Study of the cellular response to type I interferons : role of cysteine protease USP18

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TABLE OF CONTENTS

List of abreviations

AML	acute myeloid leukaemia
β-TrCP	beta-transducin repeats-containing protein
CYLD	cylindromatosis
DC	dendritic cells
DKO	double knock-out
ERK	extracellular-regulated kinase
FERM	band 4.1-ezrin-radixin-moesin domain
GM-CSF	granulocyte-macrophage colony stimulating factor
GAS	gamma-activated site
IL	interleukin
IFN	interferon
IFNAR	interferon receptor
ISG	interferon stimulated gene
ISRE	interferon-stimulated response element
IRF	interferon response factor
Jak	Janus kinase
JH	Jak homology
KL	kinase-like
KO	knock-out
LIF	leukaemia inhibitory factor
MEF	mouse embryonic fibroblast
NK	Natural killer
PTP	Protein tyrosine phosphatase
RANKL	receptor activator of nuclear κB ligand
SH2	Src-homology 2 domain
SOCS	suppressor of cytokine signalling
SUMO	small ubiquitin modifier

Th	T helper
TK	Tyrosine kinase
USP	ubiquitin specific protease
WT	wild type

Résumé

Type I and type III IFNs form two multigenic families of pathogen-induced cytokines that bind to different receptors but exhibit common bioactivities. In humans, Type I IFN comprises 17 highly related subtypes, broadly referred to as IFN α/β, all binding a ubiquitously expressed receptor complex constituted of two subunits, IFNAR1 and IFNAR2 chains. The type III IFN (3 λs) binds to a receptor complex made of cell type-restricted IFNLR1 and the broadly expressed IL-10R2. Downstream of these receptor complexes is a shared Jak/STAT pathway, involving the Janus kinases Jak1 and Tyk2 and the transcription factors STAT1/2/3. Thus, the Type I and III IFN families induce the same gene subset and exert antiviral activity through independent receptor complexes. Among the human subtypes induced *in vivo* in response to multiple stimuli, IFN β is especially potent in bioactivities requiring long term stimulation, such as proliferation inhibition. However, the molecular basis of the α2/β differential is unknown.

A critical feature of the IFN response concerns its negative regulation and indeed, its perturbation leads to auto-immune manifestations. Signaling feedback controls operate at immediate-early times and include Ser/Thr kinases and ubiquitin ligase(s) targeting the IFNAR1 receptor subunit as well as SOCS-mediated action on receptor/Jaks and STATs. An additional type of negative feedback control becomes effective at late time of IFN stimulation and involves USP18, an IFN-induced isopeptidase that cleaves ubiquitin-like ISG15 from conjugates.

In the first part of my thesis work I studied how prolonged exposure (priming) of various cell types to type I or III IFNs interferes with their subsequent ability to respond to IFNs. I found that primed cells retain sensitivity to IFN β but are desensitized to IFNs α subtypes. Differential desensitization is not consequent to down-regulation of surface receptor but is dependent of induction of the isopeptidase USP18. Using ^{125}I-radiolabeled ligands, I found that desensitized cells, *ie* expressing USP18, are impaired in their ability to bind IFN α2 but not IFN β. These data suggest that USP18, by targeting the assembly of functional IFN α binding sites, is responsible for the differential desensitization state (Francois-Newton et al., 2011).

In the second part of my thesis, I analyzed to what extent induced USP18 affects bioactivities requiring long term IFN treatment. For this, I monitored STAT activation and ISG accumulation at the mRNA and protein levels in control cells and in cells silenced for

USP18. At late stimulation times (>10 hrs), an α2/β differential ISG accumulation became manifest at both transcript and protein levels. Importantly, this α2/β differential was almost totally abrogated in cells that had been silenced for USP18. I also assessed the long term (72 hrs) response to IFNs of control and USP18-silenced cells in an antiproliferative assay and found that the α2/β differential is remarkably decreased in cells silenced for USP18. Overall, these data show that upon prolonged treatment, the dose-dependent accumulation of USP18 progressively restrains IFN α2-induced signaling (Francois-Newton et al., Biochem J. *in revision*).

In the third part of my work, I investigated whether the isopeptidase activity of USP18 is required for differential desensitization. To address this question two approaches were used. In the first one, I generated clones expressing a catalytically inactive USP18 mutant and analysed their response to IFN α2 and IFN β. I showed that the catalytic activity of USP18 is required for differential desensitization, unless the protein is very abundant. In a second approach the enzymes involved in the ISGylation machinery were silenced and the response to type I IFN was monitored. I found that the ISGylation machinery is essential for USP18 to exert its function and that the E3 enzyme EFP/TRIM25 is implicated in ISGylation of a putative USP18 substrate(s) that may contribute to efficient IFN α driven receptor complex formation. Finally, I showed that endogenous USP18 expression is fine-tuned by free ISG15. Overall, these studies demonstrate the importance of USP18 in making primed cells refractory to IFN α and in establishing differential activities of IFN α2 and IFN β.

INTRODUCTION

1. Interferons: Generalities

The term Interferon (IFN) was coined in 1957 by Isaac and Linderman to designate an "activity" produced by heat-inactivated influenza virus-infected chick embryo cells and interfering with virus replication (Isaacs and Lindenmann, 1957). Today, we distinguish three families of IFNs (type I, II and III) that do exhibit antiviral activity - though to differing extent - but also exert a wide range of additional, type-specific and powerful activities. IFNs can be roughly defined as helicoidal cytokines that participate to maintain and induce the immune response of the host to harmful pathogens. The three types of IFNs are :

Type I IFN or IFN α/β is the most potent antiviral substance and serves as an early warning molecule that signals the presence of pathogens and provides a pivotal function at the interface between innate and adaptive responses. The few examples below illustrate that, in addition to its antiviral activity, Type I IFN exerts potent immunoregulatory functions. Dendritic cells (DCs) are immune cells that act as sensors of infection and as antigen presenting cells. Treatment of immature DCs with IFN α/β results in the upregulation of maturation markers (eg CD80, CD86, CD40) and enhanced ability to stimulate B and T cells (Le Bon et al., 2001; Le Bon and Tough, 2002; Montoya et al., 2002). Along with maturation, IFN α/β treatment results in secretion of chemokines and cytokines (such as IL-15, BAFF and APRIL) (Litinskiy et al., 2002; Mattei et al., 2001). In combination, the IFN-induced DC maturation and cytokine secretion promote antibody production and class switching by B cells, and cross-priming of CD8+ T cells (Le Bon et al., 2003). Secreted IFN also contribute to the development of CD4+ T lymphocytes along the Th1-specific lineage, via the induction of the β2-chain of the IL-12R (Cella et al., 2000). By signaling through STAT4, type I IFN has also been reported to directly induce the production of IFN γ in Natural Killer cells (NK) and T cells (Nguyen et al., 2002a).

Type II IFN or IFN γ was described in 1965 as an IFN-like virus inhibitory protein produced by mitogen-activated human T lymphocytes (Wheelock, 1965). IFN γ affects diverse aspects of innate immunity, such as the activation of macrophages, and has strong effects on acquired responses, particularly in cell-mediated immunity, where it promotes the development of CD4+ Th1 cells and cytotoxic CD8+ T cells, while suppressing CD4+ Th2

cells. Studies in murine tumor models also demonstrate a role for IFN γ and T lymphocytes in the natural suppression of tumor development (Ikeda et al., 2002).

Type III IFN (IFN λs) is the most recently described group (Kotenko et al., 2003; Sheppard et al., 2003). While these proteins have little sequence similarity with Type I IFN, they share with it the ability to activate the same signaling pathway (activation of Tyk2, Jak1, STAT1, STAT2, STAT3 and formation of the transcription factor ISGF3), produce several IFN-inducible proteins (MxA, 2′–5′ oligoadenylate synthetase (OAS), PKR) and exert an antiviral activity. A major question is whether IFN λ primarily plays a back-up role to Type I IFN or whether it exerts undiscovered unique functions.

A simple way to distinguish the three IFN families is on the basis of receptor usage. Type I IFN binds to a ubiquitously expressed, heterodimeric cell surface receptor made of two single transmembrane spanning subunits, IFNAR1 and IFNAR2. The type II and type III IFN bind to receptor complexes formed by IFNGR1/IFNGR2 and IL10R2/IFNLR1, respectively (Borden et al., 2007).

In my research work, I have addressed mechanistic aspects of the regulation of signaling by human type I IFN. I have also described a signaling crosstalk between the type I and type III IFNs. In the following sections I will therefore introduce these two families of cytokines.

1.1 Type I IFN: a short history

Discovered in 1957 as an antiviral substance, type I IFN was soon after recognised for its ability to inhibit the proliferation of cells in culture and the growth of tumors in mice (Paucker et al., 1962; Gresser et al., 1969) as well as for its immunoregulatory actions. Early findings described the ability of IFN, purified from the culture medium of murine fibroblasts infected with New Castle disease virus, to enhance killing of target tumor cells by cytotoxic T cells and to induce expression of major histocompatibility complex antigens (MHC) (Lindahl et al., 1972; Lindahl et al., 1976). In 1980, using DNA recombinant technology, Taniguchi and colleagues cloned and sequenced a human IFN β-encoding cDNA from fibroblasts incubated with poly I:C (Taniguchi et al., 1980). Using the same technique, a human IFN α cDNA was cloned from leukocytes (Nagata et al., 1980).

In humans, Type I IFN includes 17 members (13 subtypes of IFN α and IFNs β/ε/κ/ω). The genes encoding Type I IFN are intronless and are located in a region spanning 400 kb on chromosome 9, with the exception of IFN κ, which is located 6 Mb away from the other type I IFN genes. *IFN* α genes share 80-95% nucleotide sequence identity and in contrast, *IFN* β gene shares only 30 % nucleotide sequence identity with *IFN* α genes (Trent et al., 1982).

To obtain insight into the selective forces that have driven the evolution of Type I IFN in humans, the group of Quintana-Murci and colleagues have recently characterized the levels of sequence-based diversity in the 17 Type I IFN genes by full resequencing of a panel of healthy individuals from sub-Saharan Africa, Europe, and Asia (Manry et al.). These analyses allowed them to study the effect of natural selection on IFN evolution since the divergence of human and chimpanzee lineages and within different human populations. The authors could show that the different subtypes of Type I IFN differ in their levels of evolutionary constraint. For example, IFN α2, IFN β and IFN ω show low levels of amino acid-altering variations, suggesting that they fulfill an essential, nonredundant function in host defense. In contrast, some IFN, such as IFN α10 and IFN ε, have accumulated missense and nonsense mutations at high frequencies within the population, suggesting redundancy in host defense (Manry et al., 2011).

My research work has focused on IFN α2 and IFN β. It is relevant that these two subtypes are non redundant since their low level of amino acid (aa) changes in human populations points to a pivotal role in host defence.

1.2 Type I IFN: induction phase

As introduced above, Type I IFN modulates numerous aspects of the cell physiology including cell proliferation and protein translation. Thus the production of these cytokines must be tightly regulated and this is achieved through stringent regulation of gene transcription. In the absence of stimulus and activated transcription factors, IFN α and IFN β gene expression is kept nearly silent, through constitutive repression by, for instance, the IFN regulatory factor IRF2 which competes with positive regulators (Paun and Pitha, 2007). As nearly all cell and tissue types are susceptible to infection, all cells of the body are capable of producing and responding to IFN. During a bacterial or viral infection, complex

signaling cascades are initiated by the detection of products that are referred as pathogen associated molecular patterns (PAMPS). Bacterial motifs or PAMPS will be recognised by transmembrane proteins known as Toll-like receptors (TLR). To date, ten TLRs have been identified in human. Each TLR recognizes specific PAMPS from bacteria, virus and fungi. For example lipopolysacharide (LPS) or flagellin, which are bacterial constituants, will be recognised by TLR4 and TLR5, respectively. The detection of viral nucleic acid is mediated by endosomal transmembrane TLRs (TLR 3, 7, 9) and also by cytoplasmic helicase receptors (RIG-I and MDA5). The detection of bacterial or viral PAMPs activates distinct signaling pathways that ultimately converge onto phosphorylation of key transcription factors (Fig. 1 upper panel). These include NF-κB, which is activated by phosphorylation-dependent destruction of its cytoplasmic inhibitor, IκB ; the AP-1 complex (composed of c-jun and ATF2) and one or more IRF family members (for e.g IRF3). When phosphorylated, these transcription factor complexes interact with the IFN β and the IFN α4 gene transcriptional control region in a concerted and highly cooperative fashion, leading to efficient recruitment of the transcriptional coactivators, the basal transcriptional machinery and RNA polymerase holoenzyme (Fig. 1, lower panel (a)). The low-level IFN β and IFN α4 that is secreted will bind to and activate the cognate type I IFN receptor in an autocrine and paracrine manner, leading to the activation of the Jak/Stat pathway, formation of the trimeric ISGF3 complex, made of phospho-Tyr-Stat1/Stat2 and IRF9, that translocates to the nucleus to induce Interferon Stimulated Genes (ISG) (Fig. 1, lower panel (b)). Among the induced ISGs is IRF7 that will contribute to further IFN β gene expression and to the induction of IFN α genes (Fig. 1 lower panel (c)) (Levy et al., 2003). Depending on the abundance in the cell of distinct transcription factors, notably IRF7, the timing and level of induction will differ. For instance, plasmacytoid dendritic cells constitutively express IRF7 that enables them to rapidly produce high levels of type I IFNs.

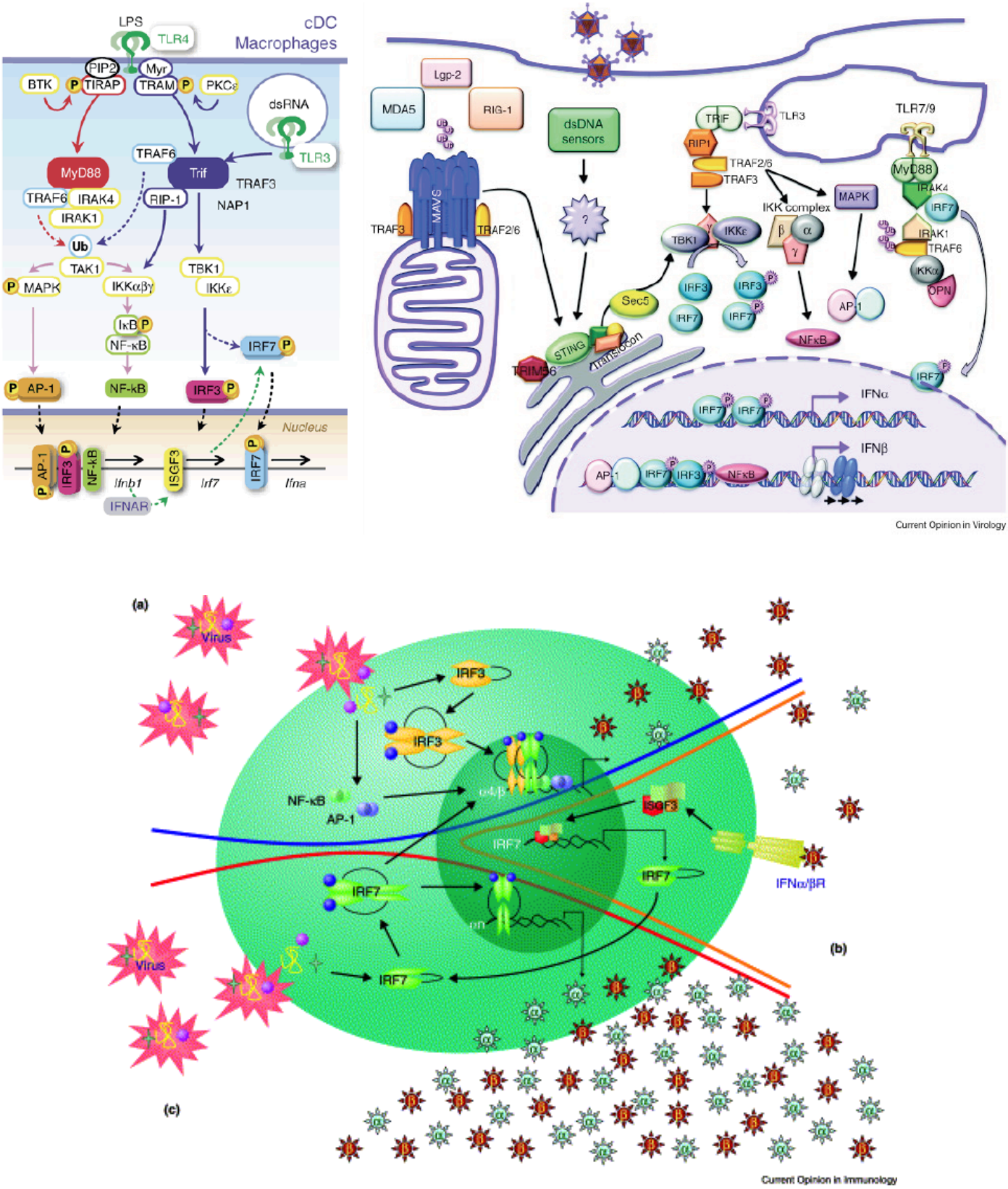


Figure 1 Schematic representation of the induction mode of type I IFN genes. Upper panel: Signaling cascade initiated by TLR (left pannel) or cytosolic sensors (right panel) upon PAMPs detection (adapted from Levy et al, curr.opinion in virology 2011). Lower panel: Type I IFN genes are differentially regulated and depending on the abundance of distinct transcription factors, three phases can be distinguished : (a) an immediate-early (sensitization) phase, (b) IRF7 induction phase, (c) delayed-early (amplification) phase (adapted from (Levy et al., 2003)).

1.3 Type I IFN: basic signaling steps

All type I IFN subtypes bind to the ubiquitously expressed receptor made up of two subunits, IFNAR1 and IFNAR2 (Fig. 2). The ligand-driven assembly of these subunits leads to the juxtaposition of the associated intracellular Janus kinases (or Jaks) Tyk2 and Jak1, which are catalytically activated by *trans*-phosphorylation (Fig. 2). Activated Jaks in turn phosphorylate the receptor cytoplasmic domains on specific tyrosine residues, which then serve as docking sites for STAT proteins (Signal Transducer and Activator of Transcription). Once recruited to the receptor, STATs become phosphorylated by Jaks on a key tyrosine residue, they dissociate from the receptor, translocate to the nucleus and bind ISRE (IFN-stimulated response element) or GAS (gamma-activated sequence) elements in the promoter of target genes (Darnell et al., 1994; Kisseleva et al., 2002). Additional non-Stat pathways have been reported to play important roles in mediating signals for the generation of IFN-responses. Various studies have shown the importance of mitogen activated protein kinases (MAPKs), especially p38 and ERK1/2, as well as the PI3K/AKT (phosphoinositol-3-kinase) pathway in transmitting signals that may be critical importance for the biological effects of IFNs. Besides regulating the transcription of ISGs in some cases, engagement of these signaling pathways by the IFN-receptor associated complexes may also play a role in mediating the translation of ISGs (Joshi et al.).

Detailed binding studies of the ligand to the ectodomains of the two receptor subunits, tethered onto solid supported bilayers have been performed in recent years. These analyses have led to propose a two-step binding mechanism, where the ligand binds first to IFNAR2, the high affinity subunit, and then the complex recruits IFNAR1 (Fig. 2) (Gavutis et al., 2006)).

A large part of my research work has focused on the differential activities of IFN α2 and IFN β. These two IFN subtypes engage each of the two receptor subunits with different affinity and I will dwell on the important consequences that the early binding steps have on their bioactivities. For this reason, I will give more insight into the early step of the signaling cascade.

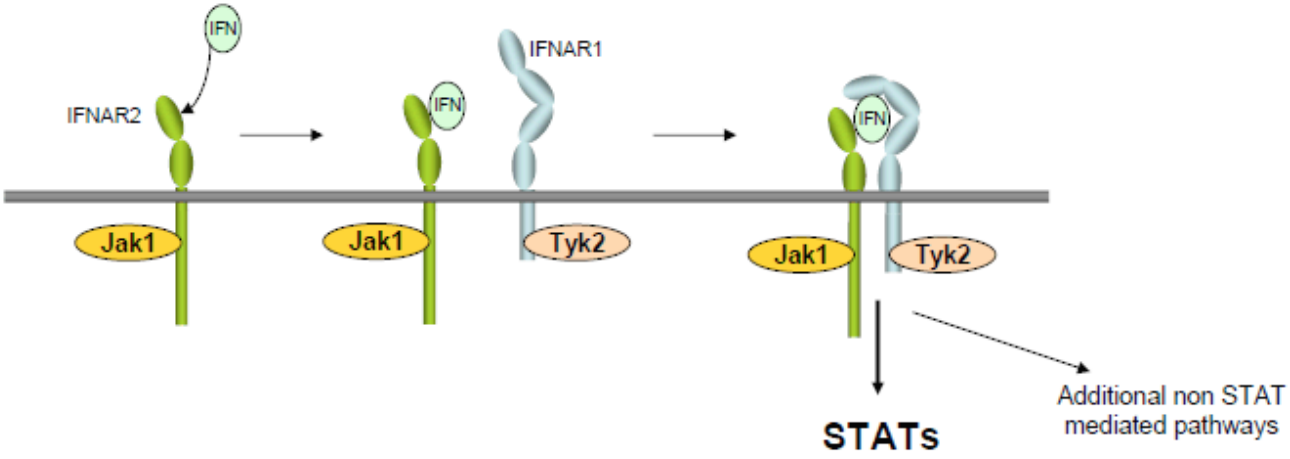


Figure 2: Schematic representation of type I IFN signaling

1.4 Type I IFN: The receptor complex

1.4.1 The Class II cytokine receptor family

IFNAR1 and IFNAR2 belong to the class II cytokine receptor family (CRF2). The CRF2 family includes also the subunits of the Type II (IFNGR-1 and IFNGR-2) and the Type III (IFNLR1/IL-28A and IL10R2) receptor, tissue factor (TF), the ligand-binding chain of the IL-10 receptor (IL-10R1) and the subunits of the IL-22 and IL-20 receptor family (Fig. 3A). CRF2 proteins are tripartite single-pass transmembrane proteins defined by structural similarities in the extracellular domain. The 200-amino-acid extracellular domain is composed of two tandem fibronectin Type III (FNIII) domains (Fig. 3B). The amino-terminal FNIII domain, distal to the membrane, is referred as D1, and the membrane-proximal domain as D2. Each FNIII domain has a structural framework of seven β-strands connected by loops and organized into two opposed β-sheets (Fig 3B). Within these domains is a pattern of hydrophobic and hydrophilic amino acids typical of β strands. In addition, the position of several conserved cysteines and a conserved tryptophan residue characterize this cytokine receptor family. Within the CRF2, IFNAR1 is unique in having four FNIII domains, denoted SD1–SD4. This structure appears to have arisen as a tandem duplication of the basic D1/D2 structure; thus, SD1 and SD3 of IFNAR1 are more closely related, as are SD2 and SD4 (Langer et al., 2004).

