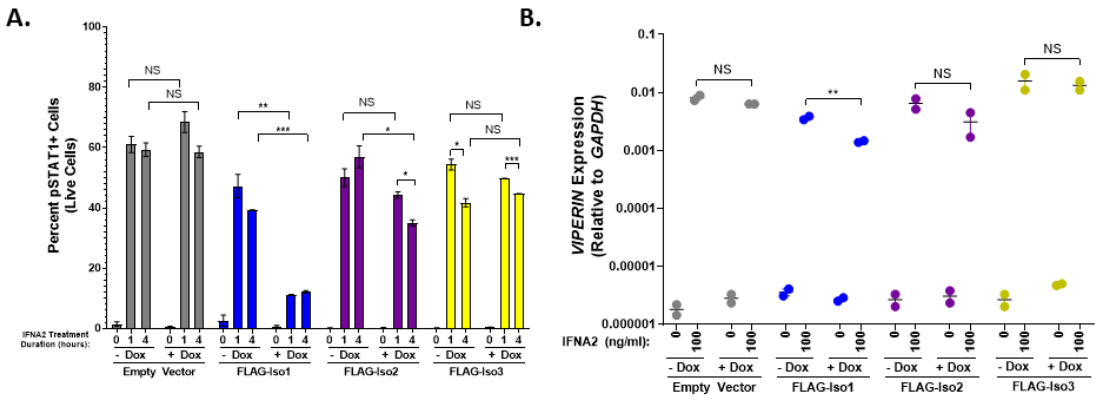


Figure 3.11: Overexpression of FLAG-Iso1 partially reduces the cellular response to the type-I IFN ligand IFNA2. (A) Quantitation of percent pSTAT1+ in HEK293T-EV and FLAG-IFNLR1 isoform cells after 0, 1, and 4 hr of IFNA2 treatment (100 ng/ml) (representative data, 2 independent experiments). **(B)** HEK293T stable lines were ± dox (100 ng/ml) treated for 24 hr, then mock or IFNA2 (100 ng/ml) treated for 24 hr, then harvested for qRT-PCR analysis of *VIPERIN* and *GAPDH* (representative data, 2 independent experiments). Statistical significance represented by bars and asterisks reflect comparisons between the indicated conditions. Error bars represent standard error of the mean. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. NS = not significant.

CHAPTER 4: DISCUSSION

In this study, we identified a unique influence of relative expression of IFNLR1 isoforms on the cellular transcriptional response to interferons. We demonstrate that minimal overexpression of FLAG-Iso1 is sufficient to markedly augment IFNL3-dependent STAT1 phosphorylation, ISRE-promoter activation, and induction of antiviral and pro-inflammatory genes, a phenotype which could not be further augmented at greater FLAG-Iso1 expression levels. Furthermore, FLAG-Iso1 overexpression partially impaired signaling induced by IFNA2. Surprisingly, we found that FLAG-Iso2 partially augmented IFNL3-dependent antiviral gene expression and phosphorylation of STAT1 at low receptor expression levels, but this augmentation was markedly reduced at greater receptor expression levels. FLAG-Iso3 expression also modestly increased IFNL3-dependent antiviral gene expression and phosphorylation of STAT1, albeit to a much lower extent than either FLAG-Iso1 or FLAG-Iso2. Strikingly, in contrast to FLAG-Iso1, neither FLAG-Iso2 or FLAG-Iso3 supported expression of pro-inflammatory genes after IFNL3 treatment, nor did they substantially impact the cellular response to IFNA2. These data suggest that relative IFNLR1 isoform expression could influence the balance of antiviral and inflammatory genes induced by interferons.

Our observation that FLAG-Iso1 overexpression robustly augments antiviral gene expression is consistent with previous reports [87, 127], suggesting that our findings are not an artifact of the addition of a FLAG tag or of the cellular system (summary of findings shown in **Figure 4.1**). In addition, we observed *de novo* expression of *CXCL10* and other pro-inflammatory genes upon FLAG-Iso1 over-expression and treatment with IFNL3, also consistent with prior reports [87]. Contrary to our hypothesis, we were unable to titrate FLAG-Iso1 to allow selective upregulation of antiviral ISGs without inducing pro-inflammatory genes, as levels of FLAG-Iso1 undetectable by western blot or flow cytometry in -dox conditions were sufficient to support a maximal response to IFNL3. This observation would be consistent with the suggested stochastic

or bimodal nature of the IFNL response, which suggests cells under-go an “all or nothing” response when stimulated with IFNs [138, 139]. These data also suggest that IFNLR1 isoform 1 abundance could be tightly regulated *in vivo* as a means to control pathway activity and prevent cells from expressing excessive and potentially deleterious pro-inflammatory genes. As it is well-established that tetracycline-inducible promoters allow some extent of construct expression even in the absence of tetracyclines [140, 141], use of alternative gene expression control strategies may prove useful in future studies to further assess whether very low levels of receptor expression allow for a titratable phenotype.

There is a high degree of cross talk between IFN systems [13]. Specifically, IFNL stimulation has been demonstrated to have a potent negative effect on the type-I IFN response through induction of negative regulators of IFN signaling [91], as we observed in IFNL3-treated FLAG-Iso1 cells. As such, we were surprised to observe that overexpression of FLAG-Iso1, even in the absence of IFNL3 pre-treatment, resulted in a marked reduction in IFNA2-dependent pSTAT1 induction. Notably, we observed a significant albeit less marked reduction in pSTAT1 in +dox FLAG-Iso1 cells after IFNL3 treatment. We speculate that overexpression of excess levels of FLAG-Iso1 could thus in-fluence the immediate cellular response to IFNs by binding and potentially sequestering signaling molecules required for propagation of the IFN response. The less notable differences observed in ISRE promoter activity and ISG expression when comparing -dox and +dox FLAG-Iso1 cells 24 hours after IFN stimulation could reflect adequacy of a partial pSTAT1 response to mediate gene expression or may relate to secondary signaling events that occur during *in vitro* culture.

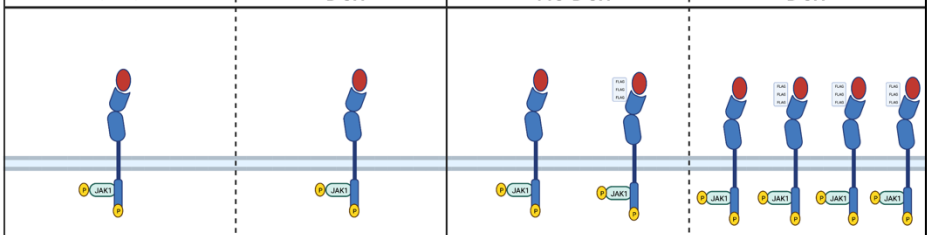
	Summary of FLAG-Iso1 Results			
	IFNL3 Treated			
	HEK293T-Empty Vector		HEK293T-FLAG-Iso1	
	No Dox	Dox	No Dox	Dox
				
pSTAT1	No change from WT	No change from WT	Increased pSTAT1	Increased pSTAT1
ISRE	No change from WT	No change from WT	Increased ISRE activity	Increased ISRE activity
Transcriptional Profile	No change from WT	No change from WT	Augmented antiviral and pro-inflammatory genes	Augmented antiviral and pro-inflammatory genes
IFNA2 response	No change from WT	No change from WT	No change from EV	Reduced

Figure 4.1: Summary of FLAG-Iso1 Results.

We were surprised to find that minimal FLAG-Iso2 overexpression partially augmented IFNL3-induced expression of antiviral ISGs without concurrent induction of pro-inflammatory cytokines and chemokines (summary of findings shown in **Figure 4.2**). While FLAG-Iso2 expression led to partial upregulation of the inflammatory transcription factor IRF1 after IFNL3 treatment, we did not observe augmentation of genes under its transcription control (e.g. *CXCL10*). Consequently, the magnitude of IRF1 induction as a function of FLAG-Iso2 expression may be insufficient to promote widespread changes in pro-inflammatory genes, or alternatively, FLAG-Iso2 may engage signaling mechanisms distinct from FLAG-Iso1. These results differ from a prior report that suggested IFNLR1 isoform 2 does not support IFNL3 signaling [126]. Consistent with this prior report, where IFNLR1 isoform 2 was constitutively expressed off the pEF2 promoter [126], we found that IFNL3 signaling was markedly reduced at high levels of FLAG-Iso2 expression in +dox conditions. We conclude that IFNLR1 isoform 2 may be capable of augmenting IFNL signaling at low, but not high levels of receptor expression.

It has been shown that overexpression of cell surface receptors can decrease the cellular response to their cognate biological stimuli [142], and that overexpression of a receptor subunit without concordant overexpression of downstream signaling molecules can lead to the formation of receptor complexes capable of binding ligand but unable to transduce signal [143]. As FLAG-Iso1 overexpression augmented the IFNL3 response, regardless of dox-induction, we find it less likely that FLAG-Iso2 overexpression diminished cell sensitivity to IFNL3 by titrating necessary signaling components. Rather, it is possible that FLAG-Iso2 influences signaling through endogenous IFNLR1 isoform 1 or can support signaling in tandem with IL10RB only at low levels. While the mechanisms underlying the effect of FLAG-Iso2 on the cellular response to IFNL3 are not clearly established by this work, our data support the hypothesis that IFNLR1

isoform 2 plays a role in modulating the sensitivity of cells to IFNLs and could thus potentially provide a tunable mechanism of IFNL regulation.

In contrast to our findings in IFNA2-treated FLAG-Iso1 cells, pSTAT1 induction was only modestly reduced in +dox FLAG-Iso2 cells and only after 4 hours of IFNA2 treatment. Notably, this reduced response was less marked than the reduced response to IFNL3 observed in +dox FLAG-Iso2 cells. Similarly, we observed no impact of FLAG-Iso2 overexpression on *VIPERIN* expression in IFNA2 relative to IFNL3-treated cells. Thus, FLAG-Iso1 overexpression has a more immediate impact on pSTAT1 induction after IFNA2 relative to IFNL3 treatment, whereas FLAG-Iso2 overexpression has the opposite impact. While the underlying mechanism by which FLAG-Iso1 and FLAG-Iso2 differentially affect signaling by IFNL3 and IFNA2 is not clearly established by this work, we hypothesize that structural differences between FLAG-Iso1 and FLAG-Iso2, with the latter missing a significant portion of its predicted JAK1 binding do-main, may contribute. Future work to examine relative differences in recruiting and/or binding of JAK1 and STAT1 to FLAG-Iso1 and FLAG-Iso2 after treatment with interferons will help further elucidate a potential mechanism for these observations.

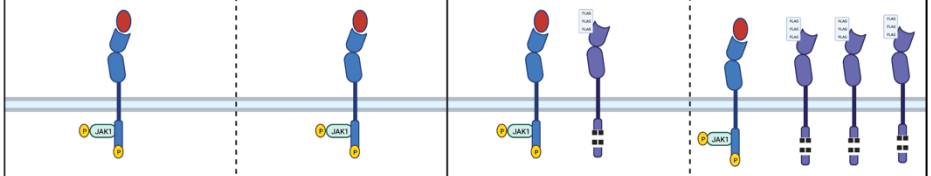
	Summary of FLAG-Iso2 Results			
	IFNL3 Treated			
	HEK293T-Empty Vector		HEK293T-FLAG-Iso2	
	No Dox	Dox	No Dox	Dox
				
pSTAT1	No change from WT	No change from WT	Increased pSTAT1	Loss of increased pSTAT1
ISRE	No change from WT	No change from WT	Increased ISRE activity	Loss of increased ISRE activity
Transcriptional Profile	No change from WT	No change from WT	Augmented antiviral without induction of pro-inflammatory genes	Loss of augmented antiviral gene expression
IFNA2 response	No change from WT	No change from WT	No change from EV	No change from EV

Figure 4.2: Summary of FLAG-Iso2 Results.

Overexpression of FLAG-Iso3 resulted in a subtle yet consistent augmentation of IFNL3-dependent STAT1 phosphorylation, ISRE-promoter activation, and induction of the antiviral gene *VIPERIN*, albeit to a much lower extent than FLAG-Iso1 or FLAG-Iso2 (summary of results shown in **Figure 4.3**). Similar to FLAG-Iso2, FLAG-Iso3 did not support induction of *CXCL10*, and NanoString analysis confirmed that FLAG-Iso3 partially augmented IFNL3-induced anti-viral genes but not pro-inflammatory genes. Prior studies investigating the influence of IFNLR1 isoform 3 on the IFNL response have shown that it can act as an inhibitor of IFNL signaling [128, 129]. In HepG2 cells, IFNLR1 isoform 3, also referred to as short IFNLR1, is secreted, can bind IFNL1, and negatively impacts IFNL1-dependent expression of MHC class-I [129]. In PBMCs, recombinant IFNLR1 isoform 3 binds to the cell surface and in-creases binding of ligand, but negatively regulates ISG induction [128]. In contrast to our work, these studies utilized recombinant, purified IFNLR1 isoform 3, which differs in concentration and absence of factors present in cellular supernatants, both of which could have influenced the observed differences. We have performed a variation of this experimental design through pre-incubation of IFNL3 ligand with conditioned media collected from Dox-induced FLAG-Iso3 cells, which was then used to stimulate WT HEK293T cells (**Figure 4.4**). Notably, we observed a blunted IFNL3-dependent pSTAT1 signal in cell treated with FLAG-Iso3 conditioned media as opposed to conditioned media collected from HEK293T-EV cells (**Figure 4.4**). While this preliminary finding needs to be further investigated, it is consistent with prior literature and reinforces the hypothesis that the effect of IFNLR1 isoform 3 on the IFNL response is dependent on several biological and experimental factors. Specifically, we speculate that the impact of IFNLR1 isoform 3 may be both receptor concentration and cell type-dependent, similar to what has been observed for the soluble IFNAR2 isoform [43, 62].

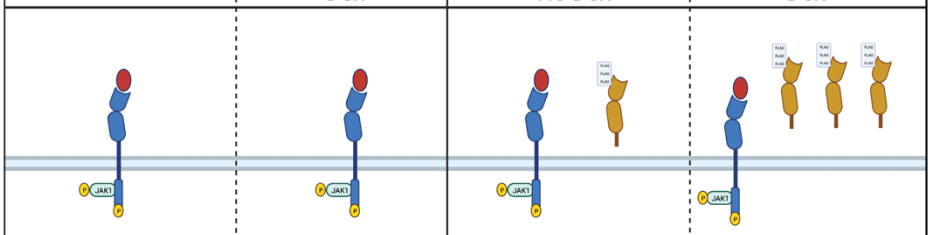
	Summary of FLAG-Iso3 Results			
	IFNL3 Treated			
	HEK293T-Empty Vector		HEK293T-FLAG-Iso3	
	No Dox	Dox	No Dox	Dox
				
pSTAT1	No change from WT	No change from WT	Increased pSTAT1	Further increase in pSTAT1
ISRE	No change from WT	No change from WT	Increased ISRE activity	Further increase in ISRE activity
Transcriptional Profile	No change from WT	No change from WT	Augmented antiviral without induction of pro-inflammatory genes	Further augmentation in antiviral gene expression
IFNA2 response	No change from WT	No change from WT	No change from EV	No change from EV

Figure 4.3: Summary of FLAG-Iso3 Results.