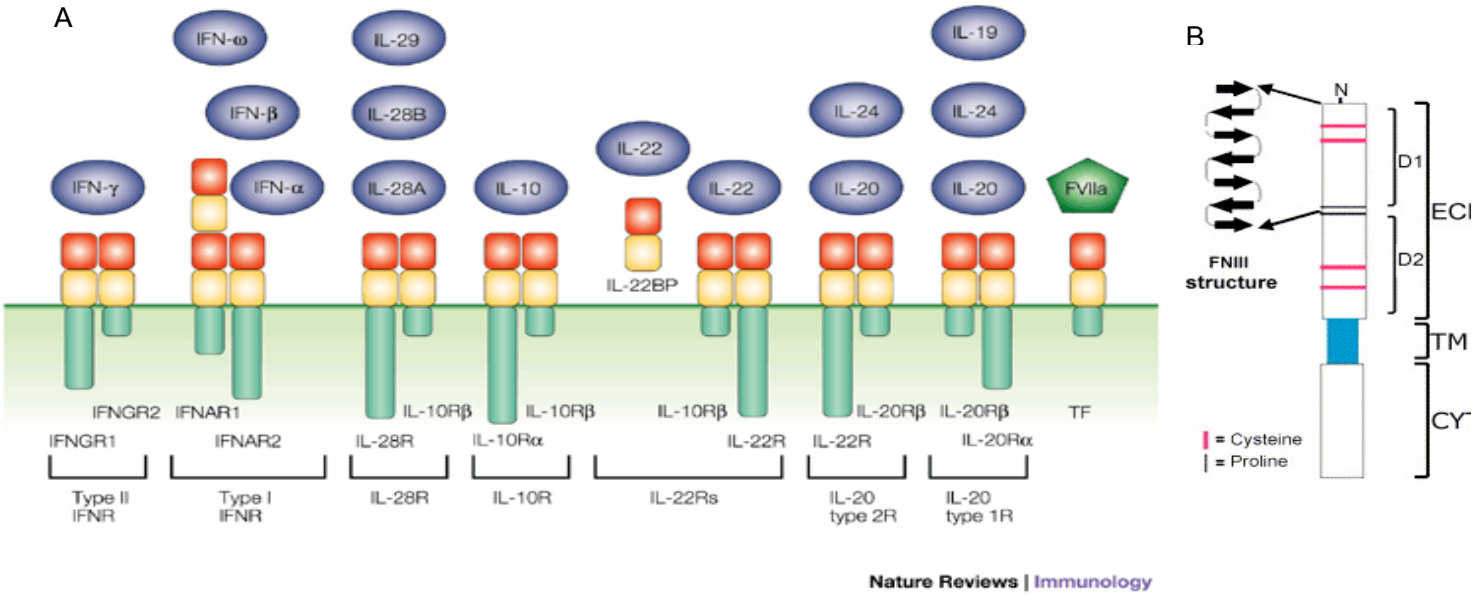


Figure 3: The CRF2 family

A) Schematic representation of the members of the CRF2 family (Renauld, 2003). B left) The structure is shown as a linear cartoon with the extracellular domain (ECD) composed of 2 FNIII domains (D1 and D2), the transmembrane segment (TM) and the cytoplasmic domain (CYT). B right) a crystallographically determined extracellular domain replaces the cartoon version (adapted from (Langer et al., 2004))

1.4.2 IFNAR1

Human IFNAR1 was cloned in 1990 using a gene transfer approach. Human genomic DNA from Daudi cells was transfected into mouse BTG 9A cells. Transfectants were selected for their sensitivity to added IFN α8 based on resistance to the cytopathic effect of Vesicular Stomatitis Virus (VSV). This technique relied on the fact that mouse cells are insensitive to human type I IFN (Uze et al., 1990).

IFNAR1 possesses a large N-glycosylated ectodomain of 409 aa with two cytokine binding modules, a single transmembrane-spanning segment of 21 aa and a cytoplasmic tail of 100 aa.

The essential role of IFNAR1 in IFN responses became soon evident from the study of IFNAR1-null mice that are totally unresponsive to all type I IFN subtypes (Muller et al., 1994)

IFNAR1 is associated to the Jak tyrosine kinase Tyk2. Using an *in vitro* binding assay, Yan et al. delimited the Tyk2-binding region and reported that a 33 aa domain between residues 479 and 511 of IFNAR1 is required to mediate the binding of Tyk2 to the receptor (Yan et al., 1996b).

The complex interplay between IFNAR1 and Tyk2 became evident with the characterization of a Tyk2-negative human fibrosarcoma derived mutant, the 11.1 cell line (Pellegrini et al., 1989). In these cells, the level of IFNAR1 at the cell surface was reduced

as compared to the parental 2fTGH cells (Pellegrini et al., 1989; Gauzzi et al., 1997). Moreover, the reconstitution of 11.1 cells with the large non-catalytic region of Tyk2 (aa 1-591) restored surface IFNAR1. Interestingly, in these cells, the IFN binding sites were not fully functional. Further studies defined the role of Tyk2 in the dynamics of IFNAR1. In transient transfection performed in 11.1 cells, it was shown that Tyk2 sustains IFNAR1 at the plasma membrane by restraining its basal internalization (Ragimbeau et al., 2003). In this study, Ragimbeau and coworkers reported that IFNAR1 deleted of its C-terminal region was stably expressed at the cell surface.

Overall, these data underline how dynamic IFNAR1 is and how its expression at the cell surface is chaperoned by the association with the tyrosine kinase Tyk2.

The cytoplasmic region of human IFNAR1 was shown to possess four tyrosines which can be rapidly phosphorylated upon treatement with IFN (Constantinescu et al., 1994; Uze et al., 1990). Tyr466 of IFNAR1 has been shown to play a role in the activation of STAT2 and STAT1 (Yan et al., 1996a). Furthermore, upon IFN addition, STAT3 was shown to associate with IFNAR1 in a tyrosine phosphorylation-dependent manner (Yang et al., 1996).

The cytoplasmic region of IFNAR1 also contains a membrane distal motif, K**Y**SSQTSQDSGN**Y**SNE, which is perfectly conserved in the mouse and bovine IFNAR1 (Basu et al., 1998; Uzé et al., 1990). Deletion of this motif was reported to increase the level of IFN-induced ISRE-dependent gel shift activity and to potentiate the antiviral activity of IFN against VSV (Basu et al., 1998).

Post translational modifications of IFNAR1 has also been shown. IFNAR1 was reported to be phosphorylated on Ser535 and Ser539 and phosphorylation of these serine residues promotes ubiquitination of IFNAR1 (will be detailed in section 3.1) (Kumar et al., 2003).

Furthermore, Claudinon et al. reported that human IFNAR1 is modified by palmitoylation, a reversible lipid modification involving specific attachment of saturated fatty acid chain to cysteines via a thioester bond (Claudinon et al., 2009). Two cysteines are present in the cytoplasmic region of IFNAR1, Cys463 and Cys502. Only the substitution of Cys463 into Ala (C463A), ablated the palmitoylation of IFNAR1. It was shown that the expression of this mutant in murine L929 cells expressing huIFNAR2 impaired STAT1 and STAT2 phosphorylation induced by huIFNα2 treatment, but did not alter Jak1 and Tyk2 activation. The authors reported that palmitoylation of IFNAR1 did not reduce its internalisation and

stability. Cys463 in IFNAR1 lies near Tyr466, a residue which was reported to be involved for STAT2 recruitment upon IFN treatment (Yan et al., 1996a). Mutation of Cys463 could thereby impair IFN-induced docking of STAT2 on IFNAR1. However, no co-immunoprecipitation between IFNAR1 and endogenous STAT2 could be detected in cells expressing wtIFNAR1. In overexpression studies performed in CHO cells, Claudinon et al. reported that the mutation C463A did not affect STAT2 interaction with IFNAR1. Additional studies are therefore necessary to conclude on the role of palmitoylated IFNAR1 in Type I IFN signaling.

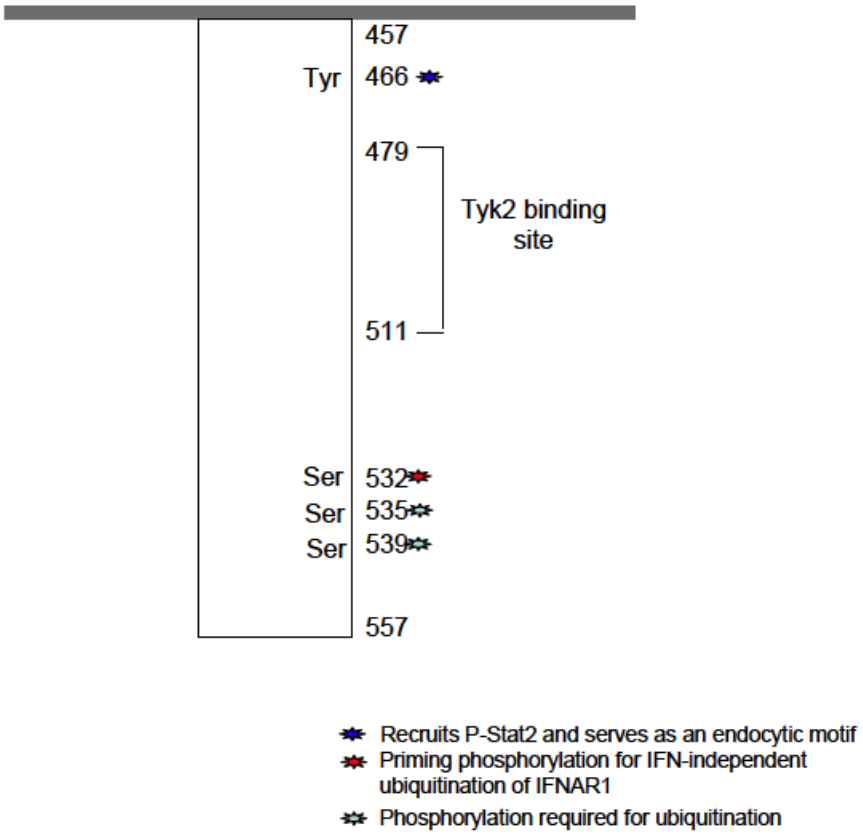


Figure 4. Schematic representation of the cytoplasmic region of human IFNAR1

1.4.3 IFNAR2

IFNAR2 is a highly glycosylated protein and exists in three different isoforms that are generated by alternative splicing, exon skipping and differential usage of polyadenylation sites (Lutfalla et al., 1990). The longest isoform is IFNAR2.2 or IFNAR2c (here referred as IFNAR2). This isoform was shown to complement the IFN-unresponsive phenotype of the 2fTGH-derived fibrosarcoma cell line, U5A (Lutfalla et al., 1995). The isoform, IFNAR2.1,

also known as IFNAR2b, possesses a truncated cytoplasmic tail and does not mediate STAT activation (Domanski and Colamonici, 1996; Pfeffer et al., 1997). The third isoform, IFNAR2.3 or IFNAR2a, lacks the transmembrane and cytoplasmic domains and is a secreted protein that can been found in various body fluids (Novick et al., 1995).

IFNAR2 has an intracellular region of 251 amino acids and interacts with the tyrosine kinase Jak1. Mutational analysis of IFNAR2 has delimited two motifs, box1- and box2-like, in the cytoplasmic tail. These motifs were reported to play an important role in the association of IFNAR2 with Jak1 (Usacheva et al., 2002b). The box1-like is an 8 aa motif rich in proline residues. When the box1 sequence of EPOR (which interacts with Jak2) is transfered into the β chain of the IL-2 receptor, IL2 induces activation of Jak2 instead of Jak1, thus establishing the ability of this motif to specify the selective recruitment of Jak2 (Jiang et al., 1996).

Usacheva et al. reported that Pro289 and Pro291 of IFNAR2 are required for Jak1-IFNAR2 interaction since mutation of either proline loosens the association between Jak1 and IFNAR2 (Usacheva et al., 2002b). These residues were reported to form part of the Box1-like motif.

Moreover, the same group delimited a 3 aa sequence, $_{303}$EVI$_{305,}$ in IFNAR2 by mutational studies. They reported that this sequence is required for Jak1-IFNAR2 interaction (Fig. 6) (Usacheva et al., 2002b). This EVI sequence ressembles the box2 motif that regulates Jak1 activity in other cytokine receptors such as IL-2Rβ (VEVI in IFNAR2 ; LEVL in IL2Rβ) (Zhu et al., 1998).

The group of Rubenstein was able to clone murine IFNAR2 by screening a mouse cDNA library with a probe corresponding to human IFNAR2 (Kim et al., 1997). Three isoforms were isolated. Two isoforms code for soluble proteins (IFNAR2a and IFNAR2b) and the last isoform codes for a transmembrane protein (IFNAR2c) which shares 49% identity with huIFNAR2c (Fig 5).

Comparison of the cytoplasmic region of murine and human IFNAR2 revealed five conserved tyrosine residues. Two tyrosine residues in huIFNAR2 and one in muIFNAR2 were not conserved. Box1- and box2-like motifs were identified in muIFNAR2, however, their sequences differe significantly from those of their human counterparts (Fig 5). Interestingly, the 30 C-terminal aa residues of the mouse and human IFNAR2 are highly conserved and this region includes one conserved tyrosine residue (Fig 5).

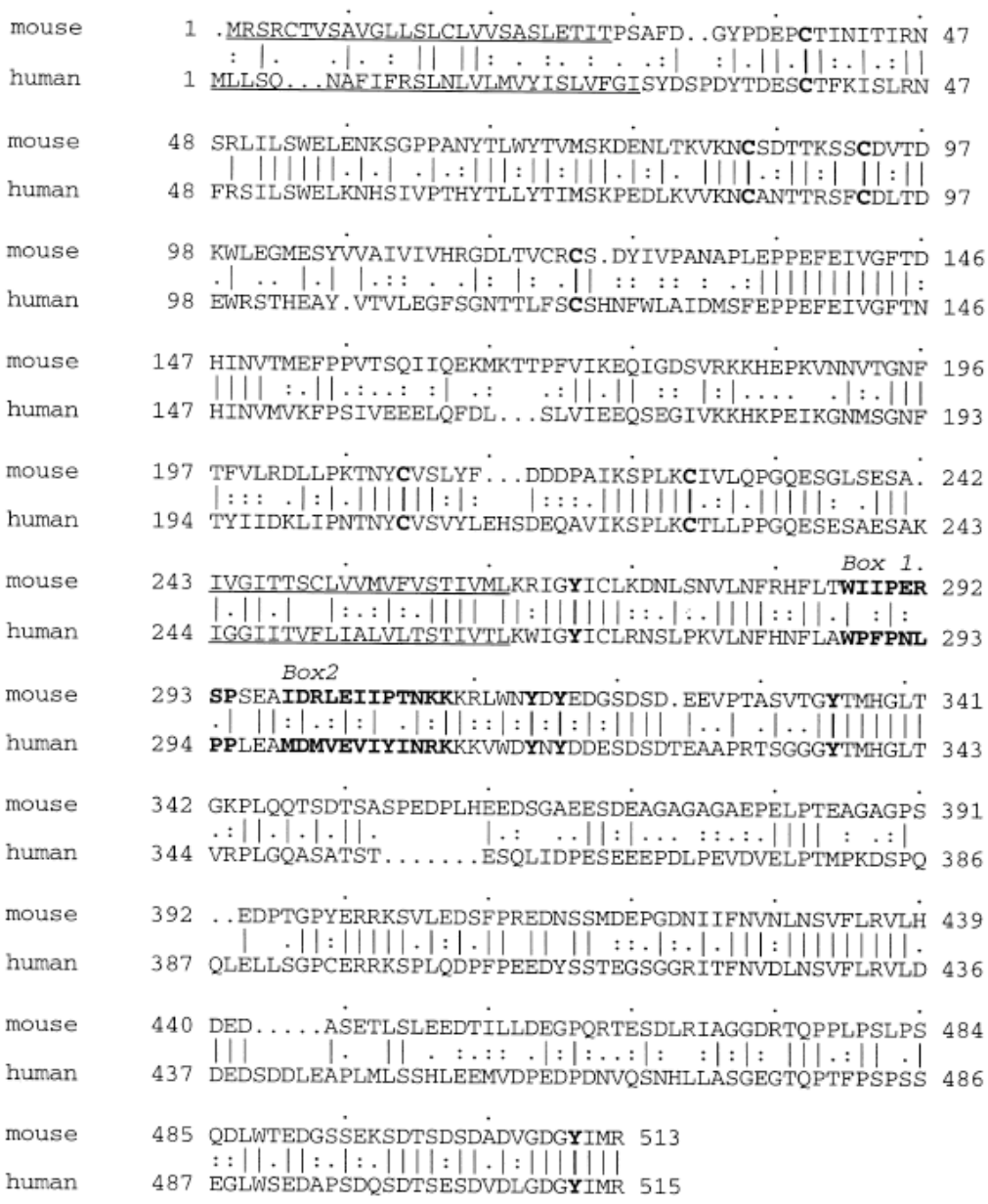


Figure 5 : Homology between the murine and human IFNAR2c at the protein level. Signal sequences and transmembrane domains of both receptor subunits are underlined. Conserved cysteines at the extracellular domains, conserved tyrosines at the cytoplasmic domains, as well as box1 and box2 motifs are shown in bold (taken from (Kim et al., 1997).

To identify post-translational modifications, immunoprecipitated IFNAR2 from IFN α-treated 293T cells was analysed by mass spectrometry (Tang et al., 2007). This analysis revealed that 7 serine residues in the cytoplasmic region of IFNAR2 can be phosphorylated and that out of 5 lysines, one lysine is acetylated (Lys 399). The acetylated Lys399 was shown to serve as docking site for IRF9, since the mutant IFNAR2 K399R lost the ability to bind IRF9. However, other groups have not yet confirmed this observation.

The group of Krowleski reported that when U5A cells reconstituted with IFNAR2 are treated with either phorbol ester or IFN α for 2 hrs, IFNAR2 is cleaved and a portion of the intracellular region of the receptor is liberated in the cytosol. The cleaved portion of IFNAR2 was reported to go to the nucleus where it represses gene transcription (Saleh et al., 2004).

The present view is that the cytoplasmic region of cytokine receptors is not well structured, but is a flexible moiety that intermingles with the amino-terminal portion of Jak proteins to acquire rigidity and conformation.

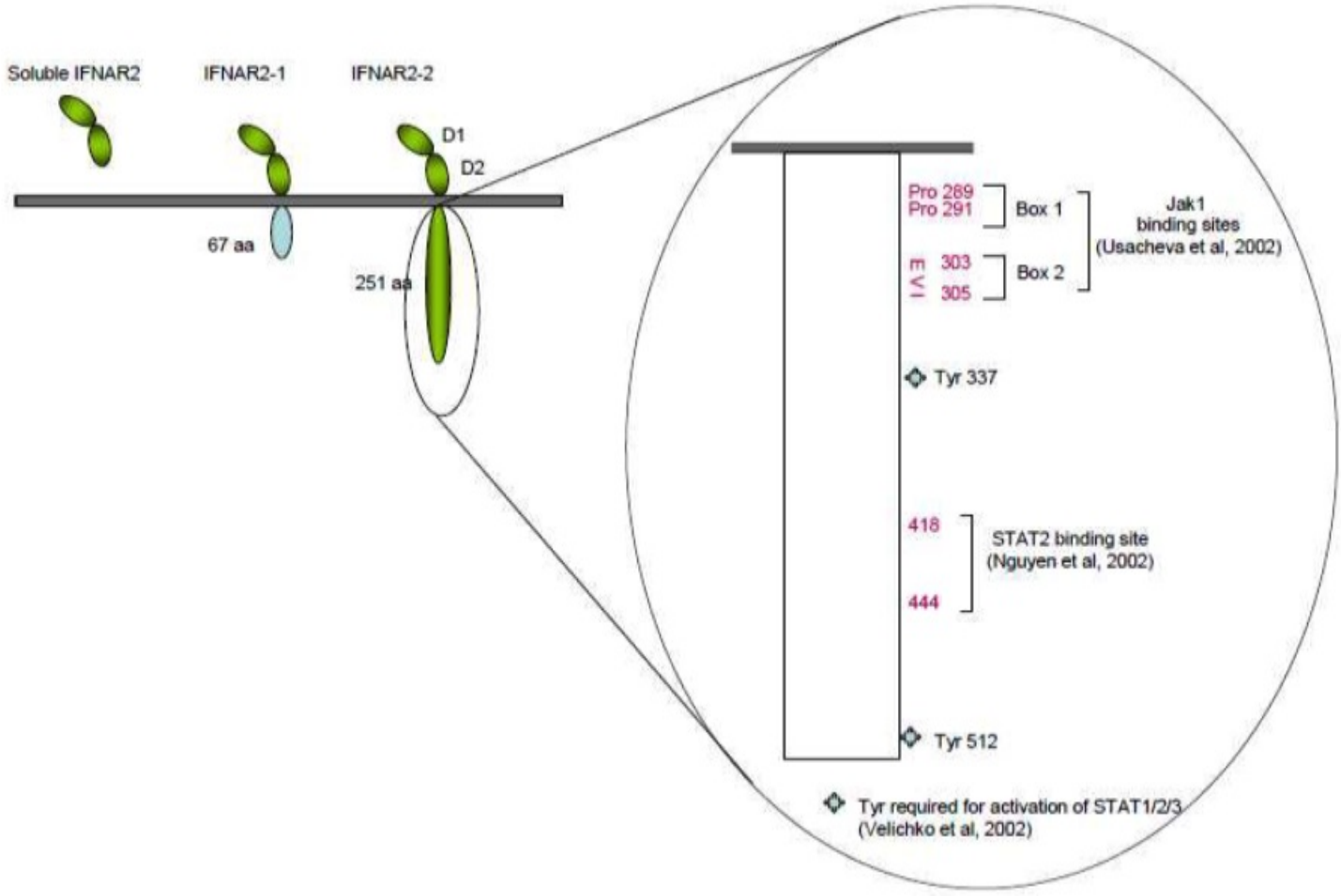


Figure 6. Schematic representation of human IFNAR2
Left: the 3 isoforms of IFNAR2 are depicted. Right: Intracellular region of human IFNAR2 and the associated proteins

1.5 The Jak/STAT signaling pathway

1.5.1 The Jak family

Jak1, Jak2, Jak3 and Tyk2 constitute the four members of the Janus or Jak family of tyrosine kinases. These enzymes are ubiquitously expressed except Jak3 which is restricted to leukocytes. They have molecular weight which range between 120-140 kDa and are multi-domain proteins that share seven Jak homology regions (JH1-JH7). They contain two adjacent kinase domains, a canonical carboxyl-terminal tyrosine kinase (JH1 or TK) and a catalytically inactive domain, referred to as kinase-like, pseudo kinase or JH2 domain (Fig. 7). Within the JH1 domain, two tyrosine residues are located in the so-called activation loop and are targets of *trans*-phosphorylation by neighbouring Jak. This phosphorylation induces conformational changes that positively regulate kinase activity and facilitate substrate binding (Yeh and Pellegrini, 1999; Ghoreschi et al., 2009).

The N-terminal half of Jak proteins is most divergent and contains a src-homology 2 (SH2) domain (JH3 and part of JH4) with unknown function and a four-point-one, ezrin, radixin, moesin (FERM) homology domain (part of JH4 and JH5-JH7). The FERM domain is a protein-protein interaction domain and is implicated in the specific interaction with cytokine receptors (Fig. 7) (Ghoreschi et al., 2009).

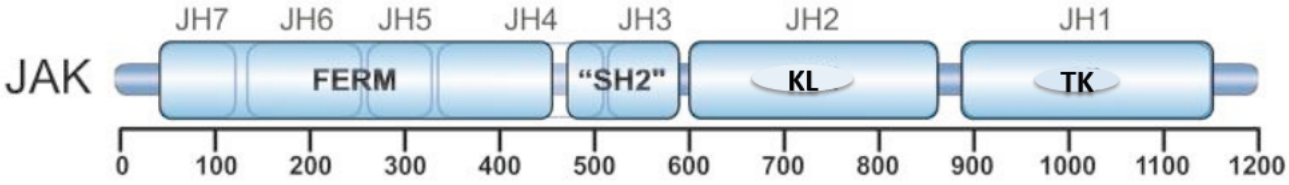


Figure 7: Domain organisation of a Jak protein (adapted from Schindler and Plumlee, 2008)

I will focus below on the two enzymes which are involved in type I IFN signaling, i.e Jak1 and Tyk2.

1.5.2 Jak1

Jak1 was first cloned in a PCR-based screen approach aimed at identifying novel tyrosine kinases (Wilks et al., 1989). It is a widely expressed protein.

The demonstration of the role of Jak1 in Type I and Type II IFN signaling came from the rescuing of the IFN-unresponsive phenotype of the 2fTGH-derived U4 mutant cells.

The Jak1 knock out mice die perinatally (Rodig et al., 1998) and more careful analysis revealed that these mice suffer from a neurological lesion that renders them unable to suckle. Identification of a similar defect in LIFRβ knockout mice (Ware et al., 1995)

suggested that loss in LIF function accounts for this neurological defect. Consistent with this, the response to LIF and IL-6 was shown to be substantially diminished in Jak1 -/- derived cells. Jak1 -/- mice have a defective lymphoid development, consistent with the fact that Jak1 binds to the ligand-specific receptor of γc-using cytokines, such as IL-2R, IL-7R, IL-9R and IL-15R and mediates signaling (O'Shea et al., 2002).

By studying truncated forms of Jak1, Usacheva and colleagues showed that Jak1 interacts with IFNAR2 via the 166 N-terminal aa comprising the JH7 and JH6 region. The JH5 and JH3 domains of Jak1 were also found to play a role in binding IFNAR2 (Usacheva et al., 2002a). Little is known of the contribution of Jak1 to the level and location of IFNAR2.

1.5.3 Tyk2

Tyk2 was the first Jak tyrosine kinase to be implicated in cytokine signaling. The group of Pellegrini and co-workers showed that Tyk2 complemented the IFN-unresponsive phenotype of 11.1 cells (Velazquez et al., 1992). These 11.1 cells are completely unresponsive to IFN α and show a weak responses to IFN β (Gauzzi et al., 1997).. Moreover, a reduction of the level of IFNAR1 in these cells leads to a loss of high-affinity IFN α binding (Gauzzi et al., 1997). In contrast to 11.1 cells, fibroblasts and macrophages derived from Tyk2-deficient mice show normal IFNAR1 surface expression and partially impaired IFN α/β signalling (Sheehan et al., 2006). In fact, strong effects of Tyk2 deficiency on IFN α/β responses are only observed at low dose of IFNs in murine fibroblasts and macrophages as monitored by antiviral activity and MHC Class I induction, respectively (Karaghiosoff et al., 2000; Shimoda et al., 2000).

Extensive analysis of Tyk2 deletion mutants using *in vitro* binding assays identified the major interaction surface of Tyk2 with IFNAR1 within the aa 21-221 (part of JH6 and JH7), (Richter et al., 1998). However, when expressed in 11.1 cells, neither this nor a larger segment comprising residues 1–385, can rescue surface IFNAR1. Thus, in addition to this minimal binding interface, other surfaces of the N moiety, including the SH2-like domain, contribute to anchoring IFNAR1 to the plasma membrane.

Tyk2 is a 134 kDa protein which is localised throughout the cell, including the nuclear compartment with the exclusion of the nucleoli. The nuclear localisation of Tyk2 requires an arginine-rich nuclear localisation signal (NLS)-like motif located within the FERM

domain. The nuclear function of Tyk2 has still not been uncovered but it does not seem to participate in the biological activities mediated by type I IFN. Indeed, expression of a myristylated form of Tyk2, anchored at the plasma membrane in 11.1 cells, was shown to rescue transcriptional and anti-vesicular stomatitis virus responses to IFN α (Ragimbeau et al., 2001).

In response to IFN α/β treatment, Tyk2 is phosphorylated on tyrosine Y1054/1055 in the activation loop ((Barbieri et al., 1994; Gauzzi et al., 1996)).

Overall, studies of deleted forms of Tyk2 expressed in 11.1 cells have highlighted distinct functions of the protein toward the expression and the binding activity of the receptor complex. Each function appears to be contributed by a different domain adding more complexity to the receptor-kinase complex. The N region, previously defined as the amino-terminal 591 residues and comprising the JH7 to JH3 regions interacts with IFNAR1 and determines its level at the cell surface (Yeh et al., 2000).

1.6 The STATs

In mammals, the STAT family of transcription factors comprises seven members (STAT1 to 6, 5a and 5b), which range in size from 750-900 amino acids (Ivashkiv and Hu, 2004) (Fig. 6). The canonical model is that upon cytokine stimulation, the Jaks phosphorylate the receptor on specific tyrosine residues, which serve as docking sites for the STAT SH2 domain (Darnell et al., 1994). After being phosphorylated by the Jaks, STAT proteins are capable of forming homo- and hetero-dimers, translocate to the nucleus and induce gene expression (Haan et al., 2000). Structural studies have shown that unphosphorylated STATs (U-STATs) can form dimers having different structural configurations with respect to the phosphorylated forms. U-STAT dimers present an anti-parallel orientation, where the SH2 domains are on the opposite end of the dimer, or a parallel orientation where the SH2 are located on the same end of the dimer (Neculai et al., 2005). On the other hand, a phosphorylated STAT dimer is always found in a parallel orientation.

Migration of native STAT1 and STAT3 from Hela and 293T cells through native gels showed that the vast majority of the STAT1 and STAT3 proteins exist as homodimers in unstimulated cells (Braunstein et al., 2003).

Expression of U-STATs 1, 2 and 3 is greatly increased in response to their activation. The *stat1* gene is strongly activated by phospho-STAT1 (P-STAT1) dimers or ISG Factor 3 (ISGF3), formed in response to type I or type II IFNs, respectively (Cheon and Stark, 2009). *Stat2* gene expression is also increased in response to type I or type II IFNs (Lehtonen et al.,

1997). Similarly, the *stat3* gene is strongly activated by the phosphorylated STAT3 dimers that are formed in response to IL-6 and other ligands that activate the gp130 common receptor subunit.

When the human mammary epithelial cells and fibroblasts were infected with lentiviruses coding for either wtSTAT1 or Y501F-STAT1, expression of several ISGs were reported to be increased. U-STAT1-induced proteins have immunoregulatory, antiviral, or unknown functions (Cheon and Stark, 2009). Similarly, U-STAT3 drives expression of a set of genes that is mostly distinct from those activated in response to P-STAT3 (Yang et al., 2005).

Figure 8: Schematic representation of STATs domains and their functions. The NH2 domain promotes formation of homotypic dimers among unphosphorylated STATs. The Coiled-Coil domain consists of a four helix bundle that associates with regulatory proteins and may also control the process of nuclear import and export. The DNA Binding Domain (DBD) mediates binding to GAS (IFN-gamma activated sequence) palindromes. The linker domain translates active dimerization to the DNA binding motif. The SH2 domain is the most highly conserved motif and mediates specific recruitment to receptor chains as well as formation of active STAT dimers. The Tyrosine Activation motif consist of conserved tyrosine. Like the SH2 domain, this motif resides on the exposed surface of the inactive homodimer, facilitating its Jak-dependent phosphorylation during receptor recruitment. Upon phosphorylation, this motif is recognised and bound by the corresponding SH2 domain of the partner STAT, directing the structural changes required for an active conformation. The Transcriptional Activation Domain (TAD) resides at the carboxy terminal and is highly variable in size and sequence between STAT family members (adapted from Schindler and Plumlee, 2008)

1.6.1 The STATs in type I IFN signaling

Type I IFN has the potential to activate all members of the STAT family. The best-studied type I IFN response that is common to all cell types involves STAT1 and STAT2.

Once phosphorylated, STAT1/STAT2 heterodimer binds to IRF9 (IFN regulatory factor 9) to form a trimeric transcriptional complex known as ISGF3 (IFN-stimulated gene factor 3) (Fig. 9). IFNs will also induce the formation of several other transcription factors that include STAT1-STAT1, STAT3-STAT3 and STAT5-STAT5 homodimers which bind to GAS-containing promoter sequences ((Kisseleva et al., 2002)).

To be activated by IFN, STAT1, STAT2 and STAT3 require the phosphorylation of IFNAR2 on Tyr 337 and 512 (Velichko et al., 2002). In addition phosphorylated IFNAR1 on tyrosine 466 was shown to be needed for the activation of STAT1 and STAT2 (Yan et al., 1996a).

Interestingly, in the absence of IFN, STAT2 is constitutively bound to IFNAR2 (residues 418-444 of IFNAR2) in a manner which does not depend on tyrosine phosphorylation or on

the SH2 domain of STAT2 (Nguyen et al., 2002b). Using STAT chimeras created by exchanging homologous sequences between STAT1 and STAT2, Li et al. implicated a domain encompassing Stat2 residues 295–315 in the binding to IFNAR2 (Li et al., 1997).

Moreover, in STAT2-deficient cells, STAT1 is considerably less phosphorylated by IFN α2 than in wt cells suggesting that STAT2 stabilises the phosphorylation of STAT1 (Leung et al., 1995).

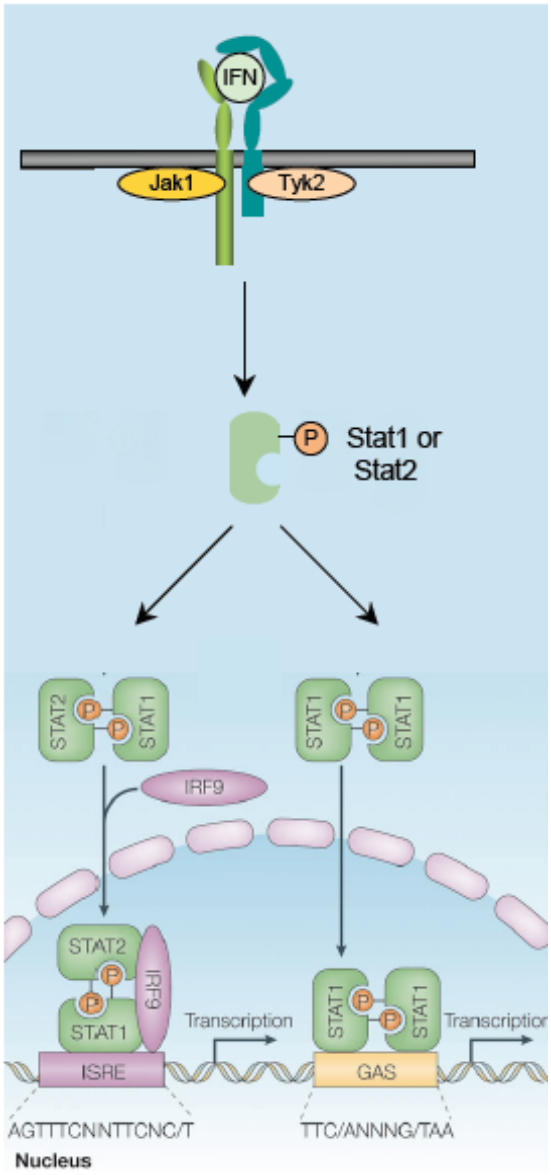


Figure 9: Schematic representation of activation of the ISGF3 transcription factor or STAT1-STAT1 homodimers upon IFN stimulation.

2. Differential activities of Type I IFN subtypes

As mentioned above, all subtypes of Type I IFN bind to the same receptor and initiate the same signaling pathway. However, subtle differences have been described in their bioactivities. For example, IFN α2 and IFN β exhibit comparable specific anti-VSV

activities as measured in the amniotic fibroblast like WISH cells. However, IFN β is much more potent than IFN α2 in inhibiting proliferation of these cells (Jaitin et al., 2006). IFN α2 and IFN β thus have differential activities for this specific function in this specific cellular context.

IFN β is more potent than IFN α2 in inducing apoptosis of human tumor cells (Vitale et al., 2006). IFN α2, but not IFN α8, was shown to increase the motility of human primary T cells (Foster et al., 2004).

Comparative gene expression profiling performed on HT-1080 cells stimulated with 300 pM of IFN α2 and IFN β for 6 hrs revealed the existence of up to 300 interferon-stimulated genes (ISG), of which 20 were found to be more highly induced by IFN β as compared to IFN α2 (de Veer et al., 2001). The differential induction of these genes was however not confirmed by other approaches such as RT qPCR (quantitative Polymerase Chain Reaction). In an elegant work, Da Silva et al. analysed the profile of genes induced in human umbilical vein endothelial (HUVEC) cells treated for 4 hrs with various doses of IFN β and IFN α2 (da Silva et al., 2002). The aim of this study was to distinguish between differences in signaling responses and differences associated to dose-dependent effects. The authors showed that genes that were more induced by IFN β than IFN α2 could be induced to similar levels by higher doses of IFN α2. Moreover, a well studied ISG encoding the chemokine CXCL11 (β-R1/ITAC) was shown to be preferentially induced by IFN β in 2fTGH, WISH and osteoclasts and to require NFkB activation (Rani et al., 1996; Rani et al., 1999). In physiological differentiation process of human monocytes, the 100 fold higher inhibition of osteoclastogenesis by IFN β with respect to IFN α2 was proposed to be mediated, at least in part, by autocrine-acting CXCL11, whose expression in monocytes undergoing osteoclastic differentiation was more efficiently upregulated by IFN β (Coelho et al., 2005).

It is to be noted that IFN subtypes exhibit different potency in biological activities that require several days of continuous IFN stimulation. However, the molecular mechanism by which, at a given concentration, a subtype is more potent than another remains ill-defined.

This question was partly answered by mutagenesis data. The first structure of a type I IFN (muIFN β) was reported in 1992 (Karpusas et al., 1997; Senda et al., 1992), followed obtention of the x-ray and NMR structures of huIFN α2 (Radhakrishnan et al., 1996). The structural information obtained, together with mutagenesis data, provided a clear framework of the location of the IFNAR2 binding site on IFN α2, and of a less defined IFNAR1

binding site (Piehler et al 2000). IFN α2 and IFN β were reported to share similar binding interfaces on IFNAR1 and IFNAR2 (Piehler and Schreiber, 1999).

2.1 Study of the different subtypes of type I IFN: Affinity dictates activity

Type I IFN subtypes share similar binding interfaces but exhibit differential activities after a prolonged exposure, three subtypes IFN α2, IFN β and IFN ω that show different potency for ISGs induction and antiproliferative activity were scrutinized for their ability to interact with the 2 subunits of the receptor. The extracellular domain of IFNAR2 and IFNAR1 was thus tethered on a solid supported lipid bilayer and differences in affinity and in association and dissociation rate constants were monitored by real-time solid phase detection ((Jaks et al., 2007). While no differences in the association kinetics could be observed for the 3 subtypes, strong differences in the dissociation kinetics were found (Table 1). For example, IFNα2 dissociated from IFNAR1 and IFNAR2 at rates 100 and 50 fold higher, respectively, than IFN β. This showed that IFNα2 dissociated from the receptor more rapidly than IFN β and thus that the ternary complex formed by IFN α2 was less stable than that formed by IFN β.

The dissociation rate of IFN ω was intermediate between IFN α2 and IFN β, i.e. 13 times lower than that of IFN α2 but 8 fold higher than that of IFN β (Table 1).

Interestingly, the affinity of the different subtypes for the receptor correlated well with their biological potency (expressed as EC50). For example, IFN α2 which has a higher dissociation rate than IFN β or IFN ω for IFNAR1 and IFNAR2 and thus which has lower affinity for the 2 subunits of the receptor, has an antiproliferative EC50 which is respectively 16 and 6 times higher than that of IFN β and IFN ω (Table 1). These data suggested that the affinities of the ligand to the receptor subunits govern differential activities.

IFN	Ifnar2-EC			Ifnar1-EC			Activities (EC_{50})		
	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_d (nM)	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_d (μM)	ISGF3 (pM)	Antiproliferative (nM)	Antiviral (pM)
IFNα2	3×10^5	0.015	5	~ 2×10^5	1	5	8	1.1	40
IFNβ	1×10^7	0.001	~ 0.1	~ 2×10^5	0.025	0.05	1.5	0.065	–
IFNω	6×10^5	0.008	1	~ 2×10^5	0.08	0.4	3	0.2	–

Table 1: Interaction constants and activities determined for the three subtypes of type I IFN (adapted from (Jaks et al., 2007).)

2.2 Mutants of IFN α2

Engineering mutant forms of IFN α confirmed the key role that these differences in affinity to the receptor subunits play in differential signaling.

Two IFN α2 mutants were described: IFN α2-HEQ and IFN α2-YNS. Three amino acids (H57, E58 and Q61) are conserved in all IFN α proteins and are located in the IFNAR1 binding interface. These residues were mutated into alanine residues (His57Ala, Glu58Ala and Gln61Ala) to yield IFN α2-HEQ (Jaitin et al., 2006) or into His57Tyr, Glu58Asn and Gln61Ser to yield IFN α2-YNS mutant (Kalie et al., 2007). The binding and biological properties of these two IFN α2 mutants were monitored. In vitro binding studies showed that when compared to IFN α2, mutant IFN α2-HEQ exhibits a higher binding affinity towards IFNAR1 (comparable to what is observed for IFN β) and IFN α2-YNS had even higher binding affinity to IFNAR1. The affinity of binding for IFNAR2 was unchanged. Interestingly, IFN α2-HEQ and IFN α2-YNS were found to be as potent as IFN β for all biological activities assessed (antiproliferative and interferon induced gene expression).
These results corroborated the first set of data (section 2.1) showing that the affinities of the ligand for the receptor govern differential activities. Moreover, these results suggested that the binding interface on IFN α2 is far from being optimal and that improvement of this interface could be possible.

2.3 Crystal structure of the ternary complex

Recently, a huge achievement was the solving of the following crystal structures (Thomas et al., 2011): the unliganded IFNAR1 ectodomain (comprising SD1 to SD3 and lacking SD4), the IFNAR2-D2 domain, the binary complex formed by IFNAR2 and IFN α2-HEQ and the ternary complex formed by the two receptor subunits and IFN α2-YNS (considered a high affinity ligand) or IFN ω (a moderate affinity ligand). These analyses confirmed that IFNAR1 and IFNAR2 bind on opposite sides of the ligand in a nearly orthogonal architecture (Fig. 10A). Although IFN α2-YNS and IFN ω do not have the same affinities for the receptor subunits, they form complexes with almost identical receptor-ligand docking modes (Fig. 10B) as can be seen when the two ternary complexes are superimposed. The ligand binds to IFNAR1 at the level of the hinge between the SD2 and SD3 domains with the SD1 domain capping the top of the IFN molecule (Fig. 10C).
In other cytokine receptor complexes, such as IFN γ and IFN λ receptor complexes, the principal interaction mode is between the cytokine and the loops projecting from the "elbow" formed between two bent fibronectin III domains (Fig. 10D). In the case of IFNAR1, the SD2-SD3 domains appear to be oriented in the opposite direction such that the loops at the extreme top and bottom ends of the fibronectin III domains form the major contacts with the IFN ligands in a manner reminiscent of pinchers (Fig. 10A-C).

Structural rearrangements of IFNAR1 appear to be required to bring key hotspot residue into contact. The energetics required for these structural rearrangements might contribute to the reduced binding affinity of IFNAR1.

Overall, these studies have confirmed previous functional analyses of mutant receptors and mutant IFNs.

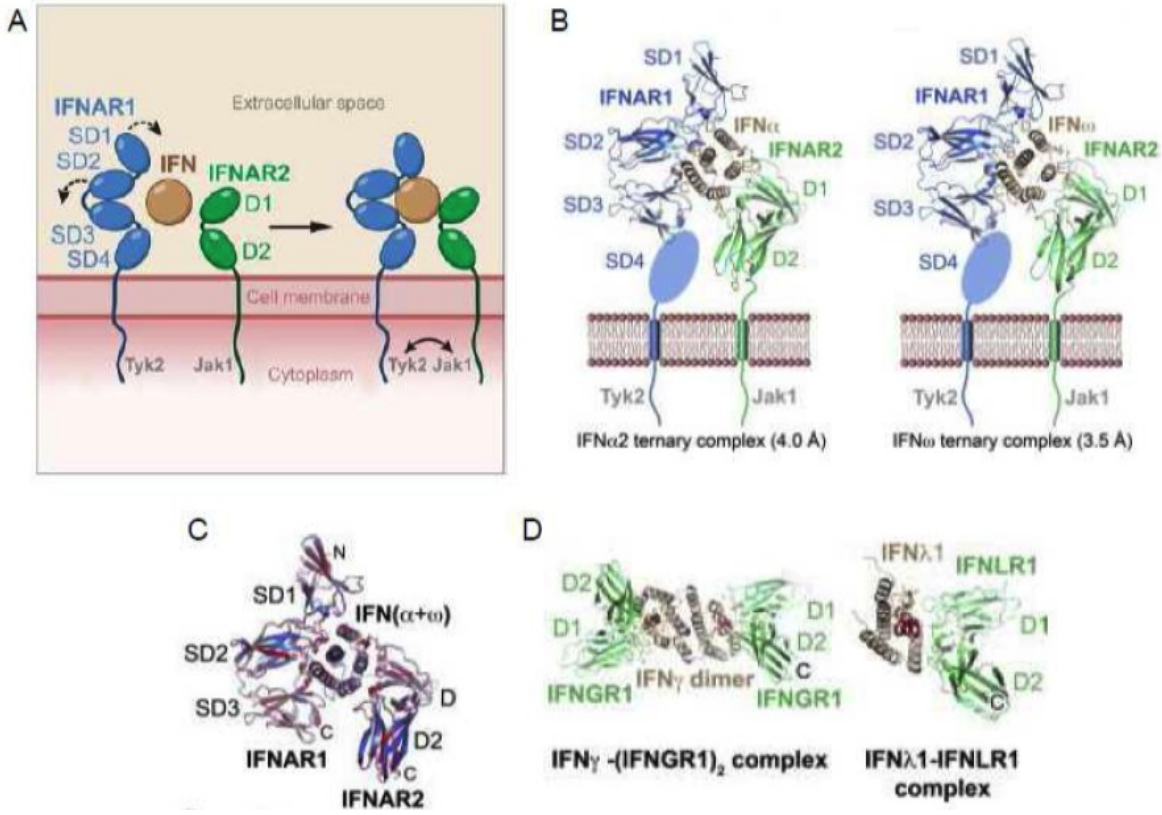


*Figure 10: Crystal structure of complexes formed by IFN with its receptor***.** (A) Schematic representation of the architecture adopted by complexes formed by the type I IFN. (B) Similar architectures of Type I IFN complexes. (C) The IFN molecules of the IFNω and IFNα2YNS ternary complexes were superimposed (D), Type II and Type III IFN Receptor complexes form distinct architectures from the one formed by Type I IFN (adapted from Thomas et al., 2011)).

On IFNAR2, the majority of the contacts with the ligand involve the membrane distal D1 domain (Figure 10A-C).

In order to analyse how different subtypes interact with the receptor, the interface contact residues and the residues conserved in IFN α2, IFN ω, IFN β, IFN ε and IFN κ were mapped onto the surface of the solved IFN ω in the ternary complex. This study revealed that IFNAR1 and IFNAR2 use a few conserved residues of the ligands as anchor points against a background of less- or nonconserved amino acids (Fig. 11).