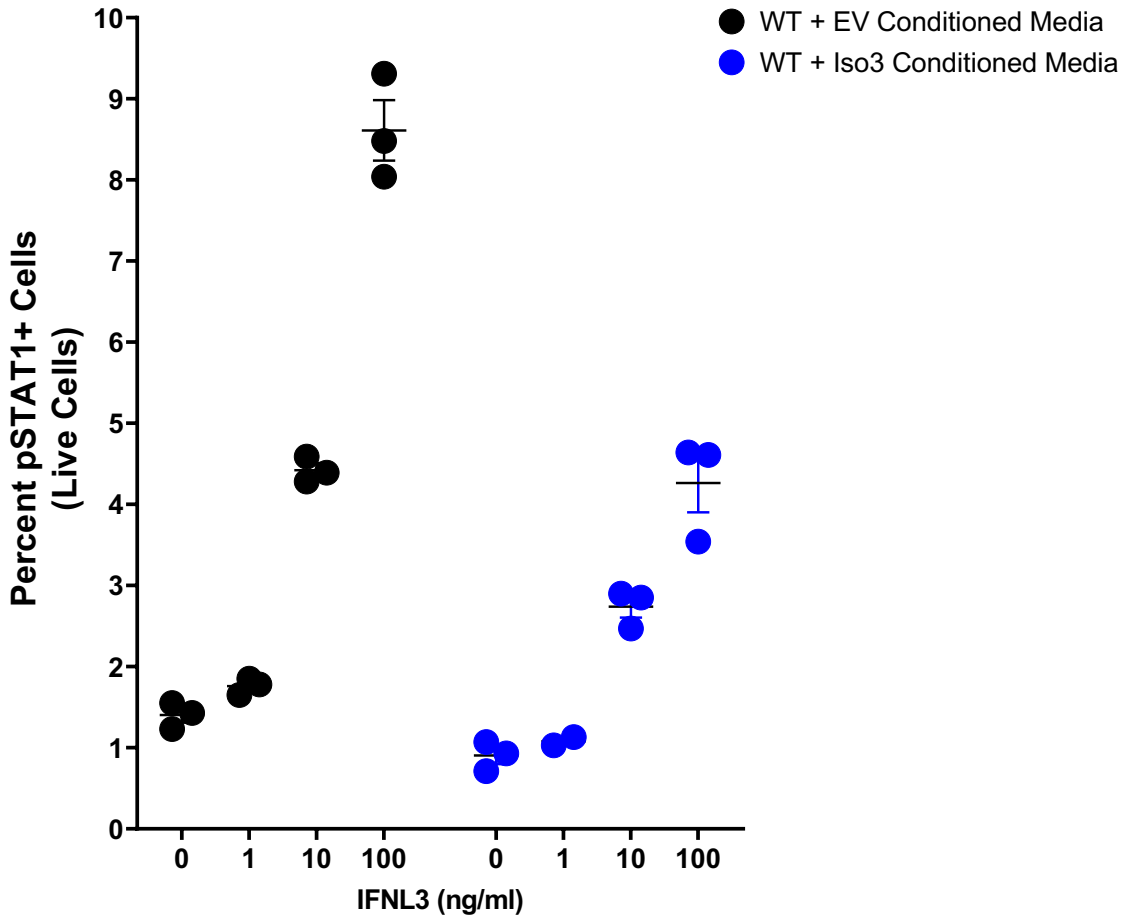


Figure 4.4: Pre-incubation of IFNL3 with FLAG-Iso3 conditioned media reduces IFNL3-dependent pSTAT1 in WT HEK293T cells. Conditioned media was collected from HEK293T-EV and FLAG-Iso3 stable lines after 48 hours of dox treatment (100ng/ml). IFNL3 (0-100ng/ml) was pre-incubated with either EV or FLAG-Iso3 conditioned media for 2 hours prior to treatment of WT HEK293T cells for 1 hour, after which cells were then assessed for pSTAT1 content.

CHAPTER 5: FUTURE DIRECTIONS

Dissecting the Underlying Mechanisms of IFNLR1 Isoform Signaling

In this work, we demonstrated that IFNLR1 isoforms uniquely and specifically modulate the IFNL response in HEK293T stable lines. As these isoforms could play a role in the natural course of viral infection, or in influencing the outcome of IFNL therapy, it is essential to continue investigating their underlying signaling mechanisms. In this section, I propose key questions and experiments that will provide additional insight into the mechanisms underlying how IFNLR1 isoforms affect IFN signaling.

Why does minimal FLAG-Iso1 expression in -dox conditions result in maximal cellular response to IFNL that cannot be titrated?

Our work investigating FLAG-Iso1's contribution to the IFNL response is consistent with the previous literature in that stable expression of FLAG-Iso1 led to greater antiviral gene expression and *de novo* expression of inflammatory genes [87, 127]. However, we were surprised to observe a maximal transcriptional response after IFNL treatment at very low receptor levels (-dox) that could not be detected by western blot or flow cytometry. This suggests that endogenous IFNLR1 is limiting and that minimal protein in -dox conditions is adequate for a marked increase in susceptibility to IFNL3.

To interpret these data, it is helpful to consider that cytokine receptor abundance and surface localization are tightly controlled to regulate cellular responses to potentially damaging stimulation [56, 144]. As such, cytokine receptors tend to exhibit low expression [130], often at abundances lower than the threshold of detection for routine laboratory measurement [131]. In our HEK293T stable cell line, we are overexpressing FLAG-Iso1 with endogenous IFNLR1 expression left intact. Consequently, the most likely explanation for our findings is that endogenous IFNLR1 is limiting and that the small amount of extra receptor present in -dox

conditions is adequate to fully sensitize cells. Alternatively, it is also possible that minimal overexpression of FLAG-Iso1 cooperates with existing expression of the endogenous IFNLR1 receptor to drive a maximal IFNL response.

To distinguish these possibilities, CRISPR-Cas9 genome editing can be used to knock-out (KO) endogenous IFNLR1 expression in WT HEK293T cells to allow expression of only FLAG-Iso1. We attempted to produce HEK293T IFNLR1 KO lines multiple times using various transfection methods (ViaFect, Neon Transfection), but the cells were consistently non-viable after puromycin selection. Future efforts to generate KO HEK293T lines could benefit from use of viral vectors to increase the efficiency and viability of IFNLR1 KO cells. Once the IFNLR1 KO lines are verified by DNA sequencing and observation of loss of response to IFNL treatment (compared to WT HEK293T cells), cells could be stably transfected with FLAG-Iso1 (HEK293T IFNLR1 KO + FLAG-Iso1). If the maximal IFNL responses we observe in the -dox conditions with minimal FLAG-Iso1 overexpression (-dox) works in conjunction with endogenous IFNLR1 receptor, I hypothesize that non-dox treated HEK293T IFNLR1 KO + FLAG-Iso1 cells will exhibit reduced IFNL responses compared to the FLAG-Iso1 overexpression line. If we continue to observe maximal IFNL responses in non-dox treated HEK293T IFNLR1 KO + FLAG-Iso1 cells, this would suggest that the minimal protein expression driven from the tetracycline promoter in the absence of dox is adequate to support a full response, showing that IFNLR1 receptor expression is truly limiting under physiological conditions, at least in this particular cell line [140, 141]. Alternative strategies to more tightly regulate construct gene expression, such as use of Tet-off system or manipulation of the *IFNLR1* promoter to regulate endogenous expression levels, could be used to further assess whether finer control of FLAG-Iso1 expression could lead to a titratable response.

Why does increased FLAG-Iso1 expression not further augment the IFNL response?

We observe a reliable increase in FLAG-Iso1 expression as a function of dox concentration. Consequently, it was surprising that greater FLAG-Iso1 receptor levels did not translate into further augmentation of the IFNL response. A simple explanation could be that the IFNL response in non-induced FLAG-Iso1 cells has already reached the cellular maximum, as it is already ~100 fold greater than the IFNA2 response. However, it is also conceivable that lack of further augmentation with greater FLAG-Iso1 abundance is due to a limiting factor present in the signaling pathway. In other words, increased FLAG-Iso1 overexpression without a concomitant increase in necessary signaling molecules (i.e. IL10RB, JAK1, STAT1/STAT2) could result in reaching a signaling ceiling. This effect is well characterized in the recent study by Sta et al. 2022, who showed that imbalanced overexpression of receptor and signaling components in T-cells can negatively impact signaling magnitude [143]. The logic underlying these findings, when applied to our overexpression of FLAG-Iso1, leads to the formulation of the following hypothesis: Further augmentation of the IFNL3 response in dox-induced FLAG-Iso1 cells is limited due to imbalanced stoichiometry between FLAG-Iso1 and one of its necessary signaling components, such as IL10RB, JAK1, or STAT1/STAT2. This hypothesis could be tested using the HEK293T FLAG-Iso1 overexpression line characterized in the results section through transiently transfecting with either IL10RB, JAK1, or STAT1/STAT2 prior to ± dox induction and ± IFNL3 treatment. If there are limiting factors, then I would expect that overexpression of IL10RB, JAK1, or STAT1/STAT2 would result in further augmentation of the IFNL3 response in +dox treated cells as opposed to -dox treated cells. Alternatively, if overexpression of signaling molecules does not augment the +dox phenotype, then it suggests that the IFN response ceiling has been met.

How does FLAG-Iso1 expression reduce the type-I IFN response?

We showed that FLAG-iso1 markedly reduced IFNA2-dependent pSTAT1 signal. As discussed previously, stimulation of the IFNL system can lead to expression of negative regulators of the type-I IFN system that are themselves ISGs, such as SOCS1, SOCS3, and USP18 [91, 93]. Unexpectedly, we observed that overexpression of FLAG-Iso1 by itself, without IFNL3 stimulation, resulted in diminished cellular responses to IFNA2. As the type-I IFN and IFNL systems share several signaling molecules, I hypothesize that increased expression of FLAG-Iso1 sequesters machinery necessary for type-I IFN signaling. Since JAK proteins have been demonstrated to constitutively associate with their cognate receptor, I further hypothesize that the diminished type-I IFN response is due to titration of JAK1 away from IFNAR2 [145]. The effect of FLAG-Iso1 overexpression on the type-I IFN response being observed at the level of STAT1 phosphorylation lends support to this hypothesis, as there may be a deficit in signal transduction between ligand binding, JAK1/TYK2 activation, and STAT1/STAT2 recruitment. This hypothesis can be tested by transient transfection of JAK1 plasmid in HEK293T-EV and FLAG-Iso1 cells prior to ± dox induction and ± IFNA2 treatment. If JAK1 overexpression rescues the IFNA2 response (no significant difference between EV or ± FLAG-Iso1), then it is suggestive that FLAG-Iso1 is titrating away JAK1 from the type-I IFN signaling pathway.

If overexpression of JAK1 does not rescue IFNA2-dependent pSTAT1 signal to that observed in HEK293T-EV cells, there are several additional hypotheses that can be considered. For example, IFNAR1/IFNAR2 subunit expression could be reduced in FLAG-Iso1 overexpressing cells. We tested this hypothesis by evaluating gene expression of each subunit and identified no change in any FLAG-Iso expressing cell type or condition. However, further investigation should evaluate protein levels using western blot, ELISA, and flow cytometry. Alternatively, FLAG-Iso1 overexpression could somehow result in disruption of IFNAR1/IFNAR2 trafficking to the cell

membrane. As we observed a decrease in IFNA2-dependent pSTAT1 signal in +dox treated FLAG-Iso1 cells, we could consider that substantial overexpression of FLAG-Iso1 could disrupt protein trafficking by causing endoplasmic reticulum stress and triggering the unfolded protein response [146]. I find this hypothesis unlikely, as we did not observe differences in cellular viability among cell lines and conditions, and overexpression in the FLAG-Iso2 and FLAG-Iso3 lines presumably had similar levels of protein being translated, without the same reduction in IFNA2-dependent pSTAT1 signal. As TYK2 has been demonstrated to facilitate trafficking of IFNAR1 to the membrane [147], it is also conceivable that overexpression of FLAG-Iso1 results in sequestration of signaling molecules like TYK2 that could lead to a reduction in IFNAR1 trafficking and membrane abundance. This hypothesis could be tested by overexpressing TYK2 followed by evaluating IFNAR1 levels via flow cytometry in dox-treated FLAG-Iso1 cells. Finally, it is conceivable that overexpression of FLAG-Iso1 results in upregulation of negative regulators of type-I IFN signaling, such as SOCS1, SOCS3, or USP18 [91-93]. As there were no significant differences in expression of any genes between FLAG-IFNLR1 isoform cells ±dox in the absence of IFNL3 treatment, including *SOCS1* and *SOCS3*, this mechanism is not likely. However, to further rule out this possibility, gene expression and protein levels of USP18, the other primary negative regulator of type-I IFN signaling, can be evaluated.

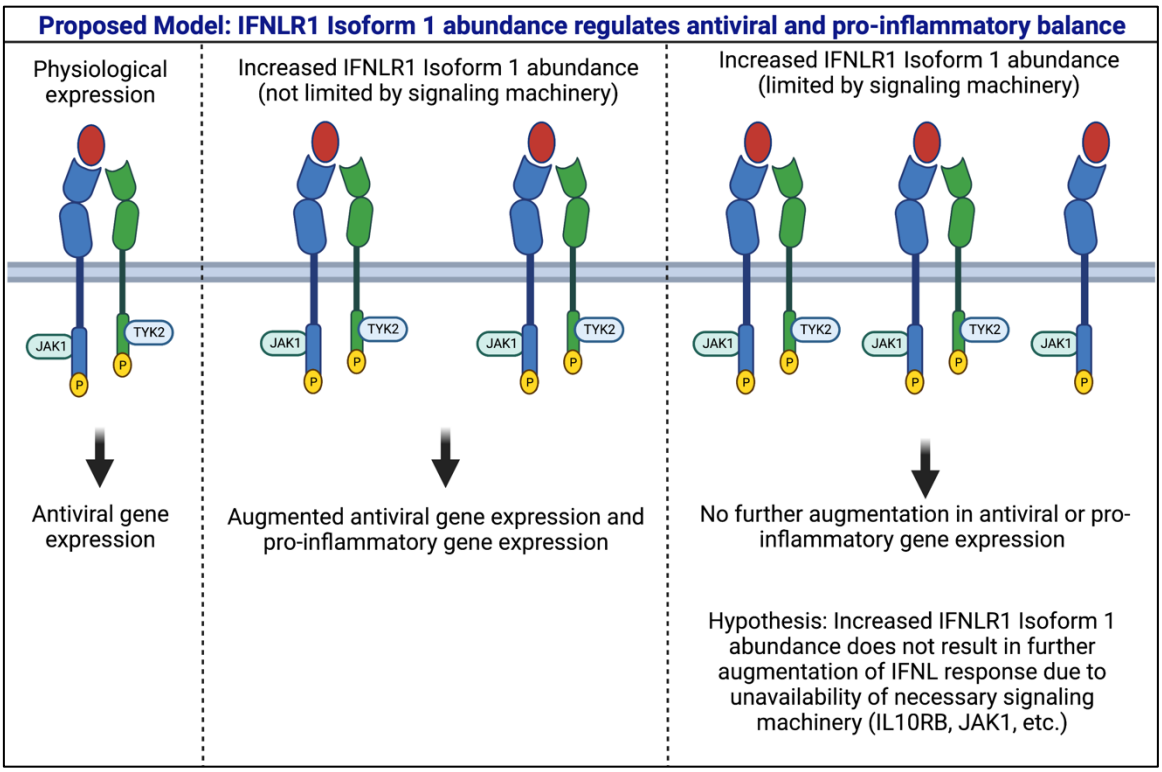


Figure 5.1: Proposed model of IFNLR1 Isoform 1 Signaling.

Can FLAG-Iso2 support IFNL signaling in the absence of endogenous IFNLR1?

We were surprised to observe that minimal overexpression (-dox) of FLAG-Iso2 augmented the IFNL3 response, whereas further expression paradoxically led to an abrogation of augmentation. This is discordant from the single study published to date investigating overexpression of IFNLR1 isoform 2 in HEK293 cells, which showed no effect on ISRE activity after treatment with IFNL3 [126]. As discussed previously, the discrepancy in findings may be explained by differences in receptor abundance, with low levels of IFNLR1 isoform 2 supporting IFNL signal augmentation (observed in our -dox model) and high levels of IFNLR1 isoform 2 leading to loss of augmentation (our +dox model and overexpression under pEF2 promoter in Lauber et al. 2015).

To explain our finding, I hypothesize that FLAG-Iso2 has capacity to form a functioning signaling complex with IFNL ligand, JAK1, and IL10RB. The extracellular and transmembrane domains of IFNLR1 isoform 1 and 2 are conserved, which are regions important for ligand binding and association with IL10RB, respectively. Furthermore, approximately half of the JAK1 binding site is maintained in IFNLR1 isoform 2's primary structure. Consequently, I hypothesize that FLAG-Iso2 could interact with the machinery needed to transduce signal. I would begin testing this hypothesis by rescuing FLAG-Iso2 expression in a HEK293T IFNLR1 KO line to express FLAG-Iso2 in the absence of endogenous IFNLR1 receptor. If FLAG-Iso2 is capable of signaling without the endogenous receptor present, I would expect to observe increased IFNL-dependent STAT1 phosphorylation, ISRE activity, and ISG expression in -dox IFNLR1 KO + FLAG-Iso2 cells relative to IFNLR1 KO + EV cells.

If FLAG-Iso2 is capable of signaling without endogenous IFNLR1 present, the next experiments would address the necessity of various signaling components. For example, I

hypothesize that IL10RB is necessary to support FLAG-Iso2 signaling. To test, I would pre-incubate cells with anti-IL10RB neutralizing antibody prior to treatment with IFNL3. If the subsequent IFNL response is abolished, then IL10RB is necessary in signaling. In addition, further testing should assess FLAG-iso2's reliance on JAK1 and TYK2 through use of small molecule inhibitors and siRNA strategies. I hypothesize that FLAG-Iso2 utilizes JAK1, as would be evidenced by observing a diminished response to IFNL3 after pre-treatment with select JAK1 inhibitors in a concentration dependent manner. As studies have demonstrated that IFNLR1 isoform 1 does not require TYK2 for signaling but may utilize it in certain circumstances where canonical signaling mechanisms are compromised [84], it would be of interest to assess FLAG-Iso2's reliance on TYK2 signaling by use of small molecule inhibition. I hypothesize that the structural deficits in FLAG-Iso2's intracellular domain would increase its need for TYK2 in its signal transduction, as evidenced by diminished IFNL3 response to IFNL3 after pre-treatment with select TYK2 inhibitors in a concentration dependent manner.

If we do not observe a significant difference in the IFNL response between IFNLR1 KO + EV or IFNLR1 KO + FLAG-Iso2 cells, then we must consider alternative hypotheses to explain how FLAG-Iso2 in -dox conditions augments the IFNL response in WT cells that express endogenous IFNLR1. As such, I propose experiments to evaluate potential interaction between endogenous IFNLR1 isoform 1 and FLAG-Iso2. For example, I hypothesize that overexpression of FLAG-Iso2 could increase the duration and proximity of IFNL3 ligand on the cell surface, thereby increasing its ability to interact with endogenous IFNLR1 [148]. To begin testing this hypothesis, I would treat HEK293T-EV and FLAG-Iso2 cells with commercially available hemagglutinin (HA)-tagged IFNL3 recombinant protein, followed by fixation and evaluation of HA signal intensity via flow cytometry, as method that has been used to indirectly assess IFNLR1 surface abundance [128]. This could also be accomplished through covalently linking IFNL3 to a fluorescent protein and

similar processing via flow cytometry. I would use doxycycline to titrate the level of FLAG-Iso2, followed by subsequent treatment with HA or fluorescently tagged IFNL3 protein and assessment via flow cytometry. If FLAG-Iso2 increases IFNL3 surface binding, it would be observed as increased IFNL3 signal as a function of FLAG-Iso2 expression. If we observe that FLAG-Iso2 increases the duration and stability of IFNL3 on the membrane, then the next step would be to identify its specific role in modulating signaling through endogenous IFNLR1. If FLAG-Iso2 stabilizes interaction of IFNL3 with endogenous IFNLR1, I hypothesize that FLAG-Iso2 would co-immunoprecipitate with IFNLR1 only in the presence of ligand. To conduct this experiment, I would synthesize an additional IFNLR1 isoform 2 construct with an alternate epitope tag, such as HA, and transiently transfect HA-Iso2 into FLAG-Iso1 stable cells. Having two distinct epitope tags would facilitate co-immunoprecipitation and provide a means to discriminate between IFNLR1 isoform 1 and 2 (other than molecular weight). Alternatively, if ligand-binding of FLAG-Iso2 is necessary to support endogenous IFNLR1 signaling, I hypothesize that disruption of the ligand binding domain in IFNLR1 isoform 2 would negate augmentation of the IFNL response. To conduct this experiment, I would delete and/or selectively mutate the ligand binding domain located within the extracellular region of IFNLR1 isoform 2 and assess its impact on the IFNL response in the presence of endogenous IFNLR1.

Why does FLAG-Iso2 result in IFNL3-dependent augmentation of antiviral genes without induction of pro-inflammatory genes?

NanoString analysis demonstrated a robust increase in *IRF1* in FLAG-Iso1 cells after IFNL3 treatment, whereas IFNL3-treated FLAG-Iso2 cells exhibited a partial increase (**Figure 3.10**). However, in contrast to FLAG-Iso1 cells, treatment of FLAG-Iso2 cells with IFNL3 did not lead to the induction of pro-inflammatory genes, such as *CXCL10*. Consequently, I hypothesize

that knocking down IRF1 expression via siRNA would abrogate pro-inflammatory gene expression in FLAG-Iso1 cells and exert no effect on the FLAG-Iso2 IFNL response.

Why does increased FLAG-Iso2 expression in +dox-treated cells reduce augmentation of the IFNL response?

Our data demonstrated that minimal expression of FLAG-Iso2 in -dox conditions augmented the IFNL3 response, but further expression in +dox conditions led to reduced augmentation. The mechanisms for this phenotype are unclear, but I hypothesize that once FLAG-Iso2 abundance is in excess (+dox) of one or more of its necessary signaling molecules, it begins to form receptor complexes that are capable of binding IFNL ligand but are signaling incompetent.

As described previously, this phenomenon has been observed in other systems where overexpression of cytokine receptors without a concomitant increase in necessary signaling molecules can lead to the formation of signaling-incompetent receptor complexes [142, 143]. To test this hypothesis, I would transfect in components of the signaling pathway, such as IL10RB, JAK1, and TKY2, followed by subsequent evaluation of the IFNL response in +dox conditions to determine whether loss of augmented IFNL signaling is prevented through overexpression of one or more signaling molecules.

Why does overexpression of FLAG-Iso2 not affect type-I IFN response?

Although FLAG-Iso2 supports partial ISG expression, similar to FLAG-Iso1, it had no impact on ability of IFNA2-dependent pSTAT1 formation, as observed with FLAG-Iso1. Described previously, I hypothesize that excess FLAG-Iso1 sequesters signaling molecules common to both pathways, such as JAK1 or TYK2. If FLAG-Iso2 can signal in the absence of endogenous IFNLR1 through the formation of competent signaling complexes with JAK1 and IL10RB, then the

question arises as to why overexpression of FLAG-Iso2 would not exert a similar effect on type-I IFN signaling. To explain this potential discrepancy, I hypothesize that the lack of effect of FLAG-Iso2 overexpression on the type-I IFN response is due to decreased affinity and/or stabilization of JAK1. More specifically, as FLAG-Iso2 is missing a significant portion of its JAK1 binding domain, including sections critical in stabilizing the interaction, I hypothesize that FLAG-Iso2 exhibits lesser ability to bind and sequester JAK1 than FLAG-Iso1. This hypothesis could be tested in several ways. First, the extent of interaction between FLAG-Iso1 or FLAG-Iso2 for JAK1 should be assessed. This can be achieved by a variety of techniques, such as co-immunoprecipitation, or quantitative measurement of interaction via surface plasmon resonance. If there is a difference in isoform affinity and interaction for JAK1, then this topic could be further explored using construction of additional expression constructs containing IFNLR1 receptor chimeras. Specifically, I would generate IFNLR1 constructs missing Box1/Box2, containing only Box1, or containing only Box2. When expressed in HEK293T cells, these constructs would not only help us understand the effect of JAK1 titration on the type-I IFN response but could provide additional insight into how Box1 and Box2 regions specifically contribute to JAK1 binding.

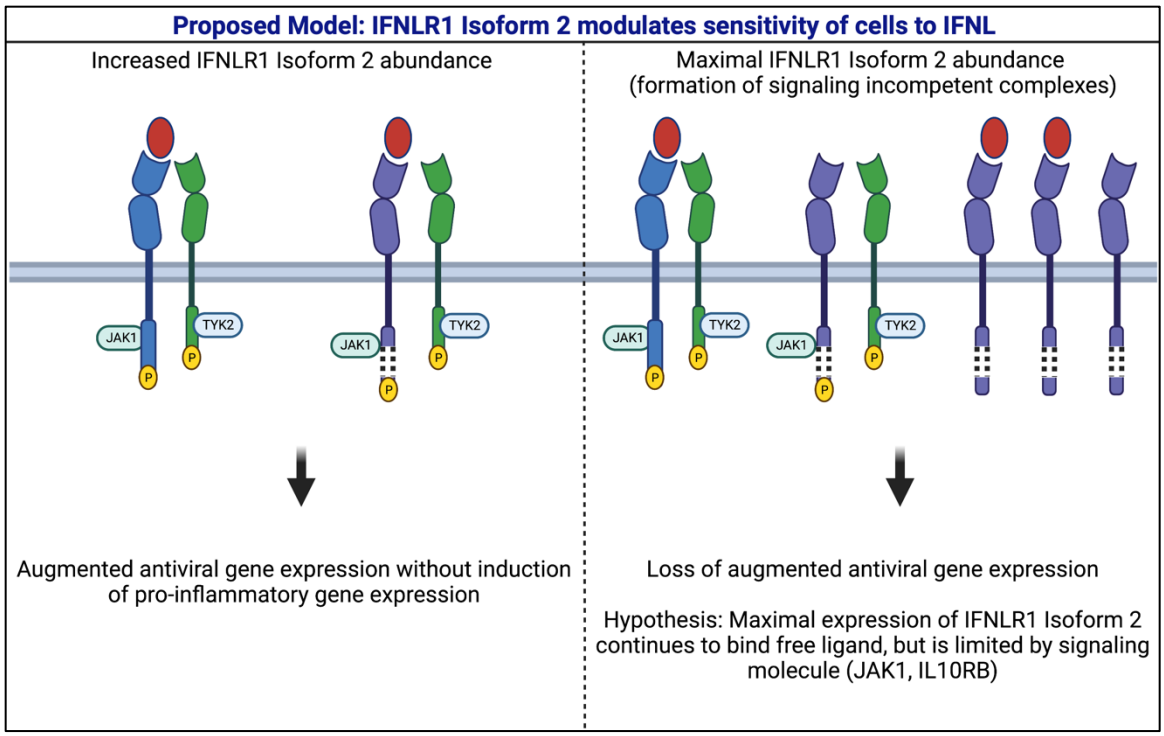


Figure 5.2: Proposed Model of IFNLR1 Isoform 2 Signaling.

Does augmentation of IFNL response after FLAG-Iso3 expression require endogenous IFNLR1?

Our initial findings demonstrating that FLAG-Iso3 overexpression partially augments IFNL signaling are surprising, as two studies have shown that IFNLR1 isoform 3 can reduce cellular response to IFNL3 [128, 129]. As discussed in the results section, there are several reasons why these seemingly different outcomes can be reconciled, likely due to the concentration of IFNLR1 isoform 3 present and cell types used for experimentation. However, our observations invite several interesting questions into the biological role of IFNLR1 isoform 3. Specifically, I would hypothesize that FLAG-Iso3 augments IFNL3 signaling through interaction with both endogenous IFNLR1 and its co-receptor IL10RB in a phenomenon known as "trans-signaling," whereby a soluble receptor is capable of interacting with its membrane-bound co-receptor [148]. This phenomenon has been best described in the field concerning IL-6 biology: In classical signaling, IL-6 binds to its membrane bound receptor IL-6R, leading to activation of its associated subunit Gp30 and subsequent signal transduction [148]. However, it was observed that cell types that do not express the membrane bound receptor can still transduce signal after exposure to IL-6 when in the presence of the soluble receptor sIL-6R, as the IL-6-sIL-6R complex could associate with membrane bound Gp30 to form an active signaling complex [148]. Using the IL-6 literature as a foundation for exploration, I would hypothesize that IFNLR1 isoform 3 can behave in a similar manner, with its presence in the extracellular space resulting in ligand binding and formation of functional signaling complexes with either endogenous IFNLR1 isoform 1 or IL10RB. This hypothesis could be tested in several ways: First, to determine whether FLAG-Iso3 augments IFNL signaling in conjunction with the endogenous IFNLR1 receptor, I would rescue and overexpress FLAG-Iso3 in a HEK293T IFNLR1 KO line. If rescue of FLAG-Iso3 does not result in augmentation of the IFNL response over the HEK293T IFNLR1 KO + EV line, then it is likely working in conjunction with endogenous receptor. Alternatively, if we observe persistent

augmentation of signal after IFNL stimulation, then I would hypothesize that FLAG-Iso3 is working in conjunction with IL10RB. To test this hypothesis, I would utilize siRNA technology to knock-down endogenous IL10RB expression in both IFNLR1 KO + EV and IFNLR1 KO + FLAG-Iso3 lines. If we observe decreased IFNL signaling after siRNA KD of IL10RB in the IFNLR1 KO + FLAG-Iso3 line compared to cells that received scrambled siRNA control vector, then FLAG-Iso3 is likely augmenting IFNL signal through an interaction with IL10RB. Further testing could involve co-immunoprecipitations of FLAG-Iso3 in the presence or absence of IFNL ligand to assess for detection of IL10RB.

If FLAG-Iso3 augments the IFNL response through trans-signaling, it would provide an interesting basis for understanding the apparent contradiction between our results and the behavior of IFNLR1 isoform 3 in the literature [128, 129]. Specifically, as the cell types used in prior reports (HepG2 cells and PBMCs) are more responsive to treatment with IFNLs than HEK293T cells, I hypothesize that the effect of excess IFNLR1 isoform 3 may be antagonistic in cell types that already exhibit robust IFNL responses, and antagonistic in cell types that are largely insensitive and may benefit more from trans-signaling. While purely speculative, this hypothesis is not far removed from work conducted in the type-I IFN field, which showed that the soluble IFNAR2a isoform can act as either an antagonist or agonist of the type-I IFN response, depending on the presence of endogenous membrane bound IFNAR2c [62]. Specifically, the type-I IFN response was diminished when IFNAR2a was present in conjunction with IFNAR2c after treatment with ligand, but partially restored in a IFNAR2 KO cell line, presumably through interaction with endogenous IFNAR1 [62].