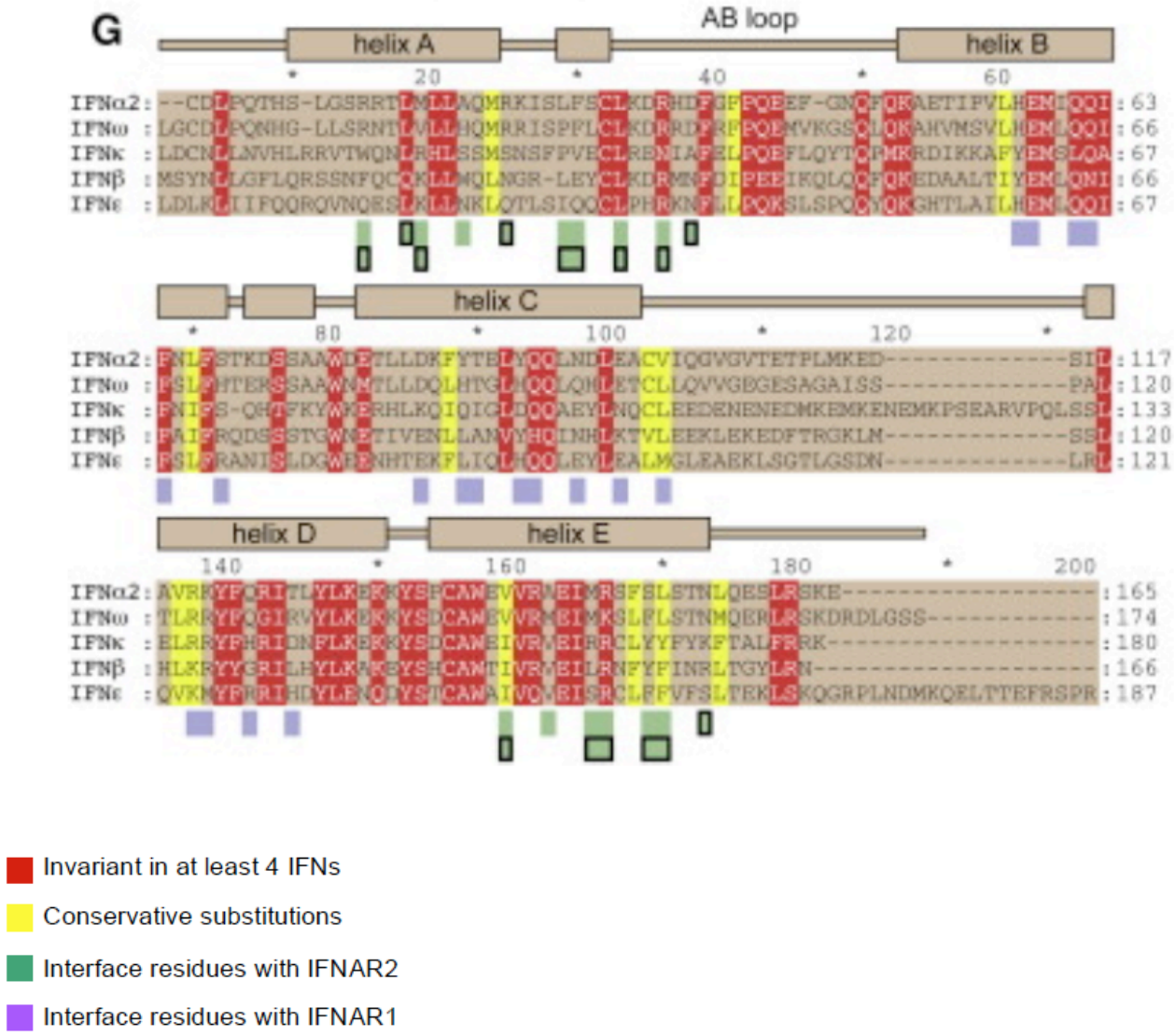


Figure 11: Sequence alignment of 5 different type I IFNs showing conserved residues in the ligand-receptor interface. Physicochemically conserved amino acids are colored yellow; residues that are invariant in at least four of five IFNs (IFNα2, IFNβ, IFNε, IFNκ, and IFNω) are shown in red. Interacting residues are denoted by rectangles below the alignment. Rectangles outlined in black mark interacting residues in the IFNα2 binary complex. The secondary structural elements of IFNω are depicted on top of the alignment (adapted from Thomas et al., 2011)).

Overall, the data obtained from the study of different IFN subtypes, of mutant forms of IFN α2 and of the crystal structure of the binary and ternary complexes confirmed that the type I IFN receptor is able to bind a large number of ligands with similar architecture and that the different chemistries of ligand interaction, through the conservation of a few residues on the ligand, ultimately dictate the stabilities of the receptor complexes and are likely to control on differential signaling (Thomas et al., 2011).

3. Negative regulation of type I IFN responses

The pleiotropic activities of IFNs must be tightly down regulated in time and space and several mechanisms have been shown to co-exist in order to attenuate IFN-initiated Jak/STAT signaling. Some of these downregulators will be induced by type I IFN and some are constitutively expressed. The following section will focus on four main ligand dependent regulatory mechanisms that modulate the IFN response through negative feedback loops.

3.1 IFNAR1 ubiquitination

One mechanism that terminates membrane-initiated signal transduction is the downmodulation of functional ligand binding sites. The cell surface level of a given receptor is the result of dynamic processes including internalisation, degradation, recycling and resplenishment, each of which can be affected by ligand binding. As briefly mentioned in the section above, within minutes of IFN binding, IFNAR1 is phosphorylated on Ser535 and Ser539 in the degron motif $_{534}$D**S**GNY**S** located in the intracellular tail of IFNAR1 (Fig. 4)(Kumar et al., 2003). Recently the Ser/Thr kinase mediating this ligand-dependent phosphorylation has been reported by the group of Fuchs to be PKD2 (Zheng et al., 2011). Phosphorylated Ser535 is recognised by β-TrCP2/HOS protein which recruits the core SCF (Skp1-Cullin-F-box) E3 ligase complex that ubiquitinates IFNAR1 on Lys501, Lys525 and Lys526. This cluster is critical for efficient ubiquitination and degradation of IFNAR1. In transient transfection experiments in 293T cells, substitution of these 3 lysines to arginine in IFNAR1^{KR} results in a a stable and ubiquitination-deficient mutant (Kumar et al., 2004).

By co-expression of IFNAR1 and various ubiquitin lysine mutants (the lysine-less ubiquitin K0, K48R and K63R ubiquitin), the group of S.Fuchs measured the involvement of ubiquitination on degradation of this receptor. These authors showed that both K48 and K63-linked polyubiquitin chains are required for maximum IFNAR1 degradation. Furthermore, they noticed that the target lysine residues are located in the proximity of a Tyr-based linear endocytic motif ($_{466}$YVFF). Substitution of Tyr466 with Phe (IFNAR1^{YF}) resulted in a protein competent in Ser535 phosphorylation, recruitment of β Trcp and ubiquitination but impaired in internalisation despite being ubiquitinated (Kumar et al., 2007). Tyr-based linear endocytic motifs are known to serve as recognition sites for the AP50 subunit of AP2 complex, thus enabling AP2-dependent tethering of cargo to clathrin molecules (Bonifacino and Traub, 2003). Indeed, immunoprecipitated IFNAR1^{Y466F} from

IFN α treated 293T cells did not efficiently interact with coexpressed AP50. Furthermore, the expression of ubiquitin mutants decreased the ligand-induced recruitment of AP50 to IFNAR1. Together, it was proposed that ubiquitination of IFNAR1 is required for exposure of the Tyr466-based linear endocytic motif within IFNAR1 to allow interaction with AP2 and thus to achieve the maximum rate of IFNAR1 internalization (Kumar et al., 2007). In the absence of IFN, Tyk2 inhibits the ubiquitin-independent internalisation of IFNAR1 by physically interacting with the latter and preventing binding of AP2 components (Kumar et al., 2008).

Surprisingly, the same group reported that when overexpressed, IFNAR1 undergoes ligand-independent phosphorylation on its degron motif and consequently is ubiquitinated and degraded (Liu et al., 2008). Moreover, the authors could show that pre-treating 293T cells with the Jak inhibitors (JI or AG490), did not decrease the serine phosphorylation of transfected IFNAR1. This result indicated that the activity of a Ser/Thr kinase responsible for phosphorylating highly expressed IFNAR1, was not regulated by Janus kinases.

The authors proposed that overexpression of IFNAR1 might overpower the ability of the cell to properly fold this protein in the endoplasmic reticulum, triggering an "unfolded protein response" (UPR). Indeed, forced expression of IFNAR1 was shown to induce the markers of the UPR, such as BiP and ATF4. Furthermore, treatment of cells with thapsigargin (TG), an inducer of UPR, conferred Ser535 phosphorylation of endogenous IFNAR1 (Liu et al., 2009b). Using an siRNA-based approach, the authors were able to show that UPR promotes ubiquitination and degradation of IFNAR1 in a PERK-(pancreatic endoplasmic reticulum kinase) dependent manner (Liu et al., 2009b).

Infection of the human fibrosarcoma cells, 2fTGH with vesicular stomatitis virus (VSV) induced expression of UPR markers and stimulated IFNAR1 phosphorylation on Ser535 and decreased total levels of IFNAR1. IFNAR1 downregulation triggered by UPR activation will therefore inhibit cellular responses to IFN α/β (Liu et al., 2009b).

Using a biochemical approach, the same group identified and characterised casein kinase 1α (CK1α) as the major kinase mediating basal phosphorylation of Ser535, ubiquitination and degradation of IFNAR1 (Liu et al., 2009a). The members of the CK1 family were reported to be constitutively active kinases (Knippschild et al., 2005). Since ligand-

independent phosphorylation of IFNAR1 could be further stimulated in cells treated with TG and TG by itself did not increase the activity of CK1α, the authors proposed that a "priming" phosphorylation event is required to trigger phosphorylation on Ser535 of IFNAR1.Using complex biochemical and pharmacological strategies, the group of Fuchs showed that the Ser/Thr kinase p38α must phosphorylate Ser532 of IFNAR1 (priming phosphorylation) to trigger CK1α to target Ser535 of IFNAR1 (Bhattacharya et al., 2010; Bhattacharya et al., 2011)

Overall, these data demonstrate how dynamic the IFNAR1 receptor is. Its cell surface level appears to be affected not only by binding to IFN but also by stress signals such as TG or by viral infection (schematically depicted in Fig.11)

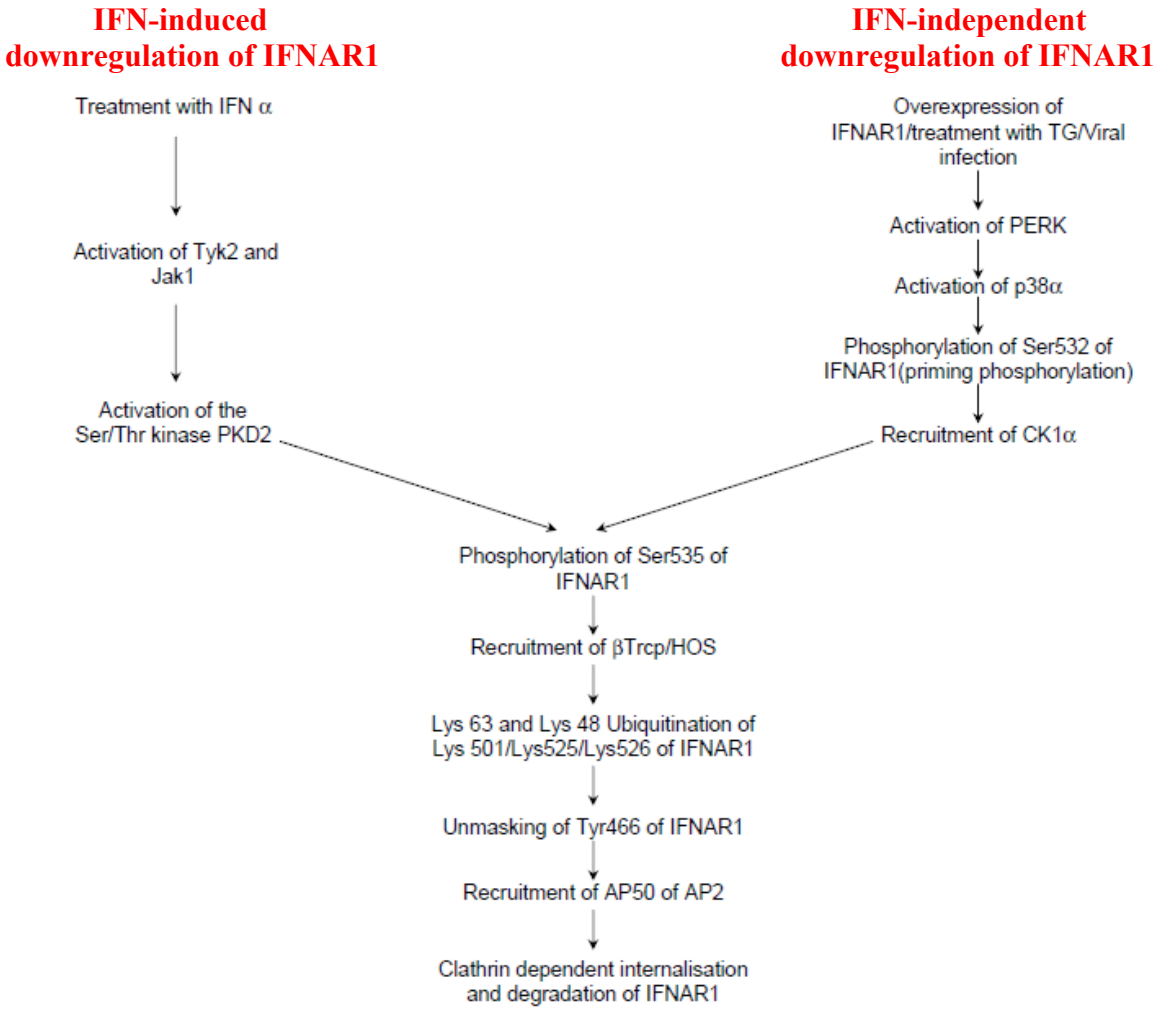


Figure 12: Schematic representation of the pathways involved in ubiquitination of IFNAR1

3.2 Control of activated receptors/Jaks: SOCS proteins

The duration of cytokine-induced signals that are transduced by the Jak/STAT pathway can be regulated by members of the SOCS (suppressor of cytokine signaling) family (CIS,

SOCS1, SOCS2, SOCS3, SOCS4, SOCS5, SOCS6 and SOCS7). All eight SOCS proteins contain a central SH2 domain and a C-terminal SOCS box domain, which interacts with elongins B and C and Cullin5 to catalyse the ubiquitination of bound signaling proteins (Babon et al., 2009). SOCS1 and SOCS3 are the two most potent suppressors of signaling. They are induced by Type I and Type II IFNs as well as by many other cytokines, including growth hormone, IL-6, IL-3, IL-13, granulocyte-macrophage colony-stimulating factor, LIF, erythropoietin, IL-12 and leptin. Elegant studies performed by Yoshimura and colleagues (Sasaki et al., 1999) showed that SOCS1 and SOCS3 contain upstream their SH2 domain, a short motif known as the KIR (kinase inhibitory region) with amino acid sequence similarity to the activation loop of Jaks. The authors proposed that this sequence similarity allows SOCS protein to suppress signaling by direct inhibition of Jak catalytic activity. The activation loop of Jaks blocks the catalytic cleft. Autophosphorylation of this loop causes its translocation away from the catalytic site and allows substrate access, thus activating the kinase. Consequently, it was proposed that the SH2 domain of SOCS1 and SOCS3 binds the phospho-tyrosine of the activation loop while the KIR acts as a pseudosubstrate to block the active site (Boyle et al., 2007).

However studies of deletion of individual SOCS genes in mice has revealed an exquisite specificity for particular cytokine-receptor combinations rather than specific Jaks. For example SOCS1-/- mice were shown to exhibit excessive responses typical of those induced by IFN γ. No dysregulation of STAT3 activation was detected suggesting that signaling of cytokines such as IL-6, LIF and oncoStatin M (OSM) that utilise STAT3 is not affected in these mice (Alexander et al., 1999). In 2006, it was reported that bone marrow macrophages from SOCS1-/- mice showed a prolonged activation of STAT1 and an enhanced induction of 2'-5'-OAS in response to IFN α. These results showed that Type I IFN signaling is also regulated by SOCS1 (Fenner et al., 2006).

SOCS3 deficient livers showed a prolonged activation of STAT1 and STAT3 after IL-6 stimulation but normal activation of STAT1 after IFN γ stimulation (Croker et al., 2003). Yet both cytokine receptor systems utilise the same Jaks (Jak1 and Jak2) (Murray, 2007). Recently, Babon et al. showed that SOCS3 targets the Jaks implicated in specific cytokine receptor systems via two interactions :

- Interaction between the SH2 domain of SOCS3 and the receptor to which the Jak is attached.

- Interaction between the KIR domain of SOCS3 and a specific motif (GQM) present in the Jak Insertion Loop.

SOCS3 might then alter the conformation of the tyrosine kinase in such a way that the distance between the terminal phosphate of ATP and the acceptor tyrosine hydroxyl group of the substrate would be affected (Babon et al., 2012).

Likewise, it had been shown that macrophages from mice harboring the Tyr441Phe mutation in the SOCS1 putative binding site in the IFNGR1 subunit, had a prolonged STAT1 activation upon IFN γ stimulation which correlated to less IFN γ-induced SOCS1 associated to IFNGR1 (Starr et al., 2009). This would suggest that SOCS1 and SOCS3 share this mechanism of receptor dependence to gain specificity and efficacy towards particular cytokines.

In the case of type I IFN signaling, it was reported that SOCS1 interacts with IFNAR1 (Piganis et al., 2011). However, using truncated and mutated forms of IFNAR1, Piganis et al could show that SOCS1 would rather interact with the activation loop of Tyk2. They also reported that SOCS1 reduces Lys-63 ubiquitination of Tyk2 and that upon IFN α stimulation, SOCS1 reduces IFNAR1 level at the cell surface. The authors postulated that interaction of SOCS1 with Tyk2 may result in the destabilization of Tyk2 and exposure of the IFNAR1 internalisation motif with subsequent IFNAR1 internalisation and reduced IFN signaling.

The regulation of cytokine signaling by the SOCS are therefore complex and their mechanism of action may vary and may depend on the cytokine receptor system.

3.3 Delayed downregulation of signaling: STAT content

It was known for many years that cultured cells become refractory to IFN α within hours and remains unresponsive for up to 3 days (Larner et al., 1986). In 2002, Dupont et al reported that when Jurkat E6.1 cells are treated with a very low concentration of IFN β (~ 2 pM) for 24 hrs (pretreatment), washed, transfected for 6 hrs with an ISRE-luciferase reporter plasmid and then rechallenged with fresh IFN β (125 pM) for 18 hrs, there is a decreased in the luciferase induction compared to cells that did not undergo a pretreatment (Dupont et al., 2002). The level of IFNAR1 was virtually unchanged after 24 hrs of IFN β stimulation with 2 pM of IFN β. Interestingly, the authors remarked that, at the lowest IFN β concentration, the level of IFNAR1 was unchanged, the IFN-dependent gene induction is reduced but the activation state of Tyk2, STAT1, STAT2 and STAT3 was intact when pretreated, washed, transfected cells were rechallenged for 15 minutes with 125 pM of IFN β. The authors also noticed that the levels of STAT1 and STAT2 were increased after

pretreatment and that this increase correlated well with the extent of IFN-dependent transcription inhibition. These results were corroborated in 2004 by another group (Radaeva et al., 2004). In this study, the authors used human hepatoma Hep3B cells and showed that prolonged IFN γ treatment (3-6 days) attenuated STAT1, STAT2 and STAT3 activation in response to 125 pM of IFN α but enhanced STAT1 activation in response to IFN γ. Such a long term treatment with IFN γ caused a considerable augmentation in the level of STAT1. Interestingly, the authors showed that the prolonged treatment with IFNγ did not inhibit level or IFNα-activation of JAK1 and Tyk2. Overexpression of STAT1 via stable transfection therefore, enhances IFN γ activation of STAT1, attenuates IFN α activation of STAT1, STAT2 and STAT3 and does not affect IFN α activation of Jaks.

These two studies suggested that the STAT1 content may regulate IFN α response, at different steps of signaling. In the first study, STATs activation was not affected while in the second study, the activation of the STATs was. In none of the two studies, the activation of the Jaks was affected.

STAT3 has also been shown to downregulate Type I IFN signaling (Wang et al., 2011). Comparative microarray analysis of STAT3 -/- and WT MEFs stimulated with 250 pM of IFN α for 2 hrs showed that the expression of a variety of ISGs is increased in STAT3-/- MEFs. Consequently, the antiviral response of STAT3-/- MEFs was higher than that of WT MEFs in response to IFN α. Interestingly, the tyrosine phosphorylation of STAT1 and STAT2 was comparable between the two MEFs. In fact the authors could show**,** using mutant forms of STAT3**,** that the latter blocks IFN signaling independently of its DNA binding domain or its transactivation domain. The mechanism by which STAT3 negatively regulate Type I IFN signaling remains unidentified. The authors excluded a previously proposed model that STAT3 sequesters STAT1 into STAT1:STAT3 heterodimers and reduces IFN α induction of DNA binding by STAT1 homodimers (Ho and Ivashkiv, 2006), since reconstitution of a STAT3 -/- MEFs with a truncated form of STAT3 lacking the SH2 domain recapitulated the negative effect of wtSTAT3.

In a mouse model infected with lymphocytic choriomeningitis virus infection (LCMV), it was reported that early type I IFN signaling drives primarily STAT4 rather than STAT1 phosphorylation in NK cells (Natural Killer), leading to enhanced IFN γ production. However as STAT1 levels increase, IFNAR stimulation will preferentially activate STAT1 resulting in enhanced NK cell killing and loss of IFN γ production (Mack et al., 2011).

Overall, these studies indicate that a change in STAT content in a given cell may influence the extent of the cell's response to Type I IFN.

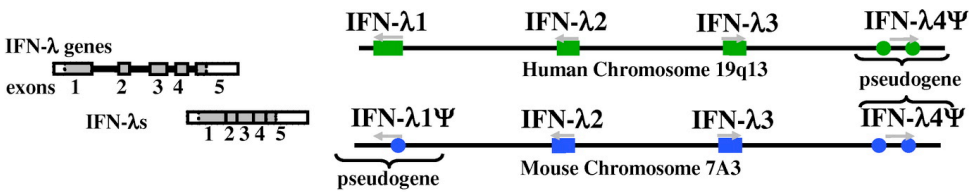
3.4 Delayed downregulation of signaling: USP18/UBP43

USP18 is a cysteine protease and a member of the ubiquitin specific protease (USP) family. USP18 is specialized in the removal of ISG15, an IFN-induced ubiquitin like molecule, from ISGylated proteins (Malakhov et al., 2002). The group of D. E Zhang was the first to demonstrate that USP18 regulates IFN α signaling (Malakhova et al., 2003). Since USP18 and ISG15 have been at the center of my thesis work for the last 4 years, a whole chapter will be devoted to these proteins.

4 **Type III IFNs or IFN λs**

In 2003, using a method that combines multiple computational techniques to detect families of uncharacterized proteins from human genomic sequence, two groups independently described three proteins functionally similar to type I IFNs (Kotenko et al., 2003; Sheppard et al., 2003). These proteins are now collectively known as type III IFNs. They form part of the class II helical cytokines (Fig. 2). They were initially named IFN λs or IL-28/29 due to their shared features with both type I IFN and the IL-10 families. Type III IFNs share with type I IFNs similar expression patterns and a common signal transduction pathway involving Tyk2, Jak1 and STAT1/2/3. Type III and type I IFNs therefore induce a similar set of genes and share many biological activities including the ability to induce an antiviral state in target cells. Structural comparison with other members of the class II cytokines shows that IFN λ is related to the IL-19 subfamily (cytokine which forms part of the IL-10 family). Type III IFNs have therefore been defined as being functionally an IFN but structurally related to the IL-10 family.