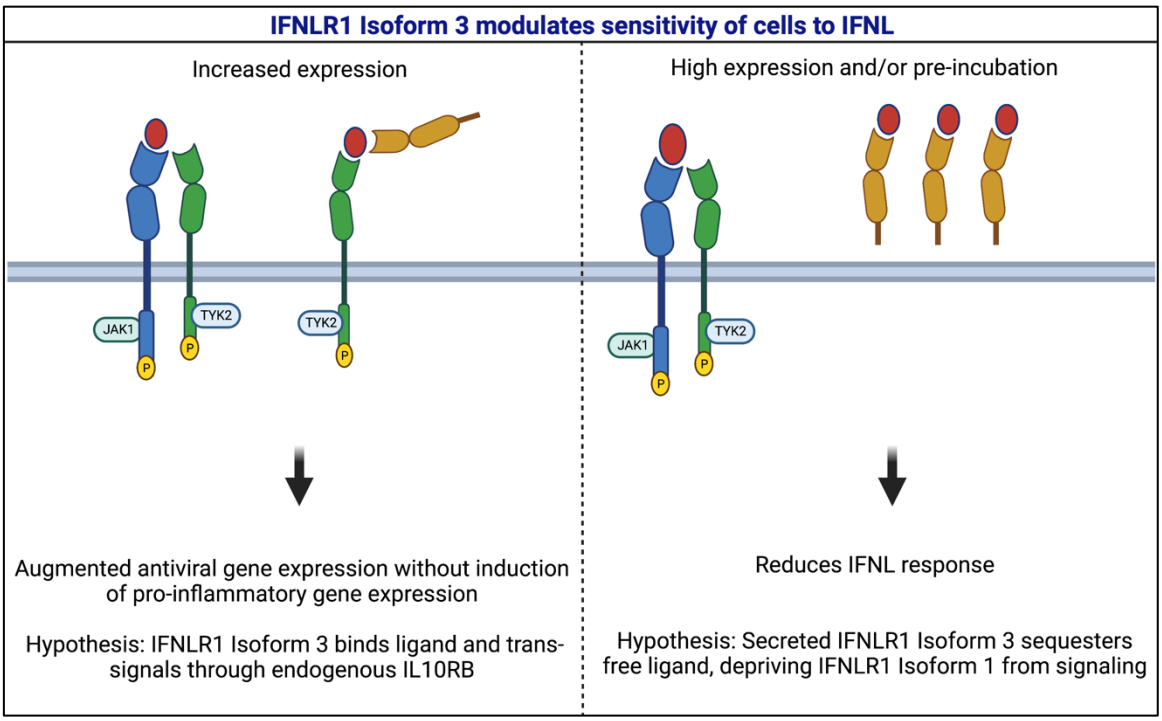


Figure 5.3: Proposed Model of IFNLR1 Isoform 3 Signaling.

Regulation of IFNLR1 Isoforms on the Cell Membrane: Trafficking, Internalization, and Intracellular Sorting

Numerous studies have shown that the cellular response to various cytokines is modulated by receptor properties including surface abundance, intracellular trafficking, endocytic signaling, and differential expression of protein isoforms [144]. For example, the type-I IFN system is heavily regulated by its receptor complex IFNAR1/IFNAR2 [56], which exhibits ligand-dependent internalization and differential sorting, with IFNAR1 trafficking to the lysosome for degradation and IFNAR2 returning to the membrane via the recycling pathway [45, 149]. I hypothesize that the IFNL system is similarly regulated, with IFNLR1 surface expression and intracellular sorting serving as an essential layer of regulation to dictate the manner by which cells respond to IFNL. Characterizing IFNLR1 trafficking, surface regulation, and intracellular sorting is important for future development and optimization of IFNL as a therapeutic option. For example, if IFNLR1 exhibits ligand-dependent internalization and degradation, the timing and dosage of IFNL treatment could be modified to prevent loss of IFNL response.

How is the surface abundance of IFNLR1 Isoforms 1 and 2 regulated?

With the expression of IFNLR1 being so low *in vivo*, in conjunction with our findings that IFNLR1 isoforms offer unique contributions to IFNL signaling, it is important to understand how surface expression of IFNLR1 receptor isoforms 1 and 2 is regulated. I hypothesize that due to differences in structural features, IFNLR1 isoform 1 and 2 will exhibit distinct internalization and intracellular sorting patterns, which may confer distinct biological effects.

We have conducted preliminary investigations into the basic nature of FLAG-Iso1 internalization. To do so, we utilized transiently transfected HepG2 cells, an immortalized cell

line derived from a hepatocellular carcinoma, with FLAG-Iso1 to assess internalization under physiological conditions in the absence of ligand. Briefly, HepG2 cells were incubated with anti-FLAG antibody at 4 ^{o}C for 30 minutes to label surface receptor, washed cells, then warmed to 37^{o}C for 30 minutes or kept at 4 ^{o}C. Cells were then permeabilized, fixed, and stained with anti-EEA1 antibody to label early endosomal structures followed by secondary antibody (see Chapter 2: Materials and Methods). Our choice to use HepG2 cells instead of HEK293T cells was due to a technical challenge; specifically, we found that HEK293T cells did not adhere well enough to the coverslip surface to withstand the number of washes necessary to perform these experiments. At 37 ^{o}C, we observed distinct formation of cytosolic punctate structures that were not observed in cells kept at 4 ^{o}C (**Figure 5.4A-C**). These punctate structures co-localize with EEA1, a marker of the early endosome, suggesting FLAG-Iso1 is internalized and localizes to the early endosome (**Figure 5.4D-E**). As FLAG-Iso2 is missing a significant section of its intracellular domain, I would hypothesize that it would exhibit altered internalization kinetics compared to FLAG-Iso1. Specifically, I hypothesize that FLAG-Iso2 would exhibit less robust internalization compared to FLAG-Iso1.

As IFNAR1 and IFNAR2 exhibit differential sorting patterns after internalization, with IFNAR1 being trafficked to the lysosome for degradation and IFNAR2 being recycled to the membrane [149], it would be of interest to dissect the sub-cellular localization of IFNLR1 isoforms 1 and 2, as well as IL10RB, both in the presence and absence of ligand. As described above, this can be tested through co-localization analysis of the FLAG epitope after internalization with sub-cellular structures, such as the early endosome and lysosome, or after treatment with inhibitors of Rab11, a protein involved in the recycling of receptor proteins to the membrane [149].

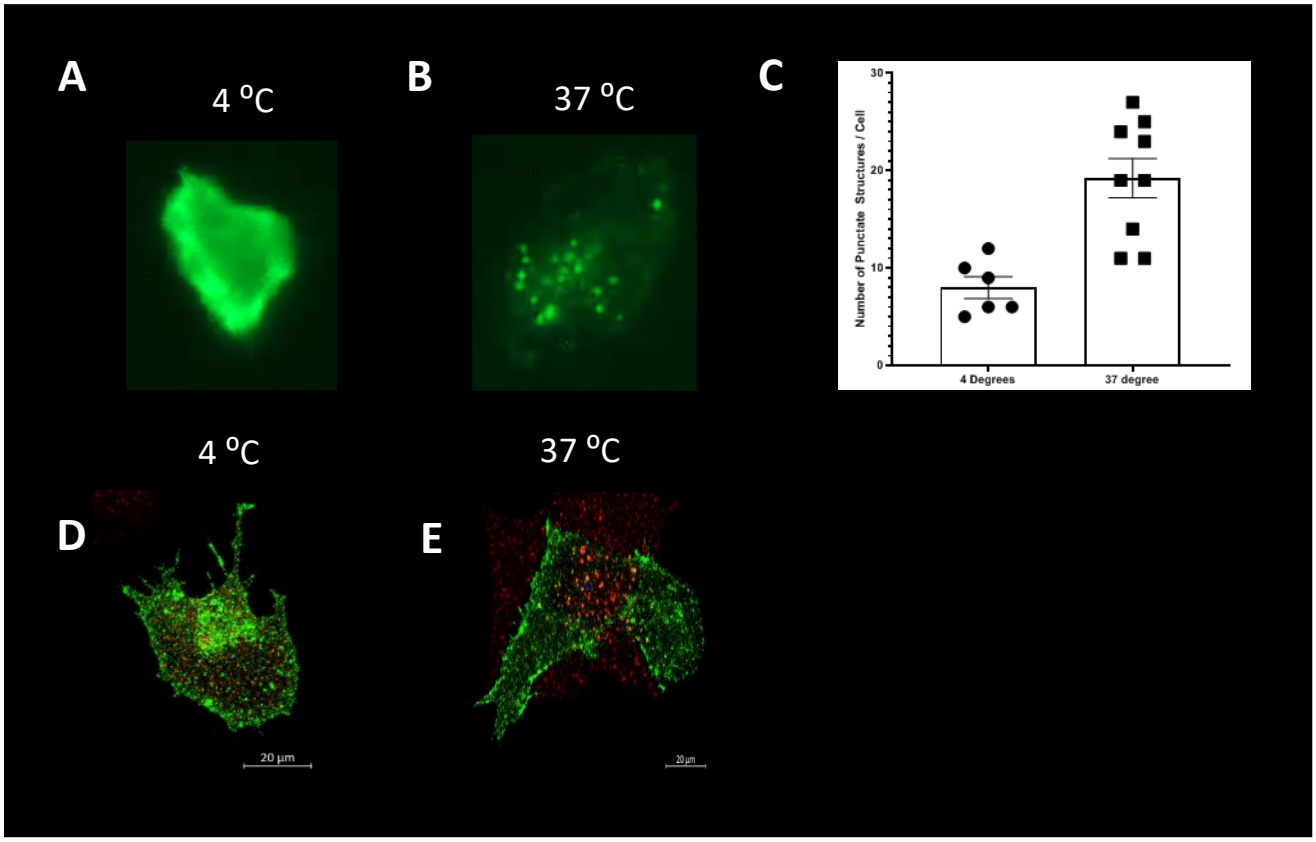


Figure 5.4: FLAG-Iso1 undergoes constitutive internalization and co-localizes with the early endosome in transiently transfected HepG2 cells. HepG2 cells transiently transfected with FLAG-Iso1 were incubated with anti-FLAG antibody at 4 °C and either maintained at 4 °C **(A)** or warmed to 37 °C **(B)** for 1 hour to allow internalization, observed as intracellular punctate structures which were quantified via ImageJ **(C).** Evaluation of co-localization of FLAG-Iso1 (green) with the marker of the early endosome EEA1 (red) after incubation at either 4 °C **(D)** or warmed to 37 °C **(E).**

Evaluating the Impact of IFNLR1 Isoform Modulation on Efficacy of Antiviral Response in Induced Pluripotent Stem Cell Derived Hepatocyte-Like Cells Infected with Hepatitis B Virus

As we have demonstrated that IFNLR1 isoforms offer unique contributions to IFN signaling, the next phase of research will involve assessing their role in supporting cellular antiviral defense and how they can be modulated to improve the therapeutic potential of IFNL.

Peg-IFNL1 has been tested head-to-head with peg-IFNA2 (a type-I IFN) in a clinical trial for patients with chronic hepatitis B viral infection [107]. This study ultimately demonstrated that that peg-IFNL1 treatment decreased several markers of HBV infection while on drug, while incurring fewer untoward effects than peg-IFNA2 treatment. However, patients who received peg-IFNL1 exhibited greater rebound of viral markers when taken off drug, compared to those on peg-IFNA2. This led the trial authors to conclude that peg-IFNL1 was inferior to peg-IFNA2 for the treatment of chronic HBV infection.

While peg-IFNL1 did not outperform type-I IFN treatment, the greater on drug suppression of viral markers is of considerable interest. In light of our insights into how IFNLR1 isoforms 1, 2, and 3 contribute to IFNL signaling, the results of this clinical trial can be viewed from a more nuanced perspective and warrant further investigation. To do so, our laboratory has adopted and established the use of induced pluripotent stem cells (iPSCs), which can be differentiated into hepatocyte-like cells (iHeps) and infected with live HBV to serve as a model of *in vitro* HBV infection [150]. In addition to testing HBV-specific hypotheses, this cell model can also serve to replicate experiments done in HEK293T cells, as they provide a translational and physiologically relevant cell type.

How does overexpression of IFNLR1 isoforms affect markers of HBV replication after IFNL treatment?

As our findings in HEK293T cells showed that overexpression of FLAG-IFNLR1 isoform 1, 2, and 3 augment the antiviral response after IFNL3 treatment, I would hypothesize that overexpression of each isoform in a WT iHep background would result in a decrease in HBV quantity after IFNL3 treatment relative to control iHeps (empty vector only). Specifically, I would hypothesize that the extent of HBV-reduction would correlate with the augmentation of the IFNL response, with overexpression of FLAG-Iso1 resulting in a larger decline in HBV quantity than FLAG-Iso2 and FLAG-Iso3.

If FLAG-Iso1 overexpression results in a decline in HBV markers, but FLAG-Iso2 or FLAG-Iso3 do not, then I would be particularly interested to assess the importance of pro-inflammatory cytokine induction in the clearance of HBV infection. As FLAG-Iso1 overexpression in HEK293T cells was the only condition that led to IFNL3-dependent transcription of pro-inflammatory cytokines, such as *CXCL10*), I would hypothesize that IRF1-driven inflammation is necessary for IFNL3-dependent reduction in HBV markers. To test this hypothesis, I would utilize siRNA targeting IRF1 prior to treating cells with IFNL3 and measuring HBV markers.

Conclusion:

Developing an understanding of each IFNLR1 isoform's signaling pathways, localization and trafficking patterns, and impact on viral replication *in vitro* will provide insights needed to build a comprehensive model of IFNL regulation through its receptor, IFNLR1. By doing so, the antiviral capacity of IFNL can be evaluated and optimized through hypothesis-driven testing in an effort to improve its clinical application.

REFERENCES

1. Riera Romo, M., Pérez-Martínez, D., and Castillo Ferrer, C., *Innate immunity in vertebrates: an overview.* Immunology, 2016. **148**(2): p. 125-39.

2. in *Autoimmunity: From Bench to Bedside*, J.M. Anaya, et al., Editors. 2013, El Rosario University Press © 2013 Universidad del Rosario.: Bogota (Colombia).

3. Marshall, J.S., Warrington, R., Watson, W., and Kim, H.L., *An introduction to immunology and immunopathology.* Allergy, Asthma & Clinical Immunology, 2018. **14**(2): p. 49.

4. Mogensen, T.H., *Pathogen recognition and inflammatory signaling in innate immune defenses.* Clin Microbiol Rev, 2009. **22**(2): p. 240-73, Table of Contents.

5. Li, D. and Wu, M., *Pattern recognition receptors in health and diseases.* Signal Transduction and Targeted Therapy, 2021. **6**(1): p. 291.

6. Carty, M., Guy, C., and Bowie, A.G., *Detection of Viral Infections by Innate Immunity.* Biochem Pharmacol, 2021. **183**: p. 114316.

7. Barton, G.M., *A calculated response: control of inflammation by the innate immune system.* The Journal of clinical investigation, 2008. **118**(2): p. 413-420.

8. Tracy, R.P., *The Five Cardinal Signs of Inflammation: Calor, Dolor, Rubor, Tumor … and Penuria (Apologies to Aulus Cornelius Celsus, De medicina, c. A.D. 25).* The Journals of Gerontology: Series A, 2006. **61**(10): p. 1051-1052.

9. Sherwood, E.R. and Toliver-Kinsky, T., *Mechanisms of the inflammatory response.* Best Pract Res Clin Anaesthesiol, 2004. **18**(3): p. 385-405.

10. Dinarello, C.A., *Historical insights into cytokines.* Eur J Immunol, 2007. **37 Suppl 1**(Suppl 1): p. S34-45.

11. Borden, E.C., Sen, G.C., Uze, G., Silverman, R.H., Ransohoff, R.M., Foster, G.R., and Stark, G.R., *Interferons at age 50: past, current and future impact on biomedicine.* Nat Rev Drug Discov, 2007. **6**(12): p. 975-90.

12. de Weerd, N.A. and Nguyen, T., *The interferons and their receptors--distribution and regulation.* Immunol Cell Biol, 2012. **90**(5): p. 483-91.

13. Stanifer, M.L., Pervolaraki, K., and Boulant, S., *Differential Regulation of Type I and Type III Interferon Signaling.* Int J Mol Sci, 2019. **20**(6).

14. Dianzani, F., *Viral interference and interferon.* Ric Clin Lab, 1975. **5**(3): p. 196-213.

15. Hilleman, M.R., *INTERFERON IN PROSPECT AND PERSPECTIVE.* J Cell Comp Physiol, 1963. **62**: p. 337-53.

16. Hoskins, M., *A Protective Action of Neurotropic Against Viscerotropic Yellow Fever Virus in Macacus Rhesus.* The American Journal of Tropical Medicine, 1935. **s1-15**(6): p. 675-680.

17. Findlay, G. and Jmaccallum, F., *An interference phenomenon in relation to yellow fever and other viruses.* Journal of Pathology and Bacteriology, 1937. **44**(2).