In humans, three IFN λs genes exist: IFN λ1 (IL-29), IFN λ2 (IL-28A) and IFN λ3 (IL-28B), clustered on chromosome 19 and in mouse two IFN λs genes exist: IFN λ2 and IFN λ3, clustered on chromosome 7. The coding region for each of these genes is divided into 5 exons. The intron-exon organization of the genes encoding the IFN-λs correlates well with the common conserved architecture of the genes encoding the IL-10-related cytokines. The amino acid identity between type I and type III IFNs is very low, ranging from 15 to 20%. IFN λ2 and IFN λ3 share 96 % sequence identity and differ for 7 amino acids (Fig. 12). The promoters of the IFN λ2 and IFN λ3 genes are very similar and share several elements with the IFN-λ1 promoter, suggesting that all 3 genes are likely to be regulated in a similar manner.



[] indicates the position of the intron

Figure 13: Alignment and schematic representation of the three members of the type III IFN family (adapted from (Kotenko et al., 2003)).

Type I and Type III IFNs share similar expression pattern owing to the presence of common regulatory elements in their promoters. Promoters of the IFN λ genes contain predicted binding sites for transcription factors of the IRF families, NF-kB, JUN, FOS and ATF. These factors are involved in the transcriptional regulation of type I IFN genes (see section 1.2). The transcriptional regulation of IFN λ1 and IFN β genes is similar, IFN λ2/λ3 have similar transcriptional regulation to most IFN α genes.

Since IRF3 is constitutively and ubiquitously expressed in cells, upon viral entry, there will be upregulation of IFN β and IFN λ1 genes while IFN λ2, IFN λ3 and αIFNs will be expressed with delayed kinetics (Levy et al., 2011).

4.1 The Type III IFN receptor

Type III IFN engages a receptor complex distinct from the one of type I IFN and is composed of IFNLR1(IL-28RA) and IL-10R2 chain, this latter being shared with receptor complexes specific for IL-10, IL-22 and IL-26. The IFNLR1 gene and the IL10R2 gene are positioned on human chromosome 1 and chromosome 21 respectively (Kotenko, 2011).

Like type I IFN, IFN λs interact with its receptor in a ratio of 1:1. Thus one molecule of IFN λ engages one molecule of IFNLR1 and one molecule of IL10R2.

The intracellular domain of IFNLR1 and IL10R2 are devoid of enzymatic activity but are associated to the Jaks, Jak1 and Tyk2 respectively.

Binding of IFN λ to its receptor leads to the activation of Jak1 and Tyk2 that will phosphorylate tyrosine residues present in the IFNLR1 intracellular domain. It was reported that in the murine T lymphoma cell line BW5147 stably transfected with either the wt or tyrosine(s) mutated huIFNLR1 subunit, phosphorylation of STAT2 and STAT5 upon IFN λ1 treatment (150 pM for 10 min) requires the presence of Tyr343 and Tyr517 on the

IFNLR1 chain. In contrast, activation of STAT1, 3 and 4 occurs independently of these tyrosine residues (Dumoutier et al., 2004).

Type III IFN similar to type I IFN is therefore able to activate STAT1 and STAT2 molecules that will form a trimeric complex with IRF9 known as ISGF3 (Figure 14). Comparative microarray analyses of the human hepatocarcinoma cell line HepG2 treated with IFN λ1 (2,5 nM) or IFN α (250 pM) for 1, 6 and 24 hrs showed that the genes induced by the two IFNs are essentially the same (Doyle et al., 2006).

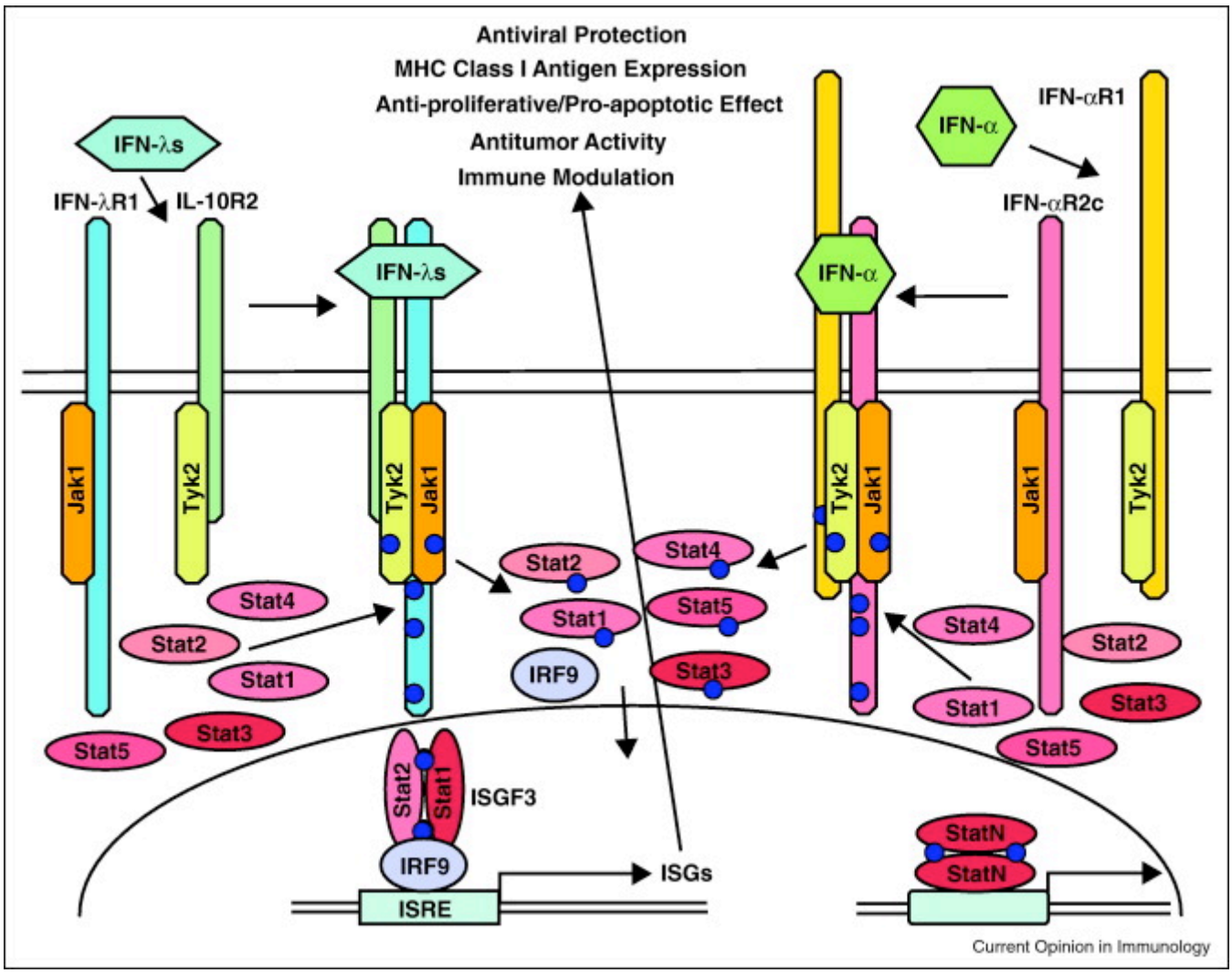


Figure 14: Schematic representation of Type III IFN and Type I IFN signaling (adapted from (Kotenko, 2011))

4.2 Biological activities

As discussed above, the type III IFN signal transduction cascade overlaps that induced by type I IFN (Figure 14) and expectedly type I and type III IFNs induce similar biological activities. Both cytokines induce an antiviral response against various viruses such as VSV and HCV (Kotenko et al., 2003; Marcello et al., 2006). Like Type I IFN, Type III IFN is able to upregulate expression of MHC class I antigens thereby enhancing the ability of the

immune system to recognise and destroy virus-infected cells. The most striking difference between Type I and Type III IFNs is their receptor distribution. While IFNAR1 and IFNAR2 are expressed in all cells, and all nucleated cells can respond to Type I IFN, expression of the IFNLR1 subunit is limited primarily to epithelial cells (Sommereyns et al., 2008). Several studies have shown that the epithelia of the intestine, lung and vagina are protected from viral infection by IFN λ treatment (Mordstein et al., 2010; Pott et al., 2011). Indeed IFN λ is predominantly induced by influenza A virus and respiratory syncytial virus infection (Jewell et al., 2010; Mordstein et al., 2010). Likewise, human hepatocytes express IFNLR1 (Doyle et al., 2006) and clinical trials of IFN λ treatment for HCV, a hepatotropic virus, are showing promising results (Muir et al., 2010). These recent studies suggest that IFN λ allows protection of anatomic compartments of the body that are open to the outside and which serve as major portals for pathogen entry.

The antiproliferative activity of IFN λ is weak but it can be promoted in cells engineered to express high level of IFNλR1 (Dumoutier et al., 2004).

The immunomodulatory role of Type I IFN is well documented (section 1.1), while IFN λ remains a matter for debate. Studies based on measurement of full length IFNLR1 mRNA in human PBMC suggest that this receptor is expressed at very low levels in immune cells (Witte et al., 2009)

4.3 Genetic analysis of type III IFNs

Analysis of genetic variations in human populations has highlighted strong positive selection on all three members of the type III IFN family in European and Asian populations (Manry et al., 2011). These IFN genes are located on a 50-kb region of chromosome 19 and display low levels of linkage disequilibrium in all human populations, suggesting independent positive selection events.

Strong signal of positive selection has been detected at the IL-29 locus. A nonsynonymous variant, 2054G>A (D188N, rs30461, predicted damaging), presented extreme levels of differentiation between Africans and Eurasians. Moreover, this variant was found to be among the most highly differentiated variant at the level of the entire genome. The group of Quintana-Murci and colleagues thus suggested that the IL-29 variation, and the D188N variant in particular, has conferred a selective advantage to Eurasian populations (Manry et al., 2011).

Thus Type III IFNs appears to be the only IFNs where selective pressures have involved processes of geographically restricted adaptation, revealing that genetic variation of these

genes has conferred a selective advantage to specific human populations. This hypothesis was corroborated by analysing the relationship between polymorphism of IL-28B and success of treatment of chronically infected HCV patients.

4.4 IL-28B gene polymorphism and Hepatitis C virus

Worldwide 170 million people are chronically infected with HCV and the current standard-of-care for patients with chronic hepatitis C is a combination of pegylated IFN α and ribavirin. The treatment achieves a sustained virological response (SVR) (i.e no detectable HCV RNA for at least six months after the end of the therapy) in approximately 55 % of patients. Several factors have been identified to play a role in the outcome of therapy, including the treatment schedule, disease characteristics, viral factors, and host factors. However, these factors only partly explain the ability of IFN and ribavirin therapy to cure HCV infection.

Human genetic factors that influence HCV treatment response have been identified in genome-wide association study (GWAS). Several groups have identified single nucleotide polymorphisms (SNPs) located in the regulatory region (rs12979860 and rs28416813) or in the 3' region of *IL28B* gene, which are strongly associated with SVR (Ge et al., 2009; Suppiah et al., 2009; Thomas et al., 2009). For example, for the rs12979860 SNP, the CC genotype was associated with a 2-fold greater SVR rate than TT genotype, CT being closer to TT than to CC. This holds true for patients of European ancestry, as well as in African-American and Hispanic patients. Interestingly, the rs12979860 SNP is in strong linkage disequilibrium with a non-synonymous coding variant in the *IL28B* gene (K70R ; rs8103142).

However, the precise mechanism by which these SNPs can influence the response to standard-of-care therapy in chronic infection to HCV is still unknown. The different SNPs could for instance influence the potency or the expression level of IL28B.

5 **USP18/UBP43**

5.1 Cloning and expression

Usp18 or UBP43 is a polypeptide with a predicted molecular weight of 43kDa. In the nomenclature system the murine protein is called Usp18 while USP18 refers to the human ortholog.

Usp18 was originally identified as an up-regulated gene in knock-in mice expressing an acute myelogenous leukemia fusion protein, AML1-ETO (Liu et al., 1999). The Usp18 cDNA codes for a 368 amino acid protein sharing 20-25 % aa identity to members of the Ubiquitin Specific Protease (USP) family. Sequence similarity is largely restricted to six motifs conserved in all USP family members: the Cys box, the QQDAQEF motif, the consensus LPQILVIHLKRF and the His box. The putative active site nucleophile is a cysteine residue in the Cys box (Cys61 in the mouse) that is found in all members of the USP family (Liu et al., 1999). In wild type adult mice, Usp18 is highly expressed in the thymus and in peritoneal macrophages.

Usp18 is also expressed in a murine monocytic leukemic cell line (M1). Liu et al showed that expression of Usp18 in M1 cells blocked the IL-6-induced differentiation of M1 cells into the macrophage lineage. These results suggest that Usp18 may play a role in hematopoiesis (Liu et al., 1999).

In 2000, the human USP18 cDNA was cloned by screening a human monocyte-derived cDNA library using as probe the murine sequence. The human cDNA codes for a 372 amino acid polypeptide that exhibits 70% aa identity with the murine Usp18 (Figure 15). In the human protein, the putative active site nucleophile is cysteine 64 in the Cys box.

The USP18 gene is located on human chromosome 22 and the Usp18 gene on murine chromosome 6 (Schwer et al., 2000).

Figure 15: Alignment of human and murine USP18 with the conserved regions and site of interaction.

To identify the complete repertoire of gene changes occurring during IFN β-induced reversion of melanoma to a more differentiated state, a rapid substraction hybridization (RaSH) approach was used. By this technique USP18 was cloned from the human melanoma cell line HO-1 treated for 24 hrs with IFN β (Kang et al., 2001).

USP18 was identified to be an IFN induced gene. All type I IFN subtypes tested (IFN α/β) induced USP18 whereas IFN γ, Interleukin-6 (IL-6) and growth factors such as TGF-β did not induce USP18 in these cells (Kang et al., 2001).

In 2002, the group of Zhang reported that LPS treatment of murine macrophage strongly activates Usp18 mRNA as early as 2 hrs after induction, reaching a maximum expression at 10 hours ((Malakhova et al., 2002).

Binding sites were identified in the *Usp18* promoter and IRF-2 and IRF-3 were shown to be involved in the induction of Usp18 by LPS. IRF-3 plays a primary role in the LPS-induced activation of *Usp18* gene and IRF-2 confers basal transcriptional activity of the *Usp18* promoter.

5.2 Structure and catalytic function

As mentioned above, USP18 is a member of the USP family which comprises the largest and most diverse family of deubiquitinases (DUBs) in mammalian cells with more than 56 distinct members. USP domain DUBs are usually large proteins (between 350 and 3400 aa) with a core catalytic domain of approximately 350 aa. Outside of their catalytic core, USP

enzymes may comprise numerous other domains, including protein interaction domains that facilitate substrate binding, or domains determining subcellular localisation (Fig. 16). Surprisingly, as opposed to other members of the USP family, USP18 possesses only its catalytic core making it the simplest USP member (Fig. 16) (Komander, 2010).

However, the crystal structure of USP18 has not been solved yet. The crystal structure of HAUSP (USP7), a deubiquitinating enzyme of the family of the USP has been crystallised alone or covalently attached to a ubiquitin aldehyde (Hu et al., 2002). This has allowed the characterisation of domains important for the activity of USPs. The catalytic core domain of HAUSP ressembles an extended right hand comprised of three domains, fingers, palm and thumb (figure 17). The thumb consists of eight alpha helices, with the N-terminal Cys box adopting an extended conformation. The palm contains eight central beta strands which are buttressed by two alpha helices and several surface loops. An anti-parallel beta sheet formed by six of the eight beta sheet packs against the globular thumb and give rise to an inter-domain deep cleft which is enriched with acidic amino acids. The Cys and His box are positioned on the opposing sides of this cleft. The fingers are comprised of four beta strands in the center and two at the tip. The primary sequence of HAUSP was aligned with other members of USP family and the sequence alignment result was crossvalidated with structural information on HAUSP. This analysis revealed that residues that contribute to the structural integrity of the fingers, the palm and the thumb are highly conserved. The finger-palm-thumb architecture of HAUSP was reported to be conserved among all USP proteins.

The active site of free HAUSP exists in an unproductive conformation. Upon binding to the ubiquitin aldehyde, structural elements surrounding the catalytic cleft undergo changes that realign the active site residues for productive catalysis. However, the overall structure and all of the secondary elements are maintained in the fingers, the palm and the thumb (Hu et al., 2002).

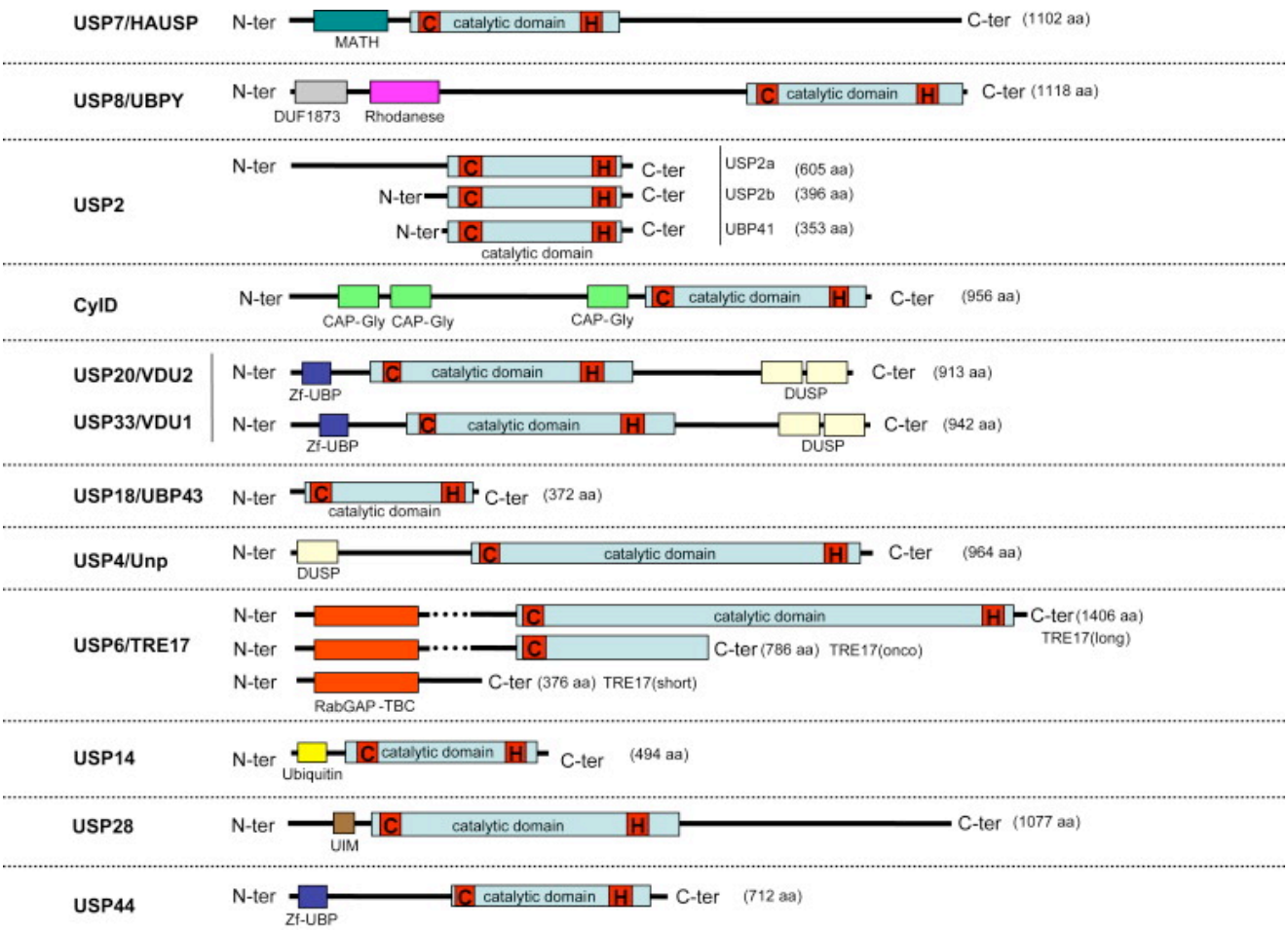


Figure 16: Domain structures of 12 members of the USP family. The catalytic domain, containing the conserved cystein (C) and histidine (H) boxes, is shown for each USP (Daviet and Colland, 2008)

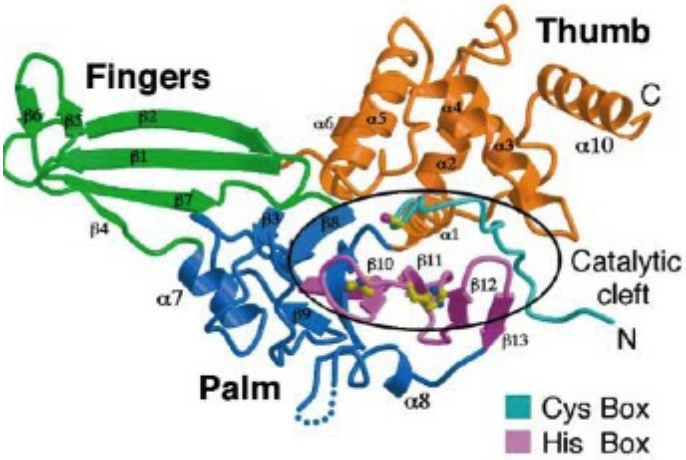


Figure 17 : Crystal Structure of free HAUSP showing the overall structure of the fingers, thumb, palm and catalytic cleft (adapted from (Hu et al., 2002).

All members of the USP family possess a Cys and an His box. The Cys box contains the catalytic cysteine residue which is thought to undergo deprotonation and to unleash a nucleophilic attack on the carbonyl carbon atom of the ubiquitin Gly 76 at the isopeptide bond (fig. 18). In analogy with other cysteine proteases, the deprotonation of this cysteine is assisted by an adjacent His residue which in turn is stabilised by a nearby side chain from an asparagine or aspartate residue. Together, these three residues constitute the **catalytic triad.**

Another catalytic feature is the formation of the oxyanion hole, which refers to the accomodation of the negative potential formed on the carbonyl oxygen atom at the scissile bond. Typically, the oxyanion is stabilised by hydrogen bonds from the backbone amide group of the catalytic cysteine as well as from neighboring glutamine, asparagine and aspartate (figure 18) (Amerik and Hochstrasser, 2004)**.**

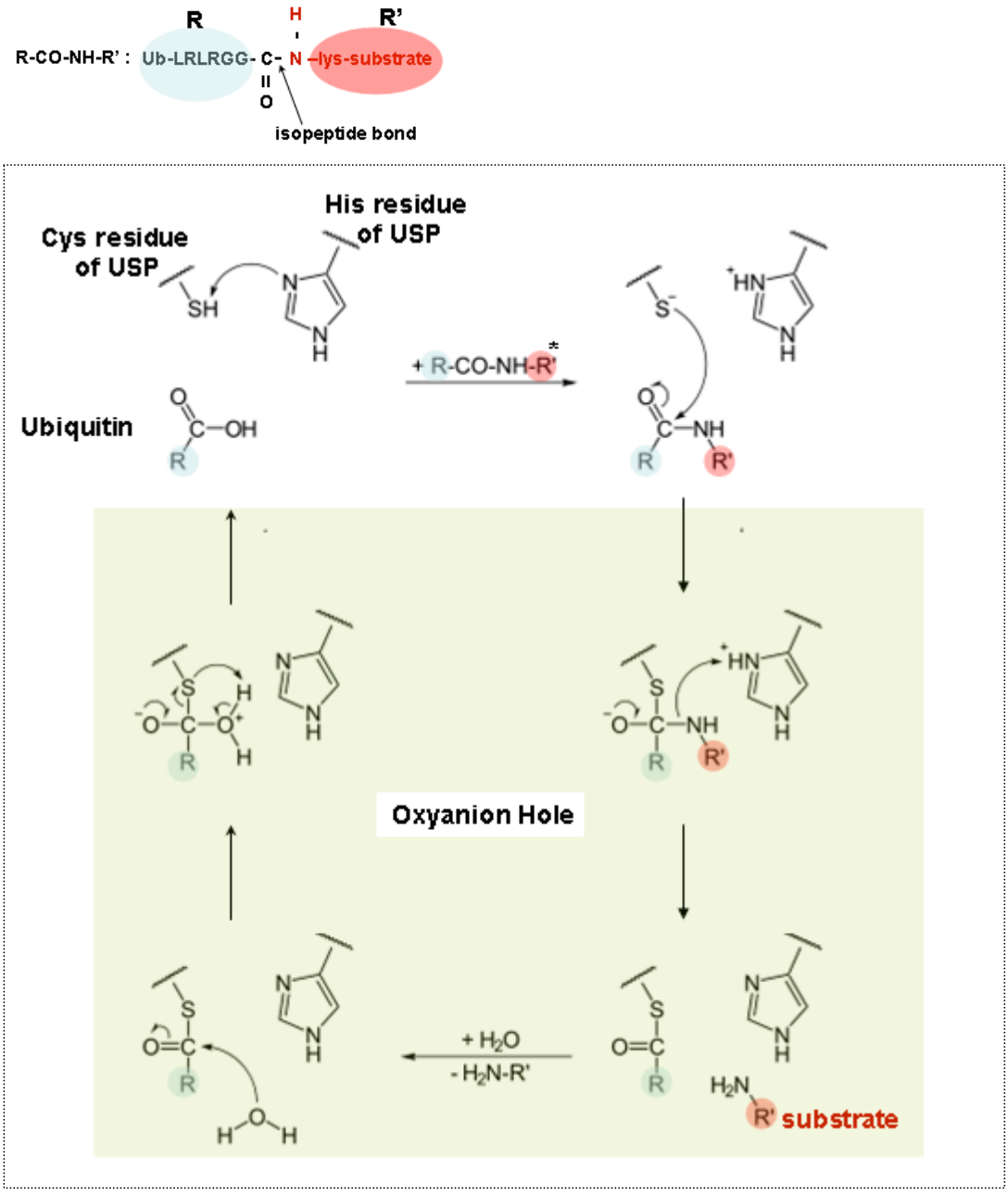


Figure 18: Biochemical reactions occurring in the catalytic cleft of USP proteins.

The alignment of USP18 with HAUSP and other USP shows that in human USP18 (refer to figure 19 below):

- Cys 64 constitutes the catalytic cysteine residue
- His 318 deprotonates Cys64 (see figure above)
- Asn 335 forms a hydrogen bond with His 318 and stabilises the histidine residue
- Asp 336 stabilises the oxyanion hole

In HAUSP, mutations of the corresponding Cys, His, Asp (In USP18, the Asp is replaced by Asn) and of the Asp which stabilises the oxyanion hole result in abrogation of the catalytic activity of the cysteine protease.

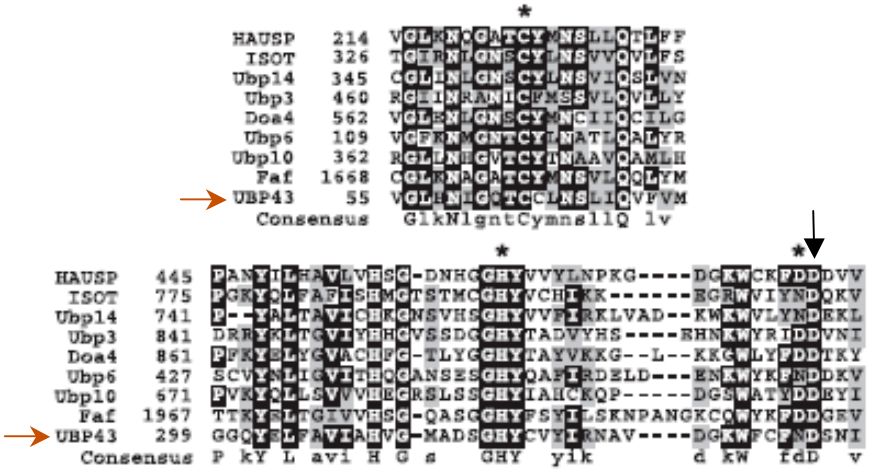


Figure 19: Alignment of USPs. Conserved residues are shaded in black and the astericks indicate the catalytic core of various USP including USP18 (UBP43). The black arrow points to the conserved aspartate which stabilises the oxyanion hole (taken from Amerik and Hochstrasser, 2004).

It was originally reported that both human and murine USP18 exhibit deubiquinating activity. Indeed, when expressed as a GST fusion protein, Usp18 was found to be able to cleave ubiquitin off a Ub-Met-β-gal fusion (Liu et al., 1999). However, the authors were unable to detect such activity when using other ubiquitin fusions. This prompted them to analyse the activity of Usp18 toward other major ubiquitin-like molecules (Ubl), such as SUMO, Nedd8 and ISG15. They used a sensitive assay whereby each Ubl, produced in E. *coli*, had a cleavable C-terminal 20 amino acid peptide extension containing a single tyrosine that could be labeled with ^{125}I and quantitated. With this method they could show that Usp18 hydrolysed modified ISG15 but not modified ubiquitin, Nedd8 or SUMO. This result suggested that Usp18 specifically cleaves the ubiquitin-like molecule ISG15 from conjugated proteins (Malakhov et al., 2002).

Briefly, ISG15 is a 15 kDa protein which bears two ubiquitin-like domains and which like ubiquitin, is conjugated to target proteins by an isopeptide bond (ISGylation). The process of ISGylation requires an ISG15-specific machinery consisting of three enzymes, as will be discussed in next chapter. Importantly, ISG15 and all the enzymes that are required to conjugate ISG15 to its substrate are induced by type I and type III IFNs.

5.3 Study of the phenotype of the Usp18 knockout mice

In an effort to explore the function of Usp18, Usp18 knockout mice were generated. These mice are viable at birth, but gradually manifest neurological disorders associated with the development of hydrocephalus. Hydrocephalus occurs as a consequence of necrosis of ependymal cells leading to aqueductal stenosis (Ritchie et al., 2002).

Despite the strong relevance of Usp18 to the development of hematopoietic system (Liu et al., 1999), analyses of peripheral blood and bone marrow cells of Usp18 null mice did not reveal any significant defect (Malakhova et al., 2003).

Knowing that Usp18 is highly induced by type I IFN, the authors hypothesized that the response of Usp18 null cells to IFN might be affected. To test this, WT and Usp18 null mice were injected daily with polyinosinic acid-polycytidylic acid (polyI-C), a potent inducer of type I IFN, and their survival was monitored. All WT mice survived the course of the treatment. In contrast, Usp18 null mice died within 72 hrs post injection. In addition, a dramatic decrease in the total number of peripheral white blood cells was observed. These observations showed that Usp18 null mice are hypersensitive to polyI-C. To assess if the hypersensitivity to polyI-C was directly related to type I IFN production, bone marrow cells from WT and Usp18 null mice were cultured under colony forming unit (CFU) assay conditions (IL-3, IL-6 and SCF) in the absence or the presence of IFN β. While wt bone marrow cells showed only 40% reduction in colony formation upon IFN stimulation, Usp18-/- cells failed to form colonies. This hypersensitivity to IFN correlated with increase of apoptosis (Malakhova et al., 2003).

Interestingly, a prolongation of STAT1 activation in response to IFN β in bone marrow cells of Usp18 null mice was also observed. Consequently, an enhanced and prolonged induction of ISGs such as OAS, IRF7 and ISG15 were reported.

In another study, GeneChip analysis was performed on bone marrow derived macrophages (BMM), isolated from WT and Usp18 null mice, treated for various times with one dose of IFN β (100 IU/ml). A significant enhancement of all ISGs was found in null BMMs cells compared to WT. Among the ISGs scored were genes involved in antigen presentation (HLA- DOA, -A, -E, Tap2), antiviral genes (OAS1B, MX1, MX2, ISG15) and genes encoding chemokines and cytokines (CXCL11, TRAIL, FAS) (Zou et al., 2007).

Usp18 null mice were reported to be more resistant than control mice to LCMV, VSV and HBV infection (Kim et al., 2008; Ritchie et al., 2004). The enhanced antiviral response of Usp18 null mice may result from an increased ISGs expression and/or an increased ISGylation of specific proteins.

As mentioned above, Usp18 is able to cleave the ubiquitin-like ISG15 from conjugated proteins. Accordingly, a high level of ISGylated proteins was detected in IFN β stimulated bone marrow cells from Usp18 null mice. On this basis it was suggested that the increased ISGylation could explain the enhanced and prolonged signaling in response to type I IFN and could somehow lead to hydrocephalus of the Usp18 null mice.

To test whether high ISGylation accounted for the enhanced and prolonged IFN signaling of Usp18 null cells, Malakhova et al used human leukemic K562 cell line which is unable to perform protein ISGylation, due to the lack of UBE1L an essential enzyme of the ISGylation machinery (see section 6.2) (Malakhova et al., 2003). When K562 cells were reconstituted with UBE1L together with an ISRE-luciferase reporter construct (therefore causing increased ISGylation), the luciferase activity was found to be significantly higher than cells transfected with the empty vector. These data suggest that Usp18 negatively regulates the type I IFN signaling by decreasing the level of protein ISGylation. Importantly, the same authors performed a high throughput western blot screening of ISGylated proteins that were purified by immunoaffinity chromatography from human thymus. Key regulators of signal transduction such as phospholipase Cγ1, Jak1, ERK1 and STAT1 were found to be ISGylated (Malakhov et al., 2003).

The conclusion that the enhanced ISGylation in Usp18 null mice was the cause of the prolonged IFN response was challenged when ISG15 null mice were generated and characterized (Osiak et al., 2005). In contrast to Usp18 null mice which developped an hydrocephalus and were more resistant to VSV and LCMV infection, ISG15 null mice showed no developmental defect and normal sensitivity to VSV and LCMV infection. However, they were also described to be more sensitive to a number of viruses (Sindbis virus, influenza A and B virus, HSV-1, and murine gamma herpesvirus 68) (Lenschow et al., 2007). More importantly, ISG15 null mice do not present any defect in STAT1 activation (Osiak et al., 2005).

As mentioned above, the hydrocephalus and the poly I-C hypersensitivity that developed in Usp18 null mice have been ascribed to an increase in protein ISGylation. On this basis, it was expected that the knock out of ISG15 in Usp18 null mice should rescue these phenotypes. However, mice deficient in both Usp18 and ISG15 (ISG15-/- Usp18-/- DKO) still developed hydrocephalus and were hypersensitive to poly I-C injection (Knobeloch et al., 2005). Furthermore, MEFs derived from DKO mice showed an enhanced and prolonged activation of Type I IFN signaling, comparable to what described in Usp18 null MEFs (Knobeloch et al., 2005).

To summarise, the occurrence of hydrocephalus, the poly I-C hypersensitivity and the enhanced activation of type I IFN response observed in Usp18 null mice appear to be independent of ISG15. Therefore, it was proposed that Usp18 regulates type I IFN signaling through a non-ISG15 mediated mechanism, as for instance by processing another ubiquitin-like molecule possibly involved in the regulation of IFN signaling.

The non-implication of ISG15 in the regulation of IFN signaling was further confirmed with the description of the UBE1L null mice (Kim et al., 2006). These mice were found to be normal, healthy and fertile. MEFS derived from these mice presented no defect in STAT1 activation by IFN β, no difference in sensitivity to IFN-induced death and no difference in anti-VSV and -LCMV responses when compared to MEFs of WT mice. No ISGylated conjugates could be detected in LPS-treated BMM of UBE1L -/- mice. Moreover, as reported for MEFs derived from ISG15-/-Usp18-/- DKO mice, MEFs derived from UBE1L-/-Usp18-/- DKO mice showed enhanced and prolonged activation of Type I IFN signaling, comparable to what described for Usp18 null MEFs (Kim et al., 2006).

The phenotypes of the ISG15 and of the UBE1L knockout mice suggested that Usp18 exerts its regulatory function on type I IFN signaling independently of ISG15 and of ISGylation. Usp18 could therefore function either independently from its catalytic activity or by processing ubiquitin or a ubiquitin-like molecule other than ISG15.

The molecular mechanism of Usp18 action in the IFN signaling pathway was studied by the group of Zhang in MEFs from Usp18-/- mice retrovirally transduced with either WT or a catalytically dead mutant form of Usp18 ($Usp18^{C61S}$). Importantly, reconstitution with either WT Usp18 or $Usp18^{C61S}$ resulted in a significant reduction of STAT1 phosphorylation, suggesting that the catalytic activity of Usp18 is dispensable, at least in the context of ectopic expression (Malakhova et al., 2006).

An alternative interpretation was that expression of Usp18 somehow affected the surface expression of the type I IFN subunits and/or activation of Tyk2 and Jak1. To test this model, human leukemic KT-1 cells were silenced for USP18 and assessed for the level and the dynamics of the type I IFN receptor subunits. The steady-state levels of IFNAR1 and IFNAR2 chains were found not to be altered by silencing. Furthermore, the half-life and the extent of ubiquitination of transfected IFNAR1 did not differ between WT and USP18 silenced cells. These results suggested that USP18 does not control the ubiquitination and proteolysis of IFNAR1 (Malakhova et al., 2006).

The authors then monitored the phosphorylation of Jak1 upon IFN β treatment in bone marrow cells derived from either WT or Usp18 -/- mice. An increase in the magnitude and

duration of IFN-induced Jak1 phosphorylation was observed in the bone marrow cells derived from Usp18-/- mice as compared with cells from wt animals, suggesting that Usp18 affects the extent of Jak1 phosphorylation.
Since Usp18 did not control IFNAR1 turnover and its expression in MEFs abrogated Jak1 activation, the authors hypothesised that USP18 might interact with one of the subunits of the receptor. In transient transfections in 293T cells, Usp18 was shown to interact with huIFNAR2 but not with muIFNAR1 or huIFNGR1.
The region of Usp18 responsible for the interaction with IFNAR2 was delineated and the C-terminus of Usp18 was shown to provide the main interaction motif for the association with IFNAR2. The authors also remarked that residues in the region of aa 312–368 might be critical for the interaction and indeed alanine substitutions of charged residues between amino acid 350-354, abrogated Usp18-huIFNAR2 interaction (Malakhova et al., 2006).

The Usp18 binding site was mapped to the membrane-proximal region of huIFNAR2 that covers the box1-box2 motifs. Of note, this region of IFNAR2 is essential for Jak1 interaction (Liu et al., 1999; Usacheva et al., 2002b), suggesting that Usp18 could compete with Jak1 for receptor binding, consequently inhibiting its activation and the downstream intracellular signaling. Indeed, from co-expression experiments, the authors showed that Usp18 inhibits the formation of the Jak1–IFNAR2 complex in a dose-dependent manner (Malakhova et al., 2006).

These results are puzzling since the amino acid residues in the box1-box2 motifs are not well conserved between mouse and human IFNAR2 (see Fig. 5) (Kim et al., 1997). Notably Pro289 of box1 is absent in muIFNAR2. Moreover, the charged residues in Usp18 (350-354 (in bold below and in alignment Fig. 5) proposed to be essential for Usp18-IFNAR2 interaction, are not conserved in the human USP18 ($_{350}$**R**Y**R**W**R**$_{354}$ in mouse versus $_{355}$NYHWQ$_{359}$ in human).
The phenotypes of the mice/cells here described are summarised in Table 2.

mice/cells	Viability	Hydrocephalus	ISGylated proteins	Survival to poly-I.C injection/apoptotic response to IFN	Antiviral response to VSV/LCMV	Stat activation
WT mice	healthy and fertile	-	+	all survive from poly-I.C injection	+	+
Usp18 -/- mice	viable at birth but reduced life expectancy	+	++	hypersensitive, die 72h postinjection	++	**enhanced and prolonged stat activation**
ISG15 -/- mice	healthy and fertile	-	No	all survive from poly-I.C injection	+	like WT
Usp18 -/- ISG15-/- mice	reduced life expectancy	+	No	hypersensitive, die 24-72h postinjection	++	**enhanced and prolonged stat activation**
UBE1L-/- mice	healthy and fertile	-	No	No hypersensitivity to IFN induced cell death (assessed in bone marrow cells)	+	like WT
Usp18 -/- UBE1L-/- mice	reduced life expectancy	+	No	hypersensitive to IFN induced cell death (assessed in bone marrow cells)	++	**enhanced and prolonged stat activation**
Usp18-/- MEFs + wtUsp18	-	-	+	-	+	like WT
Usp18-/- MEFs + Usp18C61S	-	-	+	-	+	like WT

Table 2: Summary of the phenotype of the different mice and MEFs used to characterise the role of USP18 and ISG15 in Type I IFN signaling

Immunoblot analyses of lysates from IFN-treated cells reveal two bands of approximately 37 and 34 kDa. Potu et al showed that transfection of USP18 tagged at its C-terminal gives rise to two bands whereas USP18 tagged at its N-terminal give rise to a single band. The authors concluded that the N-terminus of UPSP18 is proteolytically processed and that this processing is not generated through autocleavage since the two bands are also obtained with the catalytically inactive mutant USP18 C64S (Potu et al., 2010). Surprisingly, the group of Zhang showed that mutations of the first (ATG) in USP18 mRNA sequence do not change the expression pattern. They showed that the CUG codon at position 16 is the major translation initiation site for expression of full-length USP18 (37 kDa) and that AUG36 is the translational start site for the expression of the USP18 short form (34 kDa). The authors also showed that when CUG16 is mutated in ATG16 so that translational initiation

efficiency is optimized at this site, the level of the short form drops enormously. This suggests that the short form is mainly the consequence of leaky scanning. The authors also showed that both isoforms of USP18 are functional in deISGylase activity and in regulating type I IFN signaling (Burkart et al., 2012).

6 ISG15

6.1 Expression

Interferon-stimulated gene 15 (ISG15) is a 15 kDa protein that is generated from a precursor form of 17 kDa by cleavage of the eight C-terminal amino acids (Knight et al., 1988). The mature form of ISG15 lacks an N-terminal methionine and presents the C-ter LRLRGG sequence, which is found also in the mature ubiquitin. ISG15 was originally identified in the late 1980's as a ubiquitin cross-reactive protein (UCRP) due to its detection with some anti-ubiquitin antibodies (Haas et al., 1987). ISG15 is one of the most abundant type I IFN induced transcripts and the protein exists in three distinct states: in a free form, in a conjugated form linked to target proteins and in a secreted form.

The crystal structure of ISG15 has revealed two ubiquitin-like domains, making it a linear dimer of a ubiquitin-like protein which shares only 30% homology with ubiquitin. Compared to ubiquitin, ISG15 exhibits a relatively low cross-species conservation and is present only in vertebrates. Taken together, these properties indicate that ISG15 is not an essential housekeeping gene.

6.2 ISGylation

The process of conjugation of ISG15 to protein substrates is termed ISGylation and uses a set of modification enzymes analogous to those involved in ubiquitination (Figure 20)

Briefly, UBE1L, the E1 activating enzyme, has an ATP-binding domain and an active site cysteine residues which are both necessary for ISG15 activation. The activated ISG15 is then transferred to the active site cysteine residue of an E2 conjugating enzyme. UbcH8 is the E2 enzyme involved in the conjugation of ISG15. This enzyme can also function in the conjugation of ubiquitin (Zhang and Zhang, 2011).

UbcH7 shares 72% similarity with UbcH8 and, among all E2 enzymes, is the most closely related to UbcH8 however it does not form a thioester bond with ISG15. Kinetic analysis indicated that the K_m of UBE1L for UbcH7 is 29 fold higher than for UbcH8. Similarly, the K_m of UBE1 (the E1 for ubiquitination) for UbcH8 is 36 fold higher than for UbcH7. These

values indicated that while UBE1L preferentially transfers ISG15 to UbcH8, UBE1 preferentially transfers ubiquitin to UbcH7 (Durfee et al., 2008).

Finally, with the help of an E3 ligase, the activated ISG15 is transferred to a lysine residue of the substrate. E3 ligases play a primary role in determining substrate specificity. Two major groups of E3 ligases exist, the HECT E3s and the RING finger E3s. The HECT E3s accept ubiquitin/UBL from an E2 molecule and form a thioester intermediate between the E3 and ubiquitin/UBL while RING E3s serve as docking proteins, bringing together the E2 molecule and the substrate.

Three cellular ISG15 E3 ligases have been identified so far: HERC5 (HECT domain and RCC-1 like domain containing protein), estrogen-responsive finger protein (EFP or TRIM 25) and human homolog of drosophila ariadne (HHAR1). Herc5 is an HECT E3 ligase (Dastur et al., 2006). HERC5 was fond to be associated to polyribosome and is able to target all newly synthesized proteins. HERC5 is not substrate-specific and targets all newly synthesised proteins and thus may have important antiviral role. Indeed, viral structural proteins are among the most actively synthesised viral proteins and they often assemble into precise repeated geometric confugurations to form infectious virus particles (Durfee et al., 2010). ISGylation of few of these proteins might disrupt the assembly of virus particles. The murine ortholog of HERC5 is HERC6 (Ketscher et al., 2012; Oudshoorn et al., 2012). huHERC5 and muHERC6 are the major E3s since knockdown of these enzymes almost completely abolishes ISGylation.

EFP or TRIM25 is upregulated in response to estrogen and is a RING E3 that was reported to ISGylate 14-3-3σ protein, a negative cell cycle regulator that causes G2 arrest (Urano et al., 2002). Interestingly, it was shown that the enzymatic activity of EFP is negatively regulated by autoISGylation at lysine 117. In contrast to HERC5, EFP appears to be a substrate-specific E3 and can also serve as E3 ligase for ubiquitin as it is implicated in the K63-linked ubiquitination of Lys172 of the CARD domain of RIG-I (Gack et al., 2007).

HHAR1 is another RING E3 that was reported to ISGylate 4EHP, an mRNA 5’ cap structure-binding protein that acts as translation suppressor by competing with eIF4E. ISGylation of 4EHP increases its cap structure-binding activity (Tan et al., 2003).

It should be noted that ISG15 and the enzymes involved in ISGylation (E1, E2 and the three E3s) and deISGylation (USP18) are all induced by type I and type III IFN.