18. Lennette, E.H. and Koprowski, H., *INTERFERENCE BETWEEN VIRUSES IN TISSUE CULTURE.* J Exp Med, 1946. **83**(3): p. 195-219.

19. Isaacs, A. and Lindenmann, J., *Virus interference. I. The interferon.* Proc R Soc Lond B Biol Sci, 1957. **147**(927): p. 258-67.

20. Isaacs, A., Lindenmann, J., and Valentine, R.C., *Virus interference. II. Some properties of interferon.* Proc R Soc Lond B Biol Sci, 1957. **147**(927): p. 268-73.

21. Lampson, G.P., Tytell, A.A., Nemes, M.M., and Hilleman, M.R., *Purification and characterization of chick embryo interferon.* Proc Soc Exp Biol Med, 1963. **112**: p. 468-78.

22. Burke, D.C., *The purification of interferon.* Biochem J, 1961. **78**(3): p. 556-63.

23. Nagata, S., Mantei, N., and Weissmann, C., *The structure of one of the eight or more distinct chromosomal genes for human interferon-α.* Nature, 1980. **287**(5781): p. 401-408.

24. Taniguchi, T., Ohno, S., Fujii-Kuriyama, Y., and Muramatsu, M., *The nucleotide sequence of human fibroblast interferon cDNA.* Gene, 1980. **10**(1): p. 11-5.

25. Baglioni, C., Benvin, S., Maroney, P.A., Minks, M.A., Nilsen, T.W., and West, D.K., *Interferon-induced enzymes: activation and role in te antiviral state.* Ann N Y Acad Sci, 1980. **350**: p. 497-509.

26. Larner, A.C., Jonak, G., Cheng, Y.S., Korant, B., Knight, E., and Darnell, J.E., Jr., *Transcriptional induction of two genes in human cells by beta interferon.* Proc Natl Acad Sci U S A, 1984. **81**(21): p. 6733-7.

27. Larner, A.C., Chaudhuri, A., and Darnell, J.E., Jr., *Transcriptional induction by interferon. New protein(s) determine the extent and length of the induction.* J Biol Chem, 1986. **261**(1): p. 453-9.

28. Taylor, J., *Inhibition of interferon action by actinomycin.* Biochem Biophys Res Commun, 1964. **14**: p. 447-51.

29. Knight, E., Jr., *Interferon-Sepharose: induction of the antiviral state.* Biochem Biophys Res Commun, 1974. **56**(4): p. 860-4.

30. Schneck, J., Rager-Zisman, B., Rosen, O.M., and Bloom, B.R., *Genetic analysis of the role of cAMP in mediating effects of interferon.* Proc Natl Acad Sci U S A, 1982. **79**(6): p. 1879-83.

31. Stark, G.R. and Darnell, J.E., Jr., *The JAK-STAT pathway at twenty.* Immunity, 2012. **36**(4): p. 503-14.

32. Levy, D., Larner, A., Chaudhuri, A., Babiss, L.E., and Darnell, J.E., Jr., *Interferon-stimulated transcription: isolation of an inducible gene and identification of its regulatory region.* Proc Natl Acad Sci U S A, 1986. **83**(23): p. 8929-33.

33. Reich, N., Evans, B., Levy, D., Fahey, D., Knight Jr, E., and Darnell Jr, J., *Interferon-induced transcription of a gene encoding a 15-kDa protein depends on an upstream enhancer element.* Proceedings of the National Academy of Sciences, 1987. **84**(18): p. 6394-6398.

34. Levy, D.E., Kessler, D.S., Pine, R., Reich, N., and Darnell, J.E., Jr., *Interferon-induced nuclear factors that bind a shared promoter element correlate with positive and negative transcriptional control.* Genes Dev, 1988. **2**(4): p. 383-93.

35. Levy, D.E., Kessler, D.S., Pine, R., and Darnell, J.E., Jr., *Cytoplasmic activation of ISGF3, the positive regulator of interferon-alpha-stimulated transcription, reconstituted in vitro.* Genes Dev, 1989. **3**(9): p. 1362-71.

36. Fu, X.Y., Kessler, D.S., Veals, S.A., Levy, D.E., and Darnell, J.E., Jr., *ISGF3, the transcriptional activator induced by interferon alpha, consists of multiple interacting polypeptide chains.* Proc Natl Acad Sci U S A, 1990. **87**(21): p. 8555-9.

37. Darnell, J.E., Jr., Kerr, I.M., and Stark, G.R., *Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins.* Science, 1994. **264**(5164): p. 1415-21.

38. Schindler, C., Shuai, K., Prezioso, V.R., and Darnell, J.E., Jr., *Interferon-dependent tyrosine phosphorylation of a latent cytoplasmic transcription factor.* Science, 1992. **257**(5071): p. 809-13.

39. Velazquez, L., Fellous, M., Stark, G.R., and Pellegrini, S., *A protein tyrosine kinase in the interferon alpha/beta signaling pathway.* Cell, 1992. **70**(2): p. 313-22.

40. Firmbach-Kraft, I., Byers, M., Shows, T., Dalla-Favera, R., and Krolewski, J.J., *tyk2, prototype of a novel class of non-receptor tyrosine kinase genes.* Oncogene, 1990. **5**(9): p. 1329-36.

41. Branca, A.A. and Baglioni, C., *Evidence that types I and II interferons have different receptors.* Nature, 1981. **294**(5843): p. 768-70.

42. Uzé, G., Lutfalla, G., and Gresser, I., *Genetic transfer of a functional human interferon alpha receptor into mouse cells: cloning and expression of its cDNA.* Cell, 1990. **60**(2): p. 225-34.

43. de Weerd, N.A., Samarajiwa, S.A., and Hertzog, P.J., *Type I interferon receptors: biochemistry and biological functions.* J Biol Chem, 2007. **282**(28): p. 20053-7.

44. Ivashkiv, L.B. and Donlin, L.T., *Regulation of type I interferon responses.* Nat Rev Immunol, 2014. **14**(1): p. 36-49.

45. Schreiber, G. and Piehler, J., *The molecular basis for functional plasticity in type I interferon signaling.* Trends Immunol, 2015. **36**(3): p. 139-49.

46. Piehler, J., Thomas, C., Garcia, K.C., and Schreiber, G., *Structural and dynamic determinants of type I interferon receptor assembly and their functional interpretation.* Immunol Rev, 2012. **250**(1): p. 317-34.

47. Leonard, W.J. and O'Shea, J.J., *Jaks and STATs: biological implications.* Annu Rev Immunol, 1998. **16**: p. 293-322.

48. Gadina, M., Le, M.T., Schwartz, D.M., Silvennoinen, O., Nakayamada, S., Yamaoka, K., and O'Shea, J.J., *Janus kinases to jakinibs: from basic insights to clinical practice.* Rheumatology (Oxford), 2019. **58**(Suppl 1): p. i4-i16.

49. Usacheva, A., Sandoval, R., Domanski, P., Kotenko, S.V., Nelms, K., Goldsmith, M.A., and Colamonici, O.R., *Contribution of the Box 1 and Box 2 motifs of cytokine receptors to Jak1 association and activation.* J Biol Chem, 2002. **277**(50): p. 48220-6.

50. Lim, C.P. and Cao, X., *Structure, function, and regulation of STAT proteins.* Mol Biosyst, 2006. **2**(11): p. 536-50.

51. Paul, A., Tang, T.H., and Ng, S.K., *Interferon Regulatory Factor 9 Structure and Regulation.* Front Immunol, 2018. **9**: p. 1831.

52. Platanitis, E., Demiroz, D., Schneller, A., Fischer, K., Capelle, C., Hartl, M., Gossenreiter, T., Müller, M., Novatchkova, M., and Decker, T., *A molecular switch from STAT2-IRF9 to ISGF3 underlies interferon-induced gene transcription.* Nature Communications, 2019. **10**(1): p. 2921.

53. Sadler, A.J. and Williams, B.R., *Interferon-inducible antiviral effectors.* Nat Rev Immunol, 2008. **8**(7): p. 559-68.

54. Schneider, W.M., Chevillotte, M.D., and Rice, C.M., *Interferon-stimulated genes: a complex web of host defenses.* Annu Rev Immunol, 2014. **32**: p. 513-45.

55. Shemesh, M., Lochte, S., Piehler, J., and Schreiber, G., *IFNAR1 and IFNAR2 play distinct roles in initiating type I interferon–induced JAK-STAT signaling and activating STATs.* Science Signaling, 2021. **14**(710): p. eabe4627.

56. Zanin, N., Viaris de Lesegno, C., Lamaze, C., and Blouin, C.M., *Interferon Receptor Trafficking and Signaling: Journey to the Cross Roads.* Front Immunol, 2020. **11**: p. 615603.

57. Wang, Y., Liu, J., Huang, B.O., Xu, Y.M., Li, J., Huang, L.F., Lin, J., Zhang, J., Min, Q.H., Yang, W.M., and Wang, X.Z., *Mechanism of alternative splicing and its regulation.* Biomed Rep, 2015. **3**(2): p. 152-158.

58. Lutfalla, G., Holland, S.J., Cinato, E., Monneron, D., Reboul, J., Rogers, N.C., Smith, J.M., Stark, G.R., Gardiner, K., Mogensen, K.E., and et al., *Mutant U5A cells are complemented by an interferon-alpha beta receptor subunit generated by alternative processing of a new member of a cytokine receptor gene cluster.* Embo j, 1995. **14**(20): p. 5100-8.

59. Domanski, P. and Colamonici, O.R., *The type-I interferon receptor. The long and short of it.* Cytokine Growth Factor Rev, 1996. **7**(2): p. 143-51.

60. Cohen, B., Novick, D., Barak, S., and Rubinstein, M., *Ligand-induced association of the type I interferon receptor components.* Mol Cell Biol, 1995. **15**(8): p. 4208-14.

61. Novick, D., Cohen, B., and Rubinstein, M., *The human interferon alpha/beta receptor: characterization and molecular cloning.* Cell, 1994. **77**(3): p. 391-400.

62. Hardy, M.P., Owczarek, C.M., Trajanovska, S., Liu, X., Kola, I., and Hertzog, P.J., *The soluble murine type I interferon receptor Ifnar-2 is present in serum, is independently regulated, and has both agonistic and antagonistic properties.* Blood, 2001. **97**(2): p. 473-82.

63. McKenna, S.D., Vergilis, K., Arulanandam, A.R., Weiser, W.Y., Nabioullin, R., and Tepper, M.A., *Formation of human IFN-beta complex with the soluble type I interferon receptor IFNAR-2 leads to enhanced IFN stability, pharmacokinetics, and antitumor activity in xenografted SCID mice.* J Interferon Cytokine Res, 2004. **24**(2): p. 119-29.

64. Gilli, F., Marnetto, F., Caldano, M., Valentino, P., Granieri, L., Di Sapio, A., Capobianco, M., Sala, A., Malucchi, S., Kappos, L., Lindberg, R.L., and Bertolotto, A., *Anti-interferon-beta neutralising activity is not entirely mediated by antibodies.* J Neuroimmunol, 2007. **192**(1-2): p. 198-205.

65. Dowling, J.W. and Forero, A., *Beyond Good and Evil: Molecular Mechanisms of Type I and III IFN Functions.* J Immunol, 2022. **208**(2): p. 247-256.

66. Davidson, S., McCabe, T.M., Crotta, S., Gad, H.H., Hessel, E.M., Beinke, S., Hartmann, R., and Wack, A., *IFNλ is a potent anti-influenza therapeutic without the inflammatory side effects of IFNα treatment.* EMBO Mol Med, 2016. **8**(9): p. 1099-112.

67. Kotenko, S.V., Gallagher, G., Baurin, V.V., Lewis-Antes, A., Shen, M., Shah, N.K., Langer, J.A., Sheikh, F., Dickensheets, H., and Donnelly, R.P., *IFN-lambdas mediate antiviral protection through a distinct class II cytokine receptor complex.* Nat Immunol, 2003. **4**(1): p. 69-77.

68. Sheppard, P., Kindsvogel, W., Xu, W., Henderson, K., Schlutsmeyer, S., Whitmore, T.E., Kuestner, R., Garrigues, U., Birks, C., Roraback, J., Ostrander, C., Dong, D., Shin, J., Presnell, S., Fox, B., Haldeman, B., Cooper, E., Taft, D., Gilbert, T., Grant, F.J., Tackett, M.,

Krivan, W., McKnight, G., Clegg, C., Foster, D., and Klucher, K.M., *IL-28, IL-29 and their class II cytokine receptor IL-28R.* Nat Immunol, 2003. **4**(1): p. 63-8.

69. Donnelly, R.P. and Kotenko, S.V., *Interferon-lambda: a new addition to an old family.* J Interferon Cytokine Res, 2010. **30**(8): p. 555-64.

70. Kotenko, S.V., Krause, C.D., Izotova, L.S., Pollack, B.P., Wu, W., and Pestka, S., *Identification and functional characterization of a second chain of the interleukin-10 receptor complex.* Embo j, 1997. **16**(19): p. 5894-903.

71. Lazear, H.M., Schoggins, J.W., and Diamond, M.S., *Shared and Distinct Functions of Type I and Type III Interferons.* Immunity, 2019. **50**(4): p. 907-923.

72. Onoguchi, K., Yoneyama, M., Takemura, A., Akira, S., Taniguchi, T., Namiki, H., and Fujita, T., *Viral infections activate types I and III interferon genes through a common mechanism.* J Biol Chem, 2007. **282**(10): p. 7576-81.

73. Sommereyns, C., Paul, S., Staeheli, P., and Michiels, T., *IFN-lambda (IFN-lambda) is expressed in a tissue-dependent fashion and primarily acts on epithelial cells in vivo.* PLoS Pathog, 2008. **4**(3): p. e1000017.

74. Wack, A., Terczyńska-Dyla, E., and Hartmann, R., *Guarding the frontiers: the biology of type III interferons.* Nat Immunol, 2015. **16**(8): p. 802-9.

75. Ye, L., Schnepf, D., and Staeheli, P., *Interferon-λ orchestrates innate and adaptive mucosal immune responses.* Nature Reviews Immunology, 2019. **19**(10): p. 614-625.

76. Ye, L., Schnepf, D., Becker, J., Ebert, K., Tanriver, Y., Bernasconi, V., Gad, H.H., Hartmann, R., Lycke, N., and Staeheli, P., *Interferon-lambda enhances adaptive mucosal immunity by boosting release of thymic stromal lymphopoietin.* Nat Immunol, 2019. **20**(5): p. 593-601.

77. Ferrao, R., Wallweber, H.J., Ho, H., Tam, C., Franke, Y., Quinn, J., and Lupardus, P.J., *The Structural Basis for Class II Cytokine Receptor Recognition by JAK1.* Structure, 2016. **24**(6): p. 897-905.

78. Mendoza, J.L., Schneider, W.M., Hoffmann, H.H., Vercauteren, K., Jude, K.M., Xiong, A., Moraga, I., Horton, T.M., Glenn, J.S., de Jong, Y.P., Rice, C.M., and Garcia, K.C., *The IFN-λ-IFN-λR1-IL-10Rβ Complex Reveals Structural Features Underlying Type III IFN Functional Plasticity.* Immunity, 2017. **46**(3): p. 379-392.

79. Miknis, Z.J., Magracheva, E., Li, W., Zdanov, A., Kotenko, S.V., and Wlodawer, A., *Crystal structure of human interferon-λ1 in complex with its high-affinity receptor interferon-λR1.* J Mol Biol, 2010. **404**(4): p. 650-64.

80. Gad, H.H., Dellgren, C., Hamming, O.J., Vends, S., Paludan, S.R., and Hartmann, R., *Interferon-lambda is functionally an interferon but structurally related to the interleukin-10 family.* J Biol Chem, 2009. **284**(31): p. 20869-75.

81. Donnelly, R.P., Sheikh, F., Kotenko, S.V., and Dickensheets, H., *The expanded family of class II cytokines that share the IL-10 receptor-2 (IL-10R2) chain.* Journal of leukocyte biology, 2004. **76**(2): p. 314-321.

82. Jordan, W.J., Eskdale, J., Boniotto, M., Rodia, M., Kellner, D., and Gallagher, G., *Modulation of the human cytokine response by interferon lambda-1 (IFN-lambda1/IL-29).* Genes Immun, 2007. **8**(1): p. 13-20.

83. Fuchs, S., Kaiser-Labusch, P., Bank, J., Ammann, S., Kolb-Kokocinski, A., Edelbusch, C., Omran, H., and Ehl, S., *Tyrosine kinase 2 is not limiting human antiviral type III interferon responses.* European journal of immunology, 2016. **46**(11): p. 2639-2649.

84. Schnepf, D., Crotta, S., Thamamongood, T., Stanifer, M., Polcik, L., Ohnemus, A., Vier, J., Jakob, C., Llorian, M., Gad, H.H., Hartmann, R., Strobl, B., Kirschnek, S., Boulant, S., Schwemmle, M., Wack, A., and Staeheli, P., *Selective Janus kinase inhibition preserves interferon-λ-mediated antiviral responses.* Sci Immunol, 2021. **6**(59).

85. Zhang, D., Wlodawer, A., and Lubkowski, J., *Crystal Structure of a Complex of the Intracellular Domain of Interferon λ Receptor 1 (IFNLR1) and the FERM/SH2 Domains of Human JAK1.* J Mol Biol, 2016. **428**(23): p. 4651-4668.

86. Dumoutier, L., Tounsi, A., Michiels, T., Sommereyns, C., Kotenko, S.V., and Renauld, J.-C., *Role of the interleukin (IL)-28 receptor tyrosine residues for antiviral and antiproliferative activity of IL-29/interferon-λ1: similarities with type I interferon signaling.* Journal of Biological Chemistry, 2004. **279**(31): p. 32269-32274.

87. Forero, A., Ozarkar, S., Li, H., Lee, C.H., Hemann, E.A., Nadjsombati, M.S., Hendricks, M.R., So, L., Green, R., Roy, C.N., Sarkar, S.N., von Moltke, J., Anderson, S.K., Gale, M., Jr., and Savan, R., *Differential Activation of the Transcription Factor IRF1 Underlies the Distinct Immune Responses Elicited by Type I and Type III Interferons.* Immunity, 2019. **51**(3): p. 451-464.e6.

88. Novatt, H., Theisen, T.C., Massie, T., Massie, T., Simonyan, V., Voskanian-Kordi, A., Renn, L.A., and Rabin, R.L., *Distinct Patterns of Expression of Transcription Factors in Response to Interferonβ and Interferonλ1.* J Interferon Cytokine Res, 2016. **36**(10): p. 589-598.

89. Broggi, A., Granucci, F., and Zanoni, I., *Type III interferons: Balancing tissue tolerance and resistance to pathogen invasion.* Journal of Experimental Medicine, 2019. **217**(1).

90. Pervolaraki, K., Stanifer, M.L., Münchau, S., Renn, L.A., Albrecht, D., Kurzhals, S., Senís, E., Grimm, D., Schröder-Braunstein, J., Rabin, R.L., and Boulant, S., *Type I and Type III Interferons Display Different Dependency on Mitogen-Activated Protein Kinases to Mount an Antiviral State in the Human Gut.* Front Immunol, 2017. **8**: p. 459.

91. Arimoto, K.I., Miyauchi, S., Stoner, S.A., Fan, J.B., and Zhang, D.E., *Negative regulation of type I IFN signaling.* J Leukoc Biol, 2018.

92. Blumer, T., Coto-Llerena, M., Duong, F.H.T., and Heim, M.H., *SOCS1 is an inducible negative regulator of interferon λ (IFN-λ)-induced gene expression in vivo.* J Biol Chem, 2017. **292**(43): p. 17928-17938.

93. François-Newton, V., Magno de Freitas Almeida, G., Payelle-Brogard, B., Monneron, D., Pichard-Garcia, L., Piehler, J., Pellegrini, S., and Uzé, G., *USP18-based negative feedback control is induced by type I and type III interferons and specifically inactivates interferon α response.* PLoS One, 2011. **6**(7): p. e22200.

94. Crow, Y.J. and Stetson, D.B., *The type I interferonopathies: 10 years on.* Nature Reviews Immunology, 2022. **22**(8): p. 471-483.

95. d'Angelo, D.M., Di Filippo, P., Breda, L., and Chiarelli, F., *Type I Interferonopathies in Children: An Overview.* Front Pediatr, 2021. **9**: p. 631329.

96. Lazear, H.M., Nice, T.J., and Diamond, M.S., *Interferon-λ: Immune Functions at Barrier Surfaces and Beyond.* Immunity, 2015. **43**(1): p. 15-28.

97. Crotta, S., Davidson, S., Mahlakoiv, T., Desmet, C.J., Buckwalter, M.R., Albert, M.L., Staeheli, P., and Wack, A., *Type I and type III interferons drive redundant amplification loops to induce a transcriptional signature in influenza-infected airway epithelia.* PLoS Pathog, 2013. **9**(11): p. e1003773.

98. Caine, E.A., Scheaffer, S.M., Arora, N., Zaitsev, K., Artyomov, M.N., Coyne, C.B., Moley, K.H., and Diamond, M.S., *Interferon lambda protects the female reproductive tract against Zika virus infection.* Nature Communications, 2019. **10**(1): p. 280.

99. Pagliaccetti, N.E. and Robek, M.D., *Interferon-lambda in the immune response to hepatitis B virus and hepatitis C virus.* J Interferon Cytokine Res, 2010. **30**(8): p. 585-90.

100. Nice, T.J., Baldridge, M.T., McCune, B.T., Norman, J.M., Lazear, H.M., Artyomov, M., Diamond, M.S., and Virgin, H.W., *Interferon-λ cures persistent murine norovirus infection in the absence of adaptive immunity.* Science, 2015. **347**(6219): p. 269-73.

101. Lazear, H.M., Daniels, B.P., Pinto, A.K., Huang, A.C., Vick, S.C., Doyle, S.E., Gale, M., Jr., Klein, R.S., and Diamond, M.S., *Interferon-λ restricts West Nile virus neuroinvasion by tightening the blood-brain barrier.* Sci Transl Med, 2015. **7**(284): p. 284ra59.

102. Douam, F., Soto Albrecht, Y.E., Hrebikova, G., Sadimin, E., Davidson, C., Kotenko, S.V., and Ploss, A., *Type III Interferon-Mediated Signaling Is Critical for Controlling Live Attenuated Yellow Fever Virus Infection In Vivo.* mBio, 2017. **8**(4).

103. Lasfar, A., Zloza, A., and Cohen-Solal, K.A., *IFN-lambda therapy: current status and future perspectives.* Drug Discov Today, 2016. **21**(1): p. 167-171.

104. Muir, A.J., Shiffman, M.L., Zaman, A., Yoffe, B., de la Torre, A., Flamm, S., Gordon, S.C., Marotta, P., Vierling, J.M., Lopez-Talavera, J.C., Byrnes-Blake, K., Fontana, D., Freeman, J., Gray, T., Hausman, D., Hunder, N.N., and Lawitz, E., *Phase 1b study of pegylated*

interferon lambda 1 with or without ribavirin in patients with chronic genotype 1 hepatitis C virus infection. Hepatology, 2010. **52**(3): p. 822-32.

105. Ramos, E.L., *Preclinical and clinical development of pegylated interferon-lambda 1 in chronic hepatitis C.* Journal of Interferon & Cytokine Research, 2010. **30**(8): p. 591-595.

106. Phillips, S., Mistry, S., Riva, A., Cooksley, H., Hadzhiolova-Lebeau, T., Plavova, S., Katzarov, K., Simonova, M., Zeuzem, S., and Woffendin, C., *Peg-interferon lambda treatment induces robust innate and adaptive immunity in chronic hepatitis B patients.* Frontiers in immunology, 2017. **8**: p. 621.

107. Chan, H.L., Ahn, S.H., Chang, T.-T., Peng, C.-Y., Wong, D., Coffin, C.S., Lim, S.G., Chen, P.-J., Janssen, H.L., and Marcellin, P., *Peginterferon lambda for the treatment of HBeAg-positive chronic hepatitis B: A randomized phase 2b study (LIRA-B).* Journal of hepatology, 2016. **64**(5): p. 1011-1019.

108. Reis, G., Moreira Silva, E.A., Medeiros Silva, D.C., Thabane, L., Campos, V.H., Ferreira, T.S., Santos, C.V., Nogueira, A.M., Almeida, A.P., and Savassi, L.C., *Early treatment with pegylated interferon lambda for Covid-19.* New England Journal of Medicine, 2023. **388**(6): p. 518-528.

109. Muir, A.J., Shiffman, M.L., Zaman, A., Yoffe, B., De La Torre, A., Flamm, S., Gordon, S.C., Marotta, P., Vierling, J.M., and Carlos Lopez-Talavera, J., *Phase 1b study of pegylated interferon lambda 1 with or without ribavirin in patients with chronic genotype 1 hepatitis C virus infection.* Hepatology, 2010. **52**(3): p. 822-832.

110. Liang, T.J. and Ghany, M.G., *Current and future therapies for hepatitis C virus infection.* N Engl J Med, 2013. **368**(20): p. 1907-17.

111. Thio, C.L., *Host genetic factors and antiviral immune responses to hepatitis C virus.* Clinics in liver disease, 2008. **12**(3): p. 713-726.

112. Thomas, D.L., Astemborski, J., Rai, R.M., Anania, F.A., Schaeffer, M., Galai, N., Nolt, K., Nelson, K.E., Strathdee, S.A., and Johnson, L., *The natural history of hepatitis C virus infection: host, viral, and environmental factors.* Jama, 2000. **284**(4): p. 450-456.

113. Reddy, K.R., Hoofnagle, J.H., Tong, M.J., Lee, W.M., Pockros, P., Heathcote, E.J., Albert, D., and Joh, T., *Racial differences in responses to therapy with interferon in chronic hepatitis C.* Hepatology, 1999. **30**(3): p. 787-793.

114. Ge, D., Fellay, J., Thompson, A.J., Simon, J.S., Shianna, K.V., Urban, T.J., Heinzen, E.L., Qiu, P., Bertelsen, A.H., and Muir, A.J., *Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance.* Nature, 2009. **461**(7262): p. 399-401.

115. Rauch, A., Kutalik, Z., Descombes, P., Cai, T., Di Iulio, J., Mueller, T., Bochud, M., Battegay, M., Bernasconi, E., and Borovicka, J., *Genetic variation in IL28B is associated with chronic hepatitis C and treatment failure: a genome-wide association study.* Gastroenterology, 2010. **138**(4): p. 1338-1345. e7.

116. Suppiah, V., Moldovan, M., Ahlenstiel, G., Berg, T., Weltman, M., Abate, M.L., Bassendine, M., Spengler, U., Dore, G.J., and Powell, E., *IL28B is associated with response to chronic hepatitis C interferon-α and ribavirin therapy.* Nature genetics, 2009. **41**(10): p. 1100-1104.

117. Tanaka, Y., Nishida, N., Sugiyama, M., Kurosaki, M., Matsuura, K., Sakamoto, N., Nakagawa, M., Korenaga, M., Hino, K., and Hige, S., *Genome-wide association of IL28B with response to pegylated interferon-α and ribavirin therapy for chronic hepatitis C.* Nature genetics, 2009. **41**(10): p. 1105-1109.

118. Thomas, D.L., Thio, C.L., Martin, M.P., Qi, Y., Ge, D., O'hUigin, C., Kidd, J., Kidd, K., Khakoo, S.I., and Alexander, G., *Genetic variation in IL28B and spontaneous clearance of hepatitis C virus.* Nature, 2009. **461**(7265): p. 798-801.

119. Prokunina-Olsson, L., Muchmore, B., Tang, W., Pfeiffer, R.M., Park, H., Dickensheets, H., Hergott, D., Porter-Gill, P., Mumy, A., and Kohaar, I., *A variant upstream of IFNL3 (IL28B) creating a new interferon gene IFNL4 is associated with impaired clearance of hepatitis C virus.* Nature genetics, 2013. **45**(2): p. 164-171.

120. O'Brien, T.R. and Jackson, S.S., *What Have We Learned from Studies of IFN-λ Variants and Hepatitis C Virus Infection?* Journal of Interferon & Cytokine Research, 2019. **39**(10): p. 618-626.

121. Meissner, E.G., Bon, D., Prokunina-Olsson, L., Tang, W., Masur, H., O'Brien, T.R., Herrmann, E., Kottilil, S., and Osinusi, A., *IFNL4-ΔG genotype is associated with slower viral clearance in hepatitis C, genotype-1 patients treated with sofosbuvir and ribavirin.* The Journal of infectious diseases, 2014. **209**(11): p. 1700-1704.

122. Hamming, O.J., Terczyńska-Dyla, E., Vieyres, G., Dijkman, R., Jørgensen, S.E., Akhtar, H., Siupka, P., Pietschmann, T., Thiel, V., and Hartmann, R., *Interferon lambda 4 signals via the IFNλ receptor to regulate antiviral activity against HCV and coronaviruses.* The EMBO journal, 2013. **32**(23): p. 3055-3065.

123. O'Brien, T.R., Prokunina-Olsson, L., and Donnelly, R.P., *IFN-λ4: the paradoxical new member of the interferon lambda family.* J Interferon Cytokine Res, 2014. **34**(11): p. 829-38.

124. Onabajo, O.O., Muchmore, B., and Prokunina-Olsson, L., *The IFN-λ4 conundrum: when a good interferon goes bad.* Journal of Interferon & Cytokine Research, 2019. **39**(10): p. 636-641.

125. Obajemu, A.A., Rao, N., Dilley, K.A., Vargas, J.M., Sheikh, F., Donnelly, R.P., Shabman, R.S., Meissner, E.G., Prokunina-Olsson, L., and Onabajo, O.O., *IFN-λ4 attenuates antiviral responses by enhancing negative regulation of IFN signaling.* The Journal of Immunology, 2017. **199**(11): p. 3808-3820.

126. Lauber, C., Vieyres, G., Terczynska-Dyla, E., Anggakusuma, Dijkman, R., Gad, H.H., Akhtar, H., Geffers, R., Vondran, F.W., Thiel, V., Kaderali, L., Pietschmann, T., and

Hartmann, R., *Transcriptome analysis reveals a classical interferon signature induced by IFNlambda4 in human primary cells.* Genes Immun, 2015. **16**(6): p. 414-21.

127. Pervolaraki, K., Rastgou Talemi, S., Albrecht, D., Bormann, F., Bamford, C., Mendoza, J.L., Garcia, K.C., McLauchlan, J., Hofer, T., Stanifer, M.L., and Boulant, S., *Differential induction of interferon stimulated genes between type I and type III interferons is independent of interferon receptor abundance.* PLoS Pathog, 2018. **14**(11): p. e1007420.

128. Santer, D.M., Minty, G.E.S., Golec, D.P., Lu, J., May, J., Namdar, A., Shah, J., Elahi, S., Proud, D., Joyce, M., Tyrrell, D.L., and Houghton, M., *Differential expression of interferon-lambda receptor 1 splice variants determines the magnitude of the antiviral response induced by interferon-lambda 3 in human immune cells.* PLoS Pathog, 2020. **16**(4): p. e1008515.

129. Witte, K., Gruetz, G., Volk, H.D., Looman, A.C., Asadullah, K., Sterry, W., Sabat, R., and Wolk, K., *Despite IFN-lambda receptor expression, blood immune cells, but not keratinocytes or melanocytes, have an impaired response to type III interferons: implications for therapeutic applications of these cytokines.* Genes Immun, 2009. **10**(8): p. 702-14.