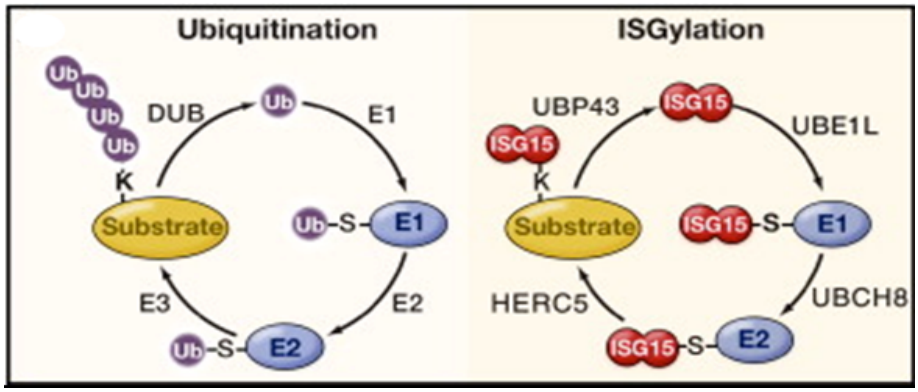


Figure 20: Schematic representation of the ubiquitination and ISGylation cycle (taken from (Skaug and Chen, 2010)

USP18 is the ISG15-deconjugating enzyme responsible for removing ISG15 from its conjugated proteins (deISGylation). In addition to the *bona fide* ISG15-specific protease, USP18 other USP like USP2, USP5 (isoT1), USP13 (IsoT3) and USP14 were shown to serve as deISGylating enzymes (Catic et al., 2007). However, the *in vivo* relevance of this has not yet been determined. It is somehow admitted that USP18 is the principal deISGylating enzyme since overexpression of USP18 decreases the level of ISGylated conjugates and, on the contrary, silencing of USP18 increases ISGylated conjugates (Malakhova et al., 2006). However, it cannot be excluded that the decrease/increase in ISGylated conjugates seen in USP18 overexpressing/silenced cells, is consequent to the decreased/enhanced cellular response to Type I IFN that drives transient induction of ISG15 and all the enzymes involved in ISGylation.

Mass spectrometry analysis revealed that more than 200 proteins are targeted by ISG15 (Giannakopoulos et al., 2005) (Zhao et al., 2005). These proteins are involved in translation, cell cycle regulation, signal transduction, glycolysis, cell motility and immune regulation. Overall it appears that ISG15 could "tag" a variety of host functions, but, unlike ubiquitination, ISGylation does not appear to target proteins for proteasome-mediated degradation (Liu et al., 2003). Till now, it has been difficult to determine the functional consequence of ISGylation, in part because only a small fraction of a given protein in the cell is modified by ISG15. In principle, ISGylation could lead to any of many effects on protein function (a gain of function, loss of function, dominant-negative). On the other hand, modification of a small fraction of proteins is unlikely to have functional consequences, unless ISGylation occurs preferentially on the active protein pool. In some cases studied, ISGylation appears to impair function by disrupting the activity of target proteins. For example Ubc13 is an ubiquitin E2 that, when complexed with another

ubiquitin-conjugating enzyme Mms2, generates atypical Lys63-linked ubiquitin conjugates. Ubc13 may be ISGylated on Lys92, which disrupts its ability to form thioester bond with ubiquitin (Zou et al., 2005). Another example, is the scaffold protein filamin B. The ISGylation of a very small fraction of the total filamin B impairs its ability to support IFN-induced Jun N-terminal kinase (JNK) activity and apoptosis (Jeon et al., 2009).

6.3 ISG15 as an antiviral agent

Although ISG15 null mice do not show any defect in their response to type I IFN, these mice have been reported to be sensitive to certain viruses such as Sindbis virus, influenza A and B virus, HSV-1, murine gammaherpesvirus 68 and chikungunya (Lenschow et al., 2007).

Since both ISG15 and Ubiquitin form an isopeptide bond with a lysine residue of the substrate, a pertinent question is whether ISG15 can somehow compete with ubiquitin for the same target residue. Many viruses, such as HIV or Ebola, use the ubiquitination machinery for efficient budding of the viral particles. In HIV, ISG15 overexpression blocks the ubiquitination of HIV Gag (encodes proteins which make up the viral core) and host Tsg 101, involved in the biogenesis of multivesicular bodies. This block in ubiquitination was found to disrupt the interaction between the two proteins, leading to less stable budding complex (Pincetic et al., 2010).

In Ebola virus, the viral matrix protein VP40 must be monoubiquitinated by the E3 ligase Nedd4 to be efficiently released. It was shown that free ISG15 interacts with Nedd4 and prevents the transfer of ubiquitin from the E2 enzyme to the active site of Nedd4 (Okumura et al., 2008).

The NS1 protein of influenza A virus (NS1A protein) has also been shown to be targeted by IFN-induced ISG15 conjugation in virus-infected cells. ISGylation of a small proportion of NSIA protein on lysine 41 was shown to disrupt the association of the NS1A RNA-binding (RBD) domain with importin-α, the protein that mediates nuclear import of the NS1A protein (Zhao et al., 2010).

Alltogether, these studies provide a first insight into mechanisms by which ISG15 may regulate viral infection.

ISG15 conjugation was also reported to positively regulate IRF3, a transcription factor involved in the induction of IFN α/β genes. ISGylation of IRF3 abolishes its binding to Pin1, a protein that promotes ubiquitination of IRF3 and its degradation. Inhibition of IRF3-

Pin1 interaction by ISG15 was shown to correlate with decreased IRF3 degradation and increased IFN β induction (Naka et al., 1997).

6.4 ISG15 as a cytokine

In addition to being a post-translational protein modifier, ISG15 has been described to act as a secreted cytokine. In its secreted form, ISG15 was shown to:

- modulate activation of monocyte-derived macrophages *in vitro* by promoting the induction of e-cadherin (Padovan et al., 2002)
- induce IFN γ production in T cells (D'Cunha et al., 1996)
- possess neutrophil chemotactic activity (Owhashi et al., 2003).

No known receptors of secreted ISG15 have yet been described.

7 USP18, ISG15 and Hepatitis C virus

Hepatitis C virus (HCV) is the cause of chronic hepatitis C (CHC), a liver disease originally described as non-A non-B hepatitis. CHC can lead to liver cirrhosis and hepatocellular carcinoma. The most effective treatment is pegylated IFN α plus ribavirin which has morbid side effects, variable cure rates and high costs.

It was hypothesized that livers from nonresponders and responders before treatment would show consistent differences in gene expression levels and that these differences could be used to predict treatment outcomes.

18 genes were shown to be upregulated in pretreatment livers of non responders. Most of these genes are ISGs. Among these upregulated ISGs are USP18 and ISG15 (Chen et al., 2005; Sarasin-Filipowicz et al., 2008). Furthermore, pretreatment liver biopsies from nonresponders showed a weak phospho-STAT1 staining in the nucleus, with no increase after 4 hrs of IFN treatment. While, pretreament liver biopsies from responders showed little-to-no phospho-STAT1 staining in the nucleus but strong staining post treatment. Overall, these results showed that a preactivated liver is a bad pronostic marker of the success of IFN a therapy in patients with chronic hepatitis C (Sarasin-Filipowicz et al., 2008).

To ascertain the role of USP18 in the refractoriness of liver cells to IFN treatment, two models were used. The first one was a human hepatocarcinoma cell line, HuH7.5 cells that is capable to reproduce the complete HCV replication cycle. Silencing USP18 in these cells potentiates the antiviral activity of IFN against HCV infection (Randall et al., 2006). The second model was a mouse model. WT and Usp18 null mice were injected with two doses

of mIFN α given 8 hr apart and STAT1 activation were assessed 1 hr after the first and the second injection. In WT mice, the first injection of IFN induced strong activation of STAT1 while the second injection had little effect on STAT1 activation. In contrast, liver cells from Usp18 null mice showed high activation of STAT1 after the first injection and were still responsive after the second injection (Sarasin-Filipowicz et al., 2009). The results obtained in both the human and murine system showed that USP18/Usp18 can induce a long lasting refractoriness to IFN α in the liver.

Since silencing USP18 leads to an increase in protein ISGylation and more importantly potentiates the antiviral effect of exogenous IFN α against HCV infection, it was hypothesized that ISG15 and/or ISGylation is "antiviral". However, the story appears far more complicated. Indeed, silencing ISG15 in HuH7 cells carrying an HCV replicon increases the antiviral effect of IFN α. Moreover, compared to cells transfected with the control siRNA, silencing ISG15 increases expression of ISGs (mRNA and protein) following a 3 days stimulation with IFN α. The authors thus suggested a novel function of ISG15 in modulating the IFN α activity against HCV (Chua et al., 2009).

Objectives

The role of USP18 in the differential desentization of human cells to type I IFNs

It has been known from 1986 that prolonged treatment of cells to IFN α renders them refractory or desensitized to further IFN treatment. This period of desensitization is quite long lasting as cells needed to be 72 hrs in the absence of IFN to recover full responsiveness. This long lasting desensitization was observed in human fibroblasts (Larner et al., 1986).

Twenty years later, the group of larner showed that human primary fibroblasts treated for 16 hours with IFN β (250 pM) followed by a 6 hr recovery period become desensitized to further IFN β (250 pM) treatment (Sakamoto et al., 2004). Poor Stat1 phosphorylation upon IFN β or IFN γ treatment could be observed in desensitized cells. TC-PTP (T-cell protein-tyrosine phosphatase), a phosphatase previously described to mediate Stat1 dephosphorylation, was implicated in the cellular desensitization. Indeed, TC-PTP -/- MEFs do not show any refractoriness to IFN β after a prior exposure to IFN as compared to naïve wt or TC-PTP-/- MEFs. However, the levels of IFNAR1 and the phosphorylation of the Jaks were not monitored in desensitized cells.

More recently, the group of Coccia in collaboration with Dr Sandra Pellegrini analysed the responsiveness of human dendritic cells (DC) to bacterial LPS, a known DC maturation factor and inducer of type I IFN (Severa et al., 2006). The authors showed that while immature DC are equally sensitive to IFN α2 and IFN β, LPS- or IFN β-matured and washed (to remove the autocrine IFN β) DC were fully sensitive to IFN β but were refractory to IFN α2. This differential desensitization to type I IFN was ascribed to a decrease in the level of IFNAR1. This was the first report of an α2/β differential desensitization.

My thesis work was based on these observations. In collaboration with Dr G.Uzé, we showed that in a model fibroblastic cell system (HLLR1-1.4 cells), the prolonged exposure to type I IFN or type III IFN interferes with their ability to respond to a second stimulation with type I IFN. We found that this desensitization state is targeted to IFN α and IFN ω subtypes, while the responses to IFN β and IFN λ1 are left nearly intact. However differential desensitization observed in fibroblasts appears independent of surface receptor downregulation. Moreover, Jak1 and Tyk2 phosphorylation is also impaired in primed,

desensitized cells suggesting that Tc-PTP is not responsible of differential desensitization. Based on these premises, we wanted to monitor the mechanism underlying differential desensitization. We got particularly interested in an IFN-induced negative regulator of type I IFN, USP18 which has been reported to block IFN signaling at an early step i.e at the level of Tyk2 and Jak1 phosphorylation.

This work is described in part I of results, in an article that is attached hereafter (Francois-Newton *et al.* PLoS One 2011; USP18-based negative feedback control is induced by type I and type III interferons and specifically inactivates interferon α response).

In part II I will try to analyse how two IFN subtypes, IFN α2 and IFN β, which initially induces the same level of Jak/Stat activation, shows different level of ISG induction and antiproliferative activity after 16 and 72 hrs respectively of continuous IFN stimulation. We found that USP18 differentially desensitize cells to Type I IFN. We therefore monitored whether USP18 was implicated in the IFN α2/β differential observed for activities requiring prolonged treatment with IFN.

This work is described in an article submitted in Biochemical Journal; USP18 establishes the transcriptional and anti-proliferative interferon α/β differential.

In Part III, a section "Additional experiments" is presented where I have addressed the mode of action of USP18 and in particular the contribution of its enzymatic activity towards differential desensitization. Two approaches were used;

1. Forced expression of catalytically inactive forms of USP18 in HLLR1-1.4 cells and monitoring their response to IFN α2 and IFN β.
2. Silencing enzymes involved in the ISGylation machinery and monitoring the response of naïve and primed cells to IFN α2 and IFN β.

These experiments will be discussed in the section "Discussion" and a "General discussion" will follow the result section

Results

Part I: USP18-Based Negative Feedback Control Is Induced by Type I and Type III interferons and Specifically Inactivates Interferon alpha response

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USP18-Based Negative Feedback Control Is Induced by Type I and Type III Interferons and Specifically Inactivates Interferon α Response

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Abstract

Type I interferons (IFN) are cytokines that are rapidly secreted upon microbial infections and regulate all aspects of the immune response. In humans 15 type I IFN subtypes exist, of which IFN α2 and IFN β are used in the clinic for treatment of different pathologies. IFN α2 and IFN β are non redundant in their expression and in their potency to exert specific bioactivities. The more recently identified type III IFNs (3 IFN λ or IL-28/IL-29) bind an unrelated cell-type restricted receptor. Downstream of these two receptor complexes is a shared Jak/Stat pathway. Several mechanisms that contribute to the shut down of the IFN-induced signaling have been described at the molecular level. In particular, it has long been known that type I IFN induces the establishment of a desensitized state. In this work we asked how the IFN-induced desensitization integrates into the network built by the multiple type I IFN subtypes and type III IFNs. We show that priming of cells with either type I IFN or type III IFN interferes with the cell's ability to further respond to all IFN α subtypes. Importantly, primed cells are differentially desensitized in that they retain sensitivity to IFN β. We show that USP18 is necessary and sufficient to induce differential desensitization, by impairing the formation of functional binding sites for IFN α2. Our data highlight a new type of differential between IFNs α and IFN β and underline a cross-talk between type I and type III IFN. This cross-talk could shed light on the reported genetic variation in the IFN λ loci, which has been associated with persistence of hepatitis C virus and patient's response to IFN α2 therapy.

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Introduction

Type I and type III (IL-28/29) IFNs form two multigenic families of pathogen-induced cytokines that exhibit common bioactivities through binding to unrelated cell surface receptors [1]. The numerous type I IFN subtypes (α/β/ω) bind to a receptor made of the ubiquitously expressed IFNAR1 and IFNAR2 chains. Conversely, the type III IFNs (λ1, 2, 3) bind to a receptor made of the broadly expressed IL-10R2 and of IFNLR1 (IL-28Rα) whose expression is cell type specific. Therefore, the response to type III IFNs is tissue specific and appears to be mainly restricted to epithelial cells [2].

Downstream of these two receptor complexes is a shared Jak/Stat pathway, involving the Janus kinases Jak1 and Tyk2 that phosphorylate Stat1, Stat2 and Stat3. Activated Stat1/2 associate to IRF9 to yield the ISGF3 complex that induces transcription of IFN-stimulated genes (ISG) [1]. Thus, in humans, the 18 subtypes (13 α, 1 β, 1 ω and 3 λ) of the type I and type III IFN systems induce a same gene subset and exhibit antiviral and antiproliferative activities through two independent cell surface receptors. In addition, the type I IFNs are recognized as mediators linking innate and adaptive immunity *via* their effect on the differentiation and maturation of dendritic cells and T cells, activities not shared with type III IFNs [3].

Among the type I IFNs, the α/ω subtypes on the one hand and the β subtype on the other are not equivalent, as they are differentially produced upon microbial infections and exhibit distinct bioactivities. The biological potency of any given subtype is determined by both receptor binding parameters and receptor density on target cells [4,5]. Hence, compared to IFN α2, IFN β binds the receptor with higher affinity, forms a longer-lived complex and is more potent at inducing translational control signals, inhibiting cell growth and osteoclastogenesis [6,7,8] (Moraga *et al.*, submitted). Importantly, IFN α2 is routinely used

in the clinic as in chronic HCV infection and several forms of cancer, whereas IFN β is approved for treatment of multiple sclerosis, considered an autoimmune disease [1].

The pleiotropic activities of IFNs must be tightly down regulated in time and space and several mechanisms have been shown to co-exist in order to attenuate IFN-initiated Jak/Stat signaling (reviewed in [9]). In an *in vivo* model, Sarasin *et al* showed that liver cells from mice repeatedly injected with murine IFN α become refractory to further IFN α stimulation [10]. The ISG-encoded isopeptidase USP18/Ubp43 was found to be essential for the establishment of the desensitized state [10,11]. USP18 can remove the ubiquitin-like ISG15 from target proteins [12] and was found to inhibit IFN-induced Jak/Stat signaling when constitutively expressed in cultured cells [13]. Interestingly, USP18 expression was recently identified as a bad prognostic marker of the success of IFN α therapy in patients with chronic hepatitis C [14,15].

Here, we have studied how IFN induced desensitization integrates into the network built by the multiple type I and type III IFNs. We found that both type I and type III IFNs can induce a long lasting desensitization state in cells of different lineages, including human primary hepatocytes. Remarkably, the refractory state is targeted to the IFN α and ω subtypes, leaving nearly intact the cells' responsiveness to β and λ IFNs. We show that USP18 is necessary and sufficient to differentially desensitize cells by disturbing the assembly of α IFNs with the receptor complex.

Altogether, our findings emphasize the existence of differential activities within the type I IFN family and underline a novel type I/III IFN cross-talk acting at the receptor level that could have important consequences in the set up of clinical protocols, especially for the treatment of HCV-infected patients who are resistant to conventional pegylated IFN α2 therapy.

Results

Type I and type III IFNs induce desensitization to IFN α

In a first set of experiments, we established to what extent cells that had responded to IFN α2, IFN β or IFN λ1 could mount a response to a second stimulation. For this, we used HLLR1-1.4 cells, a clone derived from human fibrosarcoma HT-1080 cells stably expressing the IFNLR1 receptor chain and the luciferase reporter gene controlled by an ISGF3-dependent promoter [16]. Thus, HLLR1-1.4 cells are responsive to type I IFNs as well as to type III IFNs (Fig. 1B).

HLLR1-1.4 cells were left untreated (naïve) or stimulated (primed) with IFN α2, IFN β or IFN λ1 for 24 hr, thoroughly washed and kept in fresh medium for another 24 hr (scheme in Fig. 1A). Following this resting period, the levels of Jak/Stat phosphorylation, luciferase activity and *2′–5′ oligo-adenylate-synthetase (OAS 69K)* mRNA in primed cells had nearly returned to basal levels. Naïve and primed HLLR1-1.4 cells were challenged with IFN α2, IFN β or IFN λ1 for 6 hr (Fig. 1A) and luciferase activity was quantified (Fig. 1B). The potency of IFN α2 in luciferase induction (expressed as EC_{50}) decreased 14.5–68.9 fold in primed cells as compared to naïve cells, whereas the potency of IFN β decreased only 2.1–3.2 fold (Fig. 1B). A similar trend was observed when naïve and primed cells were monitored by RT-qPCR for induction of *OAS 69K* mRNA in response to 10 pM of each IFN (Fig. 1C). As shown in Fig. 1D, upon desensitization the dose response relationship for IFN α2 had shifted down by a factor of 50-100. Interestingly, the activity of all the α/ω subtypes assayed was decreased in type I and in type III IFN-primed.

Desensitization was also evident at the level of early signaling events. Fig. 2A shows that cells primed with IFN β or with IFN λ1 were refractory to low doses (10 and 100 pM) of IFN α2 in terms of tyrosine phosphorylation of Stat1, Stat2 and Stat3 (compare lanes 2–3 with lanes 9–10 or 16–17). Conversely, in primed cells Stat1, Stat2 and Stat3 were still phosphorylated upon treatment with IFN β and IFN λ1. Moreover, in primed cells the activation of Jak1 and Tyk2, the earliest effectors of the pathway, was abrogated in the case of IFN α2, but still detectable in the case of IFN β and IFN λ1 (Fig. 2B).

To better characterize the differential desensitization state, we asked if primed cells resumed their response to IFNα2 when stimulated for longer than 30 min. Naïve and primed cells were thus stimulated with IFN α2 and IFN β for up to 8 hr. As shown in Fig. 2C, the desensitization of primed cells to IFN α2 persisted, independently of treatment duration. To define for how long primed cells remained in a refractory state, we extended the interval between priming and restimulation (washing of 24, 48 and 72 hr). As shown in Fig. 2D, primed cells had regained IFN α2 sensitivity after 72 hr in the absence of cytokine. In conclusion, the differential desensitized state of the cell persists even at 8 hr of stimulation, but is reversible as seen when cells are kept in the absence of cytokine for 3 days.

Priming with type I IFN induced a similar α2/β differential desensitized state in cell lines, such as bronchial epithelial BEAS-2B and uroepithelial Hs 789.T cells, and in foreskin fibroblasts and T cell blasts (Fig. 3A and 3B). Human primary hepatocytes respond to type I IFNs and more weakly to IFN λ1 (Fig. 3C, compare level of P-Stat2, lanes 1–8). After 24 hr of priming with either IFN α2 or IFN λ1, hepatocytes expressed higher Stat2 protein and detectable levels of USP18, both proteins being encoded by ISG (Fig. 3C, lanes 9–24). Importantly, primed hepatocytes were desensitized to IFN α2 and only marginally to IFN β (Fig. 3C, compare P-Stat2 in lanes 3, 11 and 19) and the extent of desensitization was related to the level of sensitivity to the priming cytokine. Of note, the basal phosphorylation level of Stat3 was reduced in IFN λ1-primed cells with respect to naïve or IFN α2-primed cells (compare P-Stat3 in lanes 1, 9 and 17). Overall, these results demonstrate a previously unrecognized inhibitory cross-talk between the type I and type III IFN systems.