130. Moraga, I., Spangler, J., Mendoza, J.L., and Garcia, K.C., *Multifarious determinants of cytokine receptor signaling specificity.* Adv Immunol, 2014. **121**: p. 1-39.

131. Mori, T. and Katayama, Y., *Signal amplification in flow cytometry for cell surface antigen analysis.* The Journal of Biochemistry, 2019. **166**(3): p. 205-212.

132. Li, W., Huang, X., Liu, Z., Wang, Y., Zhang, H., Tong, H., Wu, H., and Lin, S., *Type III interferon induces apoptosis in human lung cancer cells.* Oncol Rep, 2012. **28**(3): p. 1117-25.

133. Li, W., Lewis-Antes, A., Huang, J., Balan, M., and Kotenko, S.V., *Regulation of apoptosis by type III interferons.* Cell Prolif, 2008. **41**(6): p. 960-979.

134. Kozak, M., *Initiation of translation in prokaryotes and eukaryotes.* Gene, 1999. **234**(2): p. 187-208.

135. Zhang, L., Hernan, R., and Brizzard, B., *Multiple tandem epitope tagging for enhanced detection of protein expressed in mammalian cells.* Mol Biotechnol, 2001. **19**(3): p. 313-21.

136. Fisher, J.B., Pulakanti, K., Rao, S., and Duncan, S.A., *GATA6 is essential for endoderm formation from human pluripotent stem cells.* Biol Open, 2017. **6**(7): p. 1084-1095.

137. Evans, J.G., Novotny, L.A., and Meissner, E.G., *Influence of Canonical and Non-Canonical IFNLR1 Isoform Expression on Interferon Lambda Signaling.* Viruses, 2023. **15**(3): p. 632.

138. Bhushal, S., Wolfsmüller, M., Selvakumar, T.A., Kemper, L., Wirth, D., Hornef, M.W., Hauser, H., and Köster, M., *Cell polarization and epigenetic status shape the*

heterogeneous response to type III interferons in intestinal epithelial cells. Frontiers in immunology, 2017. **8**: p. 671.

139. Levin, D., Harari, D., and Schreiber, G., *Stochastic receptor expression determines cell fate upon interferon treatment.* Molecular and cellular biology, 2011. **31**(16): p. 3252-3266.

140. Das, A.T., Tenenbaum, L., and Berkhout, B., *Tet-On Systems For Doxycycline-inducible Gene Expression.* Curr Gene Ther, 2016. **16**(3): p. 156-67.

141. Loew, R., Heinz, N., Hampf, M., Bujard, H., and Gossen, M., *Improved Tet-responsive promoters with minimized background expression.* BMC Biotechnol, 2010. **10**: p. 81.

142. Nakayama, T., Wiest, D.L., Abraham, K.M., Munitz, T.I., Perlmutter, R.M., and Singer, A., *Decreased signaling competence as a result of receptor overexpression: overexpression of CD4 reduces its ability to activate p56lck tyrosine kinase and to regulate T-cell antigen receptor expression in immature CD4+CD8+ thymocytes.* Proc Natl Acad Sci U S A, 1993. **90**(22): p. 10534-8.

143. Sta, L., Voisinne, G., Cotari, J., Adamer, M.F., Molina-París, C., and Altan-Bonnet, G., *Tuning of cytokine signaling through imbalanced abundances of receptors and kinases.* bioRxiv, 2022: p. 2022.10.07.511173.

144. Cendrowski, J., Mamińska, A., and Miaczynska, M., *Endocytic regulation of cytokine receptor signaling.* Cytokine Growth Factor Rev, 2016. **32**: p. 63-73.

145. Morris, R., Kershaw, N.J., and Babon, J.J., *The molecular details of cytokine signaling via the JAK/STAT pathway.* Protein Sci, 2018. **27**(12): p. 1984-2009.

146. Hetz, C., *The unfolded protein response: controlling cell fate decisions under ER stress and beyond.* Nature Reviews Molecular Cell Biology, 2012. **13**(2): p. 89-102.

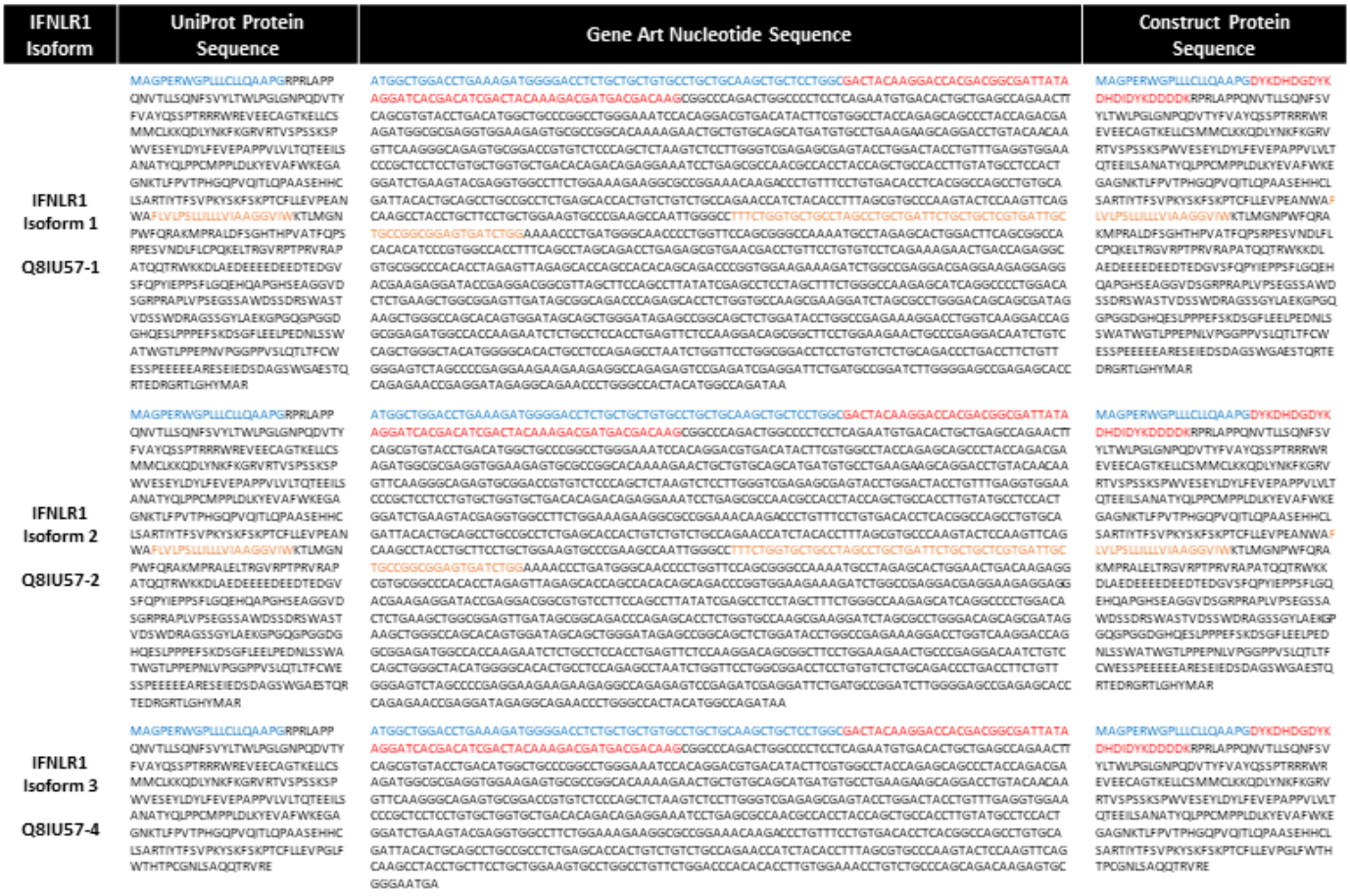
147. Ragimbeau, J., Dondi, E., Alcover, A., Eid, P., Uzé, G., and Pellegrini, S., *The tyrosine kinase Tyk2 controls IFNAR1 cell surface expression.* Embo j, 2003. **22**(3): p. 537-47.

148. Scheller, J., Chalaris, A., Schmidt-Arras, D., and Rose-John, S., *The pro-and anti-inflammatory properties of the cytokine interleukin-6.* Biochimica et Biophysica Acta (BBA)-Molecular Cell Research, 2011. **1813**(5): p. 878-888.

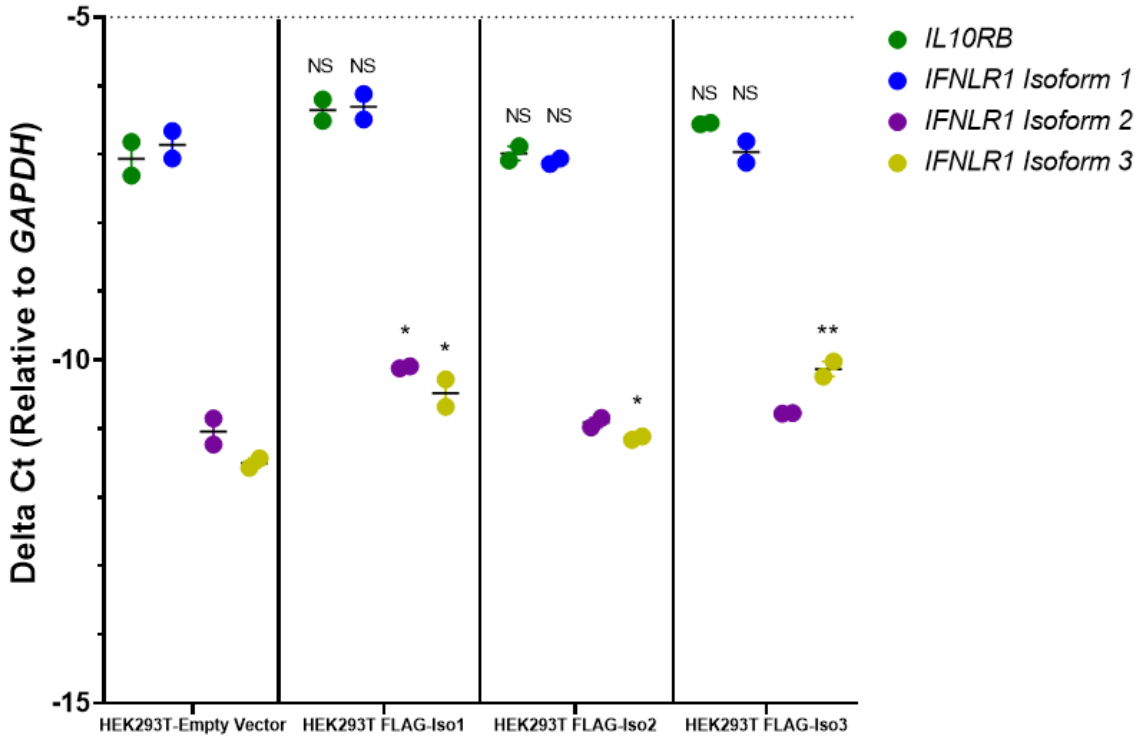
149. Chmiest, D., Sharma, N., Zanin, N., Viaris de Lesegno, C., Shafaq-Zadah, M., Sibut, V., Dingli, F., Hupé, P., Wilmes, S., Piehler, J., Loew, D., Johannes, L., Schreiber, G., and Lamaze, C., *Spatiotemporal control of interferon-induced JAK/STAT signalling and gene transcription by the retromer complex.* Nat Commun, 2016. **7**: p. 13476.

150. Sakurai, F., Mitani, S., Yamamoto, T., Takayama, K., Tachibana, M., Watashi, K., Wakita, T., Iijima, S., Tanaka, Y., and Mizuguchi, H., *Human induced-pluripotent stem cell-derived hepatocyte-like cells as an in vitro model of human hepatitis B virus infection.* Sci Rep, 2017. **7**: p. 45698.

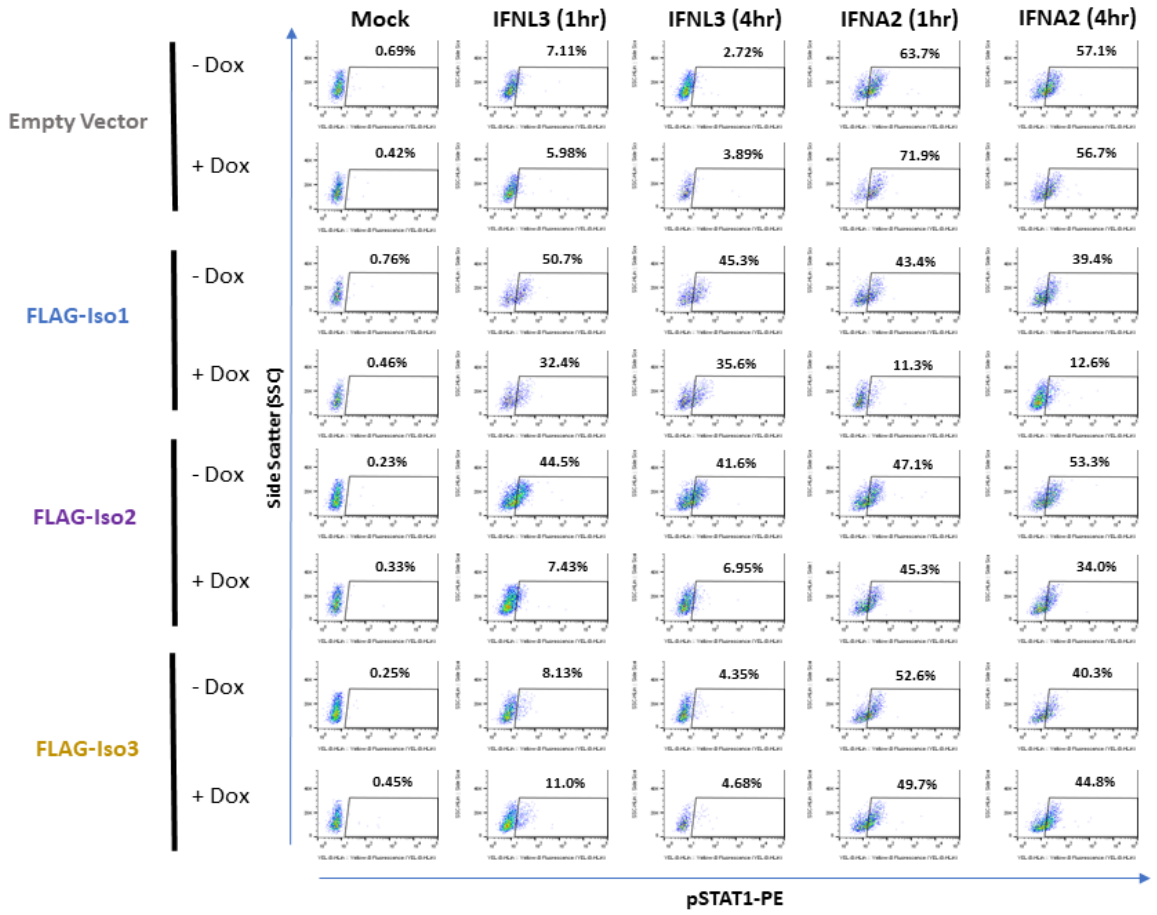
SUPPLEMENTAL FIGURES:



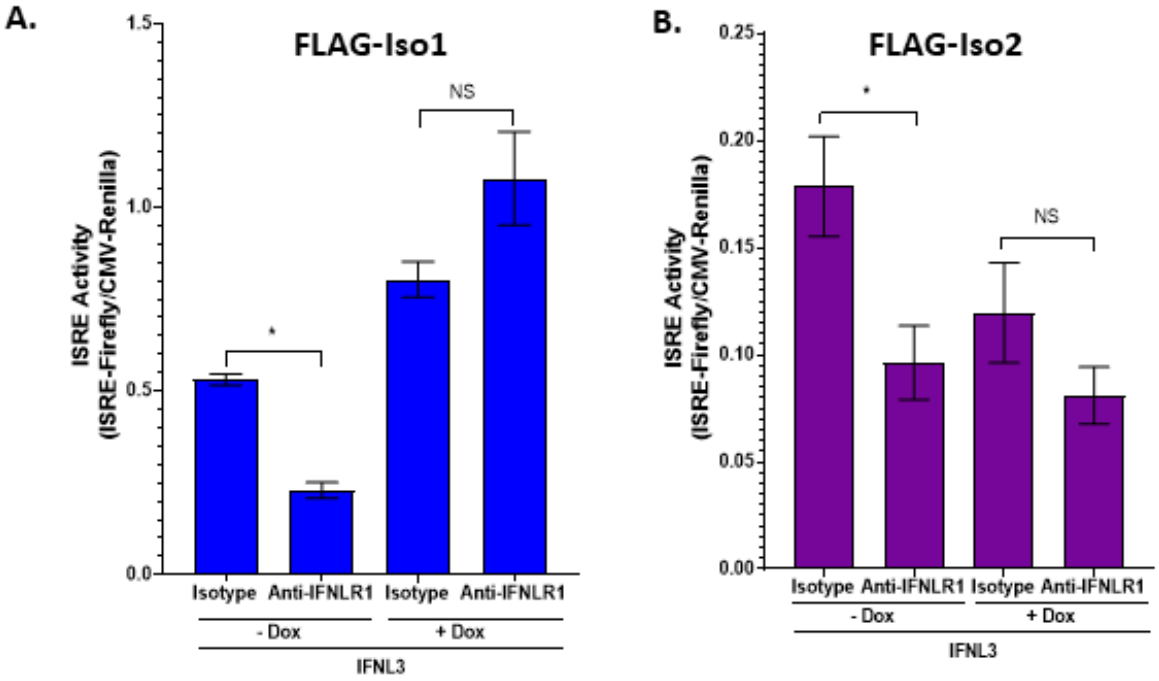
Supplemental Figure 1: Protein and nucleotide sequences of IFNLR1 isoforms. Unaltered protein sequences downloaded from UniProt database (left column), codon-optimized nucleotide sequences used for Gene Art synthesis (middle column), and FLAG-tagged IFNLR1 isoform protein sequences (right column). Signal sequence is denoted in blue; 3x-FLAG tag is denoted in red; the transmembrane domain is denoted in orange.



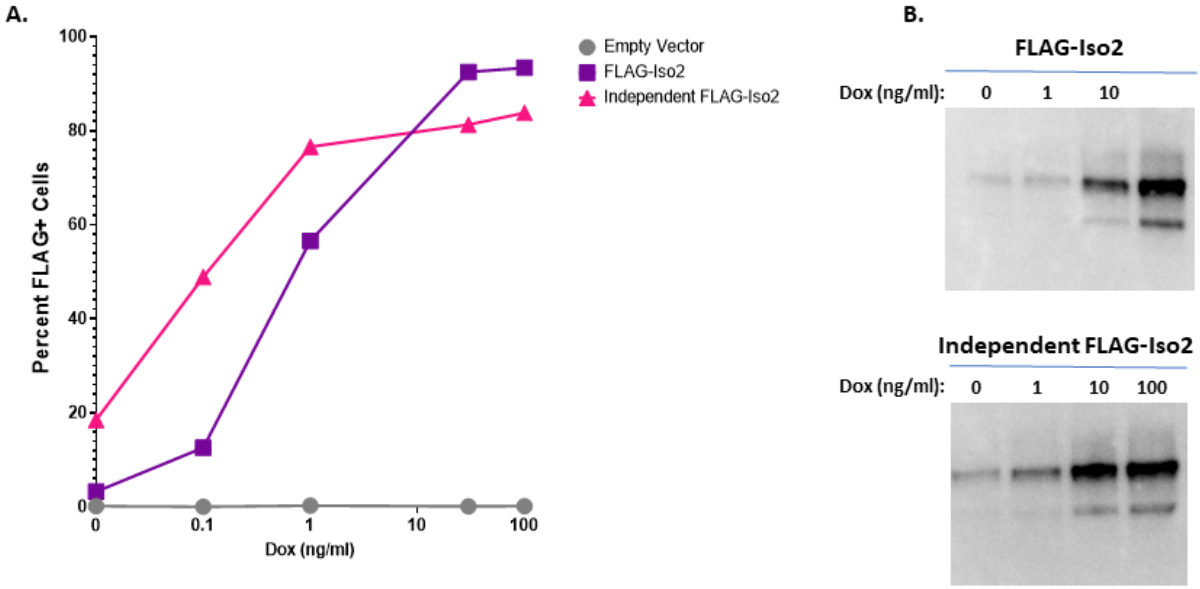
Supplemental Figure 2: Endogenous IL10RB and IFNLR1 isoform expression in HEK293T stable lines. Cellular gene expression was quantitated by qRT-PCR. Statistical significance represented by asterisks reflect comparisons between EV and FLAG-IFNLR1 isoform cells. Error bars represent standard error of the mean. * = $p < 0.05$, ** = $p < 0.01$. NS = not significant.



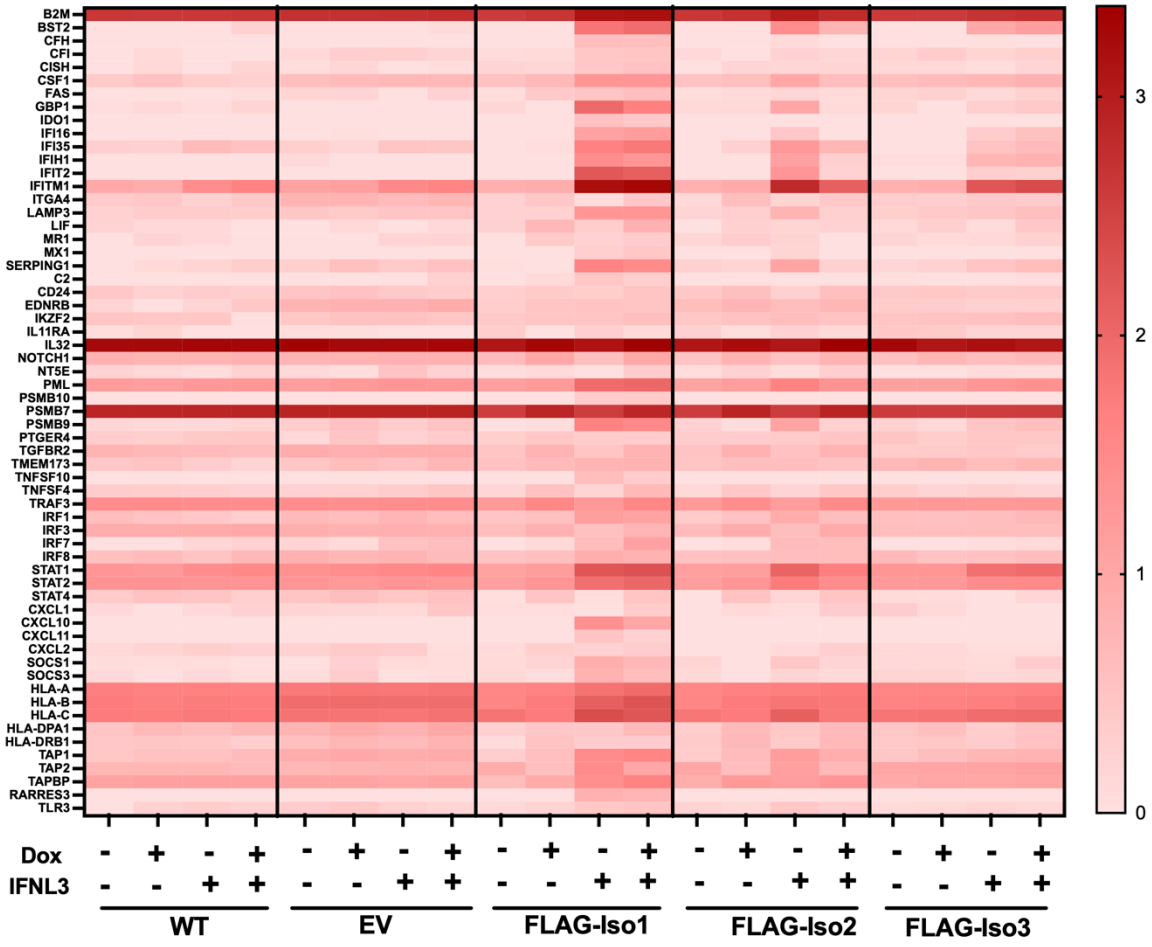
Supplemental Figure 3: Representative flow cytometry plots used for quantification in Figures 3, 4, 6, and 9.



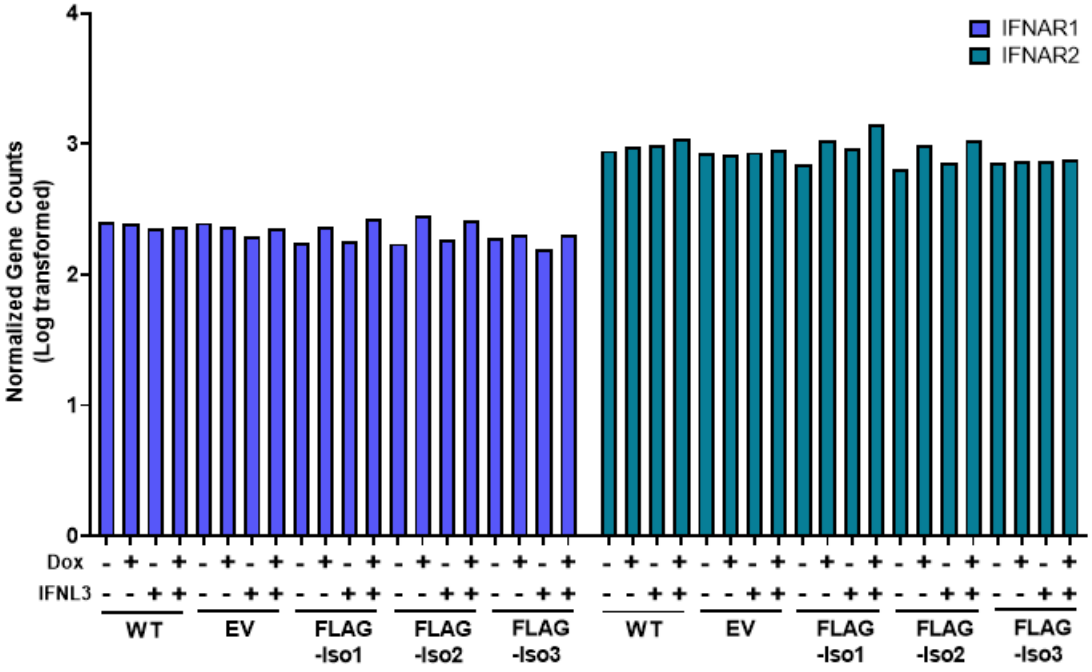
Supplemental Figure 4: Effect of pre-incubation with anti-IFNLR1 antibody on the IFNL3 response in FLAG-Iso1 and FLAG-Iso2 lines. IFNLR1 FLAG-Iso1 **(A)** and FLAG-Iso2 **(B)** cells were treated ± dox (100 ng/ml) for 24 hrs prior to incubation with anti-IFNLR1 antibody or isotype control (anti-IL-28RA antibody, control IgG, R&D Systems, 4ug/ml) for 1.5 hrs, after which the cells were stimulated with IFNL3 (100 ng/ml) for 24 hrs and harvested for dual luciferase assay (representative data, 2 independent experiments). Error bars represent standard error of the mean. * = $p < 0.05$. NS = not significant.



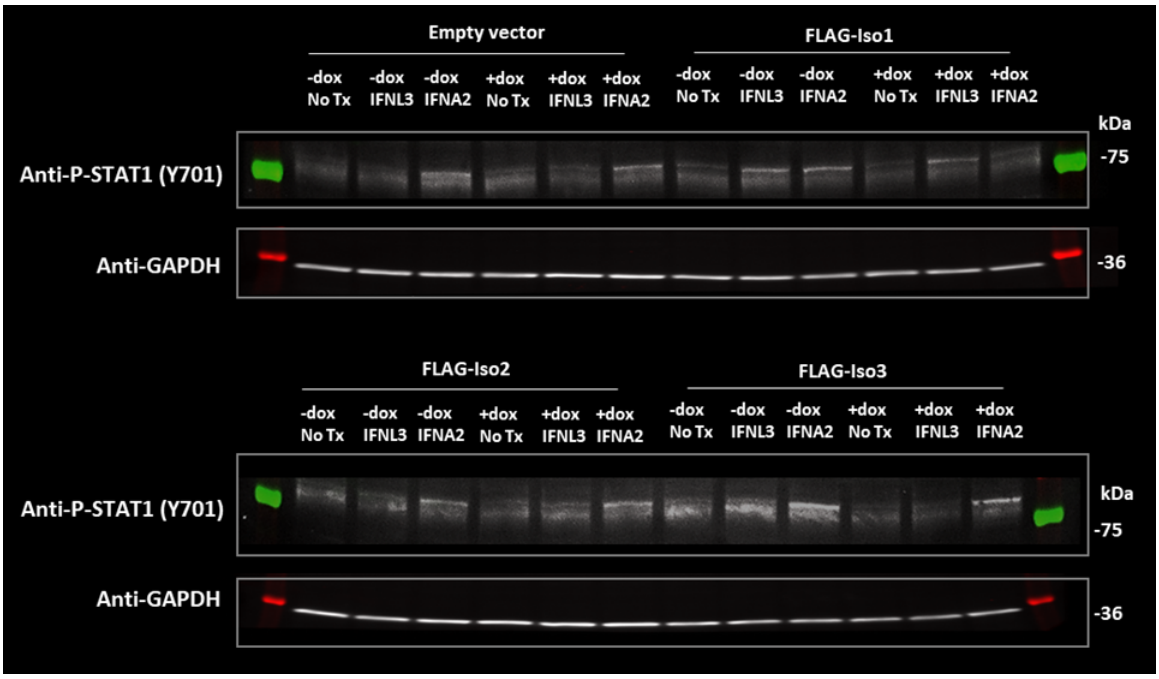
Supplemental Figure 5: Dox-titratable FLAG-Isoform 2 expression in independent HEK293T stable lines. (A) HEK293T-EV, the FLAG-Iso2 line characterized in Figure 4, and an independent FLAG-Iso2 line were treated ± dox (0, 0.1, 1, 10, or 100 ng/ml) for 24 hr prior to analysis of FLAG expression by flow cytometry. **(B)** FLAG-Iso2 expression in each line after treatment ± dox (0, 1, 10, 100 ng/ml) for 24 hr is shown by western blot of cell lysates using anti-FLAG antibody.



Supplementary Figure 6: Differentially expressed genes among HEK293T cells line. HEK293T WT and stable lines were induced ± dox (100 ng/ml) for 24 hrs prior to treatment with ± IFNL3 (100 ng/ml) for an additional 24 hrs. RNA was collected and gene count quantitated by NanoString analysis (nCounter Human Immunology v2 Panel). Genes shown were differentially regulated (>2-fold change) in one or more cell lines after doxycycline and/or IFNL3 treatment. Data are shown as log transformed normalized counts.



Supplemental Figure 7: ***IFNAR1*** **and** ***IFNAR2*** **RNA expression levels are unchanged among cell lines and conditions.** Data were derived from NanoString analysis (described in Figure 8 and Supplemental Figure 6).



Supplemental Figure 8: Western blot analysis of pSTAT1 in HEK293T stable lines. HEK293T-EV and FLAG-IFNLR1 isoform lines were ± dox treated (100 ng/ml) for 24 hrs prior to mock, IFNL3 (100ng/ml), or IFNA2 (100 ng/ml) stimulation for 1 hr. Protein was collected from cell lysates and assessed by western blot. The larger of the two observed bands is interpreted to reflect pSTAT1 signal.