Desensitized cells are impaired in their ability to bind IFN α2

As shown above, cell desensitization to IFN α2 is manifest at the level of Janus kinase activation and thus may result from reduced surface level of the receptor chains or impaired binding of IFN α2. FACS analysis clearly showed that naïve and primed (*i.e.* desensitized) cells expressed equivalent levels of IFNAR1 and IFNAR2 (Fig. 4A). Therefore, we tested whether the ligand binding property of the receptor differed between naïve and primed cells. For this, we iodinated IFN α2 and, in place of IFN β which cannot be iodinated without loss of bioactivity, we made use of an engineered mutant of IFN α2 (IFN α2-HEQ) whose affinity for IFNAR1 is similar to that of IFN β and which recapitulates IFN β unique biological activities [6]. Accordingly, in primed HLLR1-1.4 cells, IFN α2-HEQ was as potent as IFN β in inducing Stat phosphorylation (Fig. 4B). On this basis, we compared the binding of ^{125}I-IFN α2 and ^{125}I-IFN α2-HEQ to naïve and to primed HLLR1-1.4 cells. Fig. 4C shows that the binding of ^{125}I-IFN α2 was reduced in both IFN β-primed cells and IFN λ1-primed cells with respect to naïve cells. The reduction was most apparent for low ^{125}I-IFN α2 concentrations, matching the decrease in specific biological activity (Fig. 1D). In contrast, the binding of ^{125}I-IFN α2-HEQ was only marginally reduced in primed cells with respect to naïve cells (Fig. 4D). In conclusion, despite unaltered levels of IFNAR1 and IFNAR2, cells primed

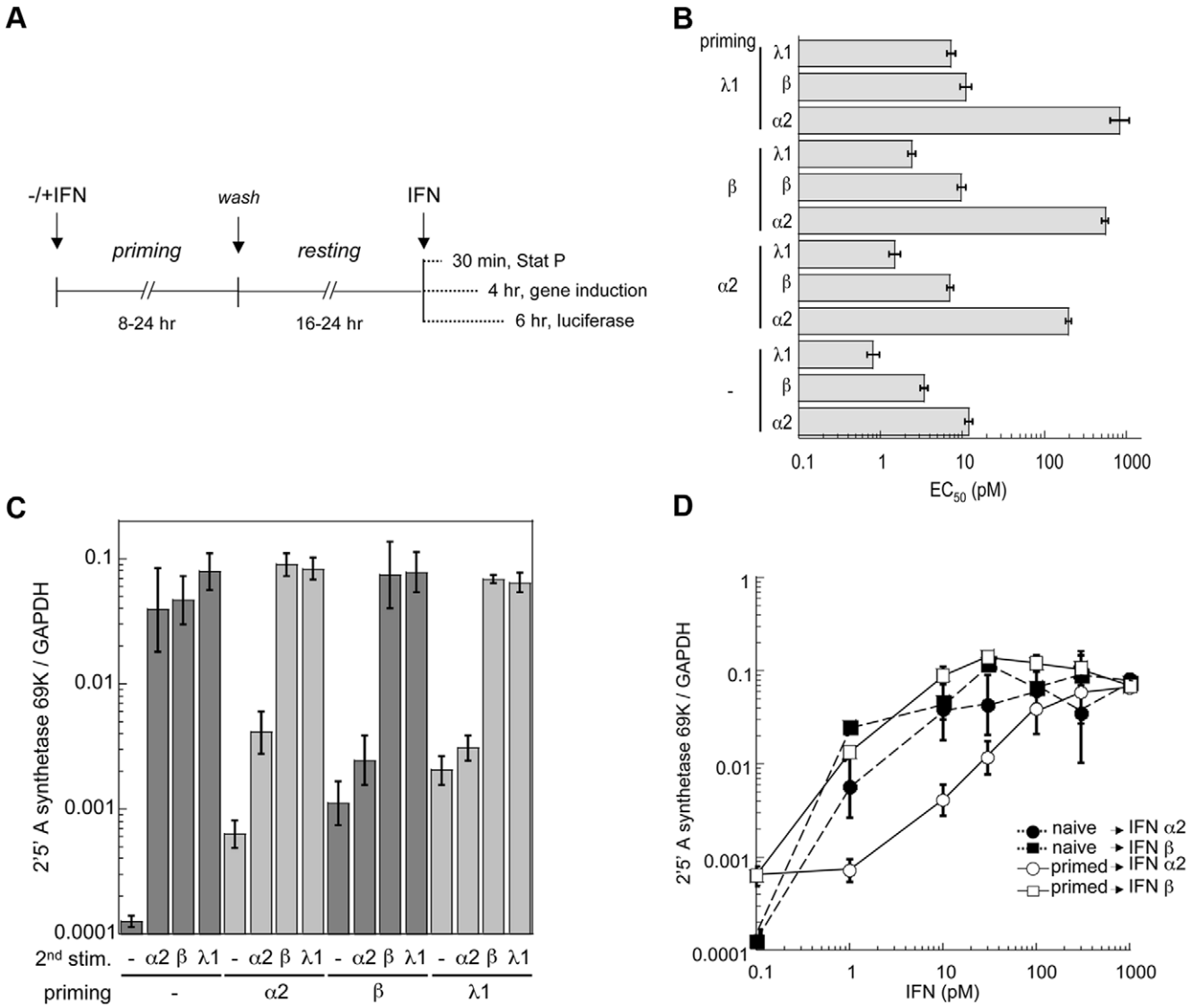

Figure 1. Differential desensitization studies in HLLR1-1.4 cells. (A) Protocol used to measure desensitization. Unless otherwise indicated, cells were primed with IFN α2 or IFN β (500 pM) or IFN λ1 (50 pM). The priming phase varied between 8 and 24 hr and the resting phase between 16 and 24 hr. Cells were then challenged with IFN for different times depending of the read out. (B) Graphic representation of the EC_{50} (pM) as determined by the luciferase activity induced by IFN α2, IFN β or IFN λ1 in naïve or primed cells. EC_{50} were calculated from the non-linear regression fits of the luciferase activity induced by IFN in a concentration range covering 2.4 log. Priming and resting times lasted 24 hr each. Bars represent the 95% confidence limits. (C) Level of *OAS-69K* mRNA induced by IFN α2 (10 pM), IFN β (10 pM) or IFN λ1 (50 pM) in naïve and primed cells as determined by RT-qPCR. Data are expressed as ratios to GAPDH levels. Priming and resting times lasted 24 hr each. Bars represent the 95% confidence limits (Student's t-test). (D) Dose response induction profile of *OAS-69K* mRNA in naïve (closed symbols) and IFN α2 primed cells (open symbols) stimulated for 4 hr with different doses of IFN α2 (circles) or IFN β (squares) as determined by RT-qPCR. Priming and resting times lasted 24 hr each. Data are expressed as ratios to GAPDH levels. Bars represent the 95% confidence limits (Student's t-test).
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with either type I or type III IFNs (*i.e.* desensitized) are unable to assemble a functional IFN α2 receptor complex.

Expression of USP18 is necessary and sufficient to cause differential desensitization

USP18 has been shown to downmodulate type I IFN activity through binding to IFNAR2 [13], and we therefore tested its role in differential desensitization. In HLLR1-1.4 cells *USP18* mRNA was induced by α2, β and λ1 IFNs, but not by other cytokines such as IFN γ or IL-6 (Fig. 5C). Accordingly, cell priming with IFN γ or IL-6 did not induce USP18 nor lead to a desensitized state (Fig. 5A and 5B). USP18 protein accumulated with similar kinetics in cells treated with IFN β or IFN λ1, reaching maximum level between 8 and 16 hr of stimulation (Fig. 5D). As found in primary hepatocytes (Fig. 3C), two USP18-specific bands of comparable intensity were consistently detected.

To study the involvement of USP18, its expression was silenced in HLLR1-1.4 cells (Fig. 5E). Remarkably, cells wherein USP18 was efficiently silenced were not desensitized to IFN α2. Of note, the level of Stat1/2 phosphorylation was higher in USP18-silenced/primed cells (lanes 14, 15 and 17, 18) than in USP18-silenced/naive cells (lanes 11, 12) and most likely this is consequence of the higher content of these Stats in primed cells. To determine if USP18 was sufficient to establish differential desensitization, HLLR1-1.4 cells were stably transfected with a

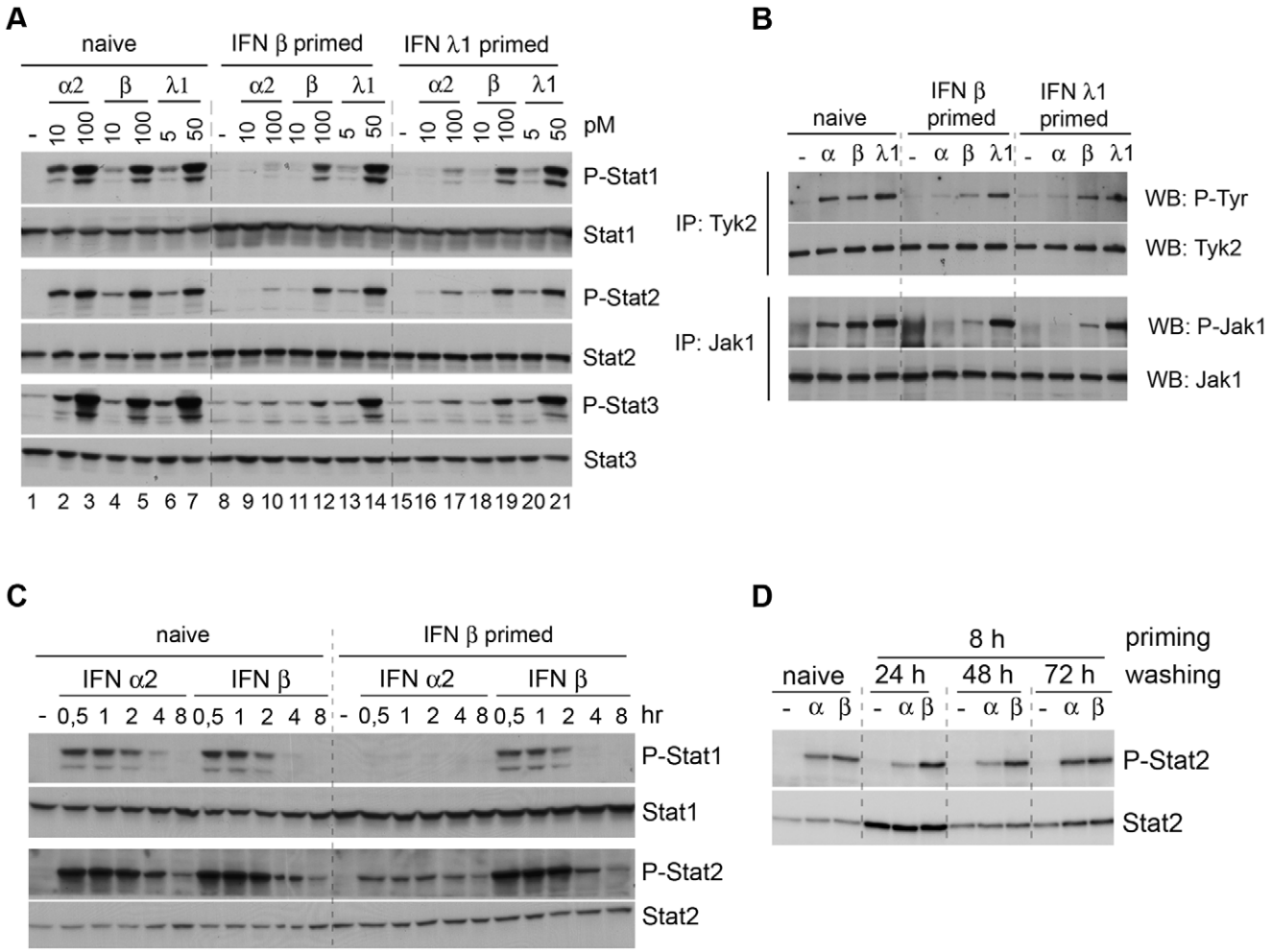

Figure 2. Differential desensitization studies in HLLR1-1.4 cells. (A) Level of phosphorylation of Stat1, Stat2 and Stat3 induced at 30 min by the indicated doses of IFN in naïve cells and in primed cells. Priming was for 8 hr and resting was for 16 hr. Cell lysates (30 μg) were fractionated on a 7% SDS polyacrylamide gel and immunoblotted with the indicated Abs. (B) Level of tyrosine phosphorylation of immunoprecipitated Tyk2 and Jak1 induced at 30 min by IFN α2 (100 pM), IFN β (100 pM) or IFN λ1 (50 pM) in naïve and primed cells. Priming was for 8 hr and resting was for 16 hr. Lysates (2 mg) were immunoprecipitated with Tyk2 Abs (top) or Jak1 Abs (bottom). The top membrane was incubated with phospho-tyrosine 4G10 mAb (P-Tyr) and the bottom membrane with phospho-Jak1 Abs. Protein content was assessed by re-blotting with Tyk2 or Jak1 specific Abs. (C) Kinetics of Stat1, Stat2 and Stat3 phosphorylation in naïve and primed cells. Cells were stimulated with 50 pM of IFN α2 or IFN β, as indicated. Priming was for 8 hr and resting was for 16 hr. Lysates (30 μg) were fractionated on a 7% SDS polyacrylamide gel and immunoblotted with the indicated Abs. (D) Level of phosphorylation of Stat2 induced at 30 min by 100 pM of IFN in naïve cells and in primed cells. Priming was for 8 hr and the resting phase varied from 24 hr to 72 hr. Cell lysates (30 μg) were fractionated on a 7% SDS polyacrylamide gel and immunoblotted with the indicated Abs.
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USP18 expression vector or an empty vector. In the 10 positive clones analysed, the level of ectopic USP18 was higher (4 to 50 fold) than endogenous USP18 present in 8 hr-primed cells. Clone HU13 was chosen as it expressed the least USP18 (Fig. 6A). We checked the integrity of the deISGylase activity of USP18 expressed in clone HU13 by comparing the steady state level of ISGylated conjugates in IFN β-treated parental and HU13 cells (data not shown). The response to IFN α2, IFN β and IFN λ1 was measured. Compared to naïve HLLR1-1.4 cells, clone HU13 was severely impaired in its phosphorylation response to IFN α2. Conversely, responses to IFN β and IFN λ1 were preserved or slightly reduced (Fig. 6A–C). By directly comparing luciferase induction in a control clone (HP1) and in HU13 clone, it appeared that constitutive USP18 in HU13 shifted the dose response relationship so that higher concentration of IFN α2 was required to trigger a response of the same magnitude (Fig. 6D). All IFN α subtypes tested and IFN ω were found to be between 25 to 85-fold less potent on HU13 cells than on control HP1 cells, whereas the activities of IFN β and IFN λ1 were marginally reduced (Fig. 6E). The specific activity of IFN α2-HEQ, engineered for higher binding towards IFNAR1 (see above), was reduced only by a factor of 10 on HU13 cells (Fig. 6E), in accordance with the level of activity of this mutant on cells desensitized by IFN priming (Fig. 4B). On the other hand, the specific activity of IFN α2-α8tail, an IFN α2 mutant engineered for higher IFNAR2 binding [7], was decreased by a factor of 45 on HU13 cells as compared to HP1 cells (Fig. 6E). These results indicate that the affinity of a given IFN α subtype towards IFNAR1 determines the degree of USP18-dependent desensitization to that subtype.

As shown for desensitization caused by IFN priming (Fig. 4A), desensitization caused by expression of USP18 was not dependent on a change of IFNAR1 or IFNAR2 cell surface expression (Fig. 7A). In studies analogous to those performed on IFN-primed cells, we measured binding of radiolabeled ligands on HU13 and

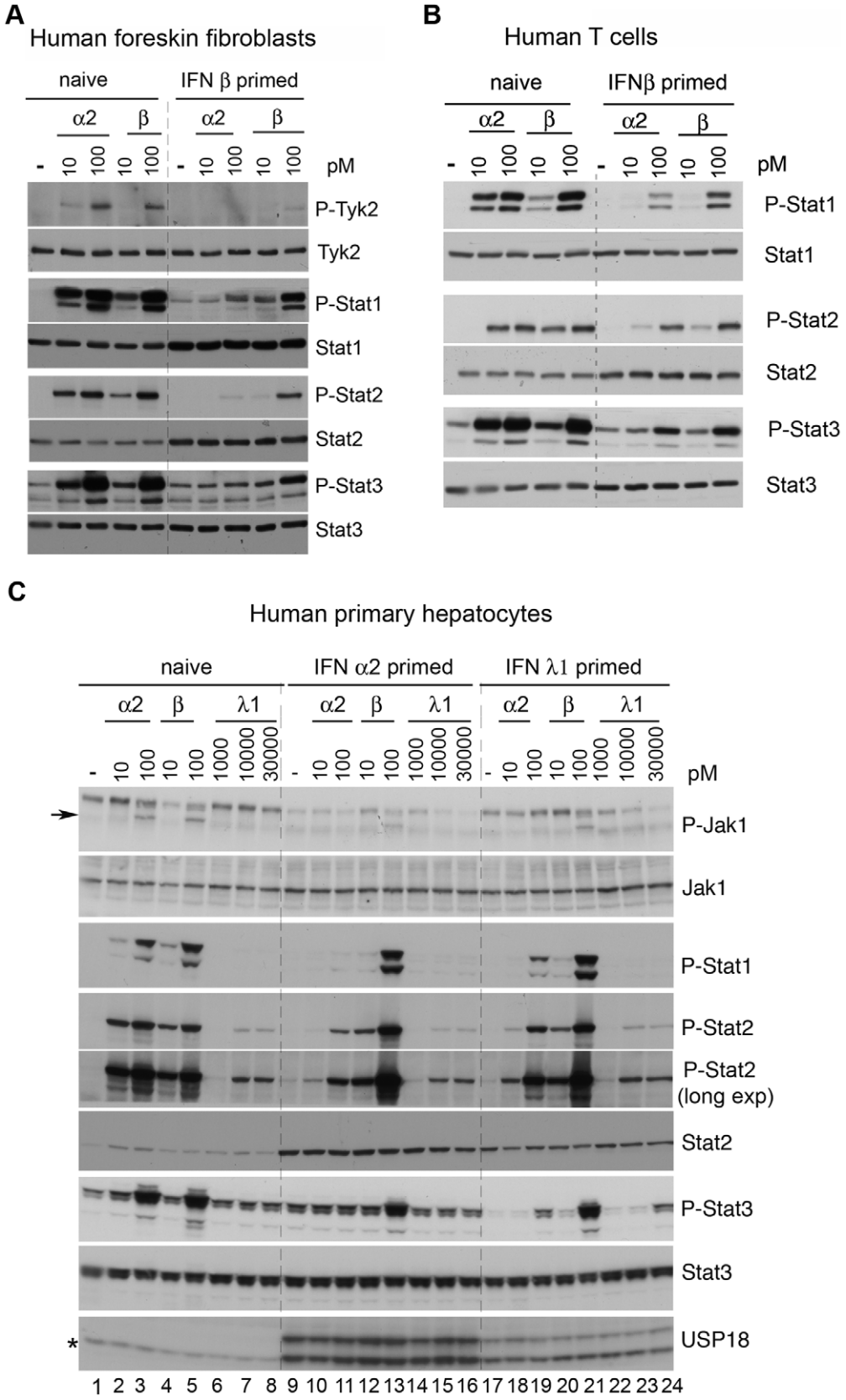

Figure 3. Differential desensitization of human primary cells. (A) Human foreskin fibroblasts and (B) human T cells were either left untreated (naïve) or primed for 8 hr. Cells were washed, maintained in medium without IFN for 16 hr and stimulated for 30 min with 10 and 100 pM of the indicated IFN. Cell lysates (30 μg) were analysed with the indicated Abs. (C) Human primary hepatocytes were left untreated (naïve) or primed with 500 pM of IFN α2 or 30 nM of IFN λ1 for 24 hr. Cells were washed, maintained in medium without IFN for 24 hr and stimulated for 30 min with the

indicated IFN doses. Cell lysates (50 μg) were analysed with the indicated Abs to evaluate tyrosine phosphorylation and content of Jak1 and Stats. The arrow points to the band corresponding to phosphorylated Jak1. The level of USP18 (bottom panel) was assessed in a 10% SDS PAGE. Of the two USP18 bands (apparent MW of 38 and 35 kDa), the faster migrating one results from proteolytic processing [46]. This latter comigrates with a non specific cross-reacting band detected in naïve cells and indicated by the asterisk (bottom panel).
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naïve HLLR1-1.4 cells. Binding of ^{125}I-IFN α2 on HU13 cells was clearly reduced (Fig. 7B) and this to the same extent as for IFN-primed HLLR1-1.4 cells (see Fig. 4C). As expected, the binding of ^{125}I-IFN α2-HEQ was similar for the two clones (Fig. 7C). In conclusion, these data demonstrate that the sole expression of USP18 recapitulates the binding alteration observed in IFN-primed (*i.e.* desensitized) cells.

Discussion

The major findings of our study are hereafter summarized: i) type I IFN and type III IFN desensitize cells to several α IFNs but only marginally to IFN β; ii) cells of different lineages - including primary hepatocytes - undergo differential desensitization; iii) the extent of desensitization is controlled by the level of an ISG, USP18; vi) forced expression of USP18 in naive cells blunts IFN α response at the level of its assembly to the receptor complex.

USP18 is a cysteine protease specialized in the removal of ISG15 from ISGylated proteins. However, the phenotypic alterations caused by USP18 deletion in the mouse have been dissociated from ISG15-dependent mechanisms [17,18,19]. One group has proposed that USP18 attenuates IFN α signaling regardless of the isopeptidase activity of the protein by competitively displacing Jak1 from its interaction with IFNAR2 [13]. We

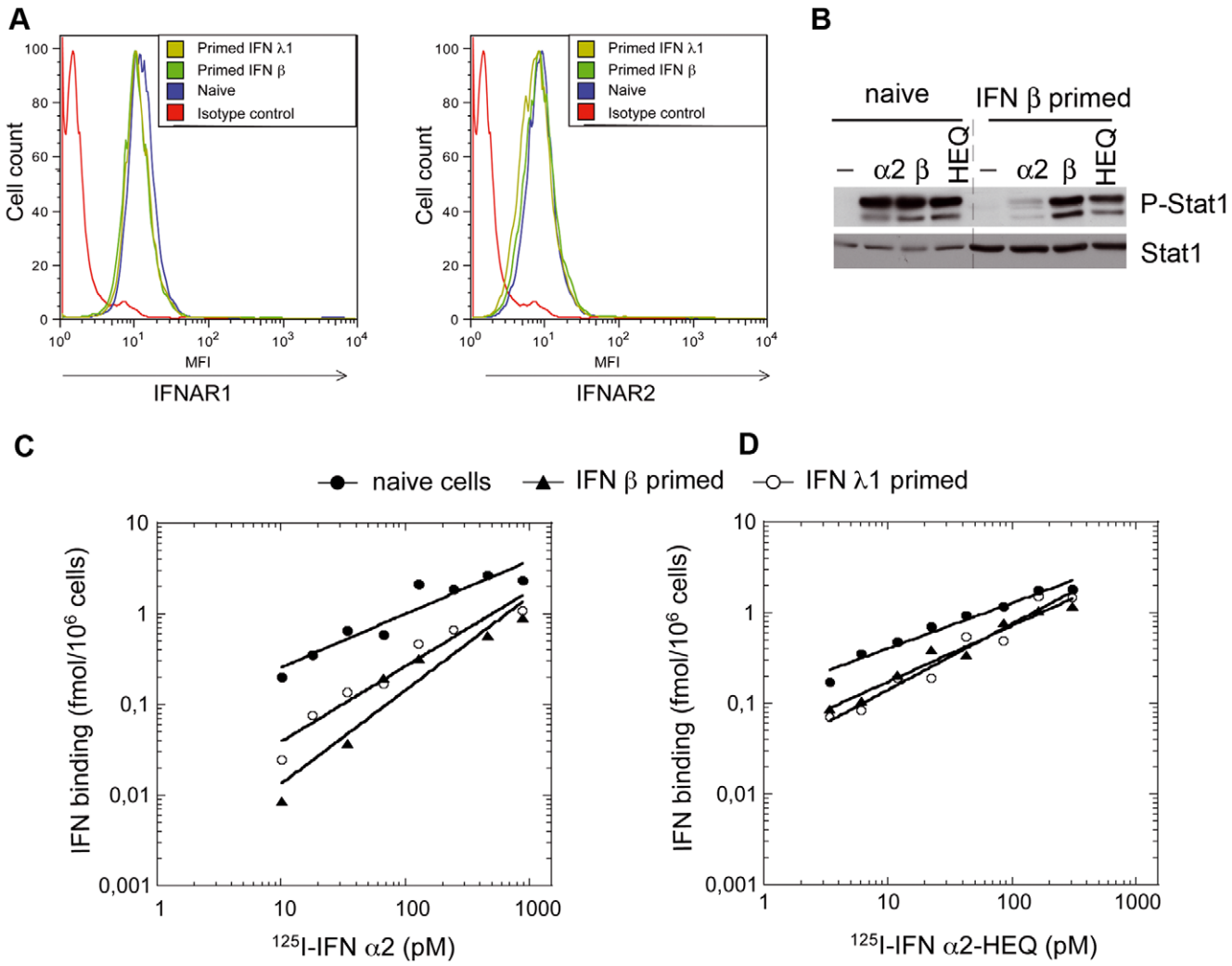

Figure 4. Analysis of the type I IFN receptor in naïve and primed HLLR1-1.4 cells. (A) Surface level of IFNAR1 and IFNAR2 in naïve cells and in IFN β or IFN λ1-primed cells as determined by FACS. Cells were primed for 24 hr, washed and maintained in medium without IFN for 24 hr. Cells were then stained with AA3 mAb (IFNAR1) or CD118 mAb (IFNAR2) followed by biotinylated rat anti-mouse Ab and streptavidin-PE. (B) Level of phosphorylation of Stat1 in naïve and primed cells stimulated for 30 min with 100 pM of IFN α2, IFN β or IFN α2-HEQ. Lysates (30 μg) were immunoblotted with the indicated antibodies. Priming was for 8 hr followed by 16 hr resting in medium without IFN. (C and D) Binding of ^{125}I labelled IFN α2 (C) or IFN α2-HEQ (D) at 37°C for 1 hr to naïve (closed circles), IFN β-primed cells (triangles) or IFN λ1-primed cells (open circles). Cells were primed for 8 hr and maintained without IFN for 16 hr.
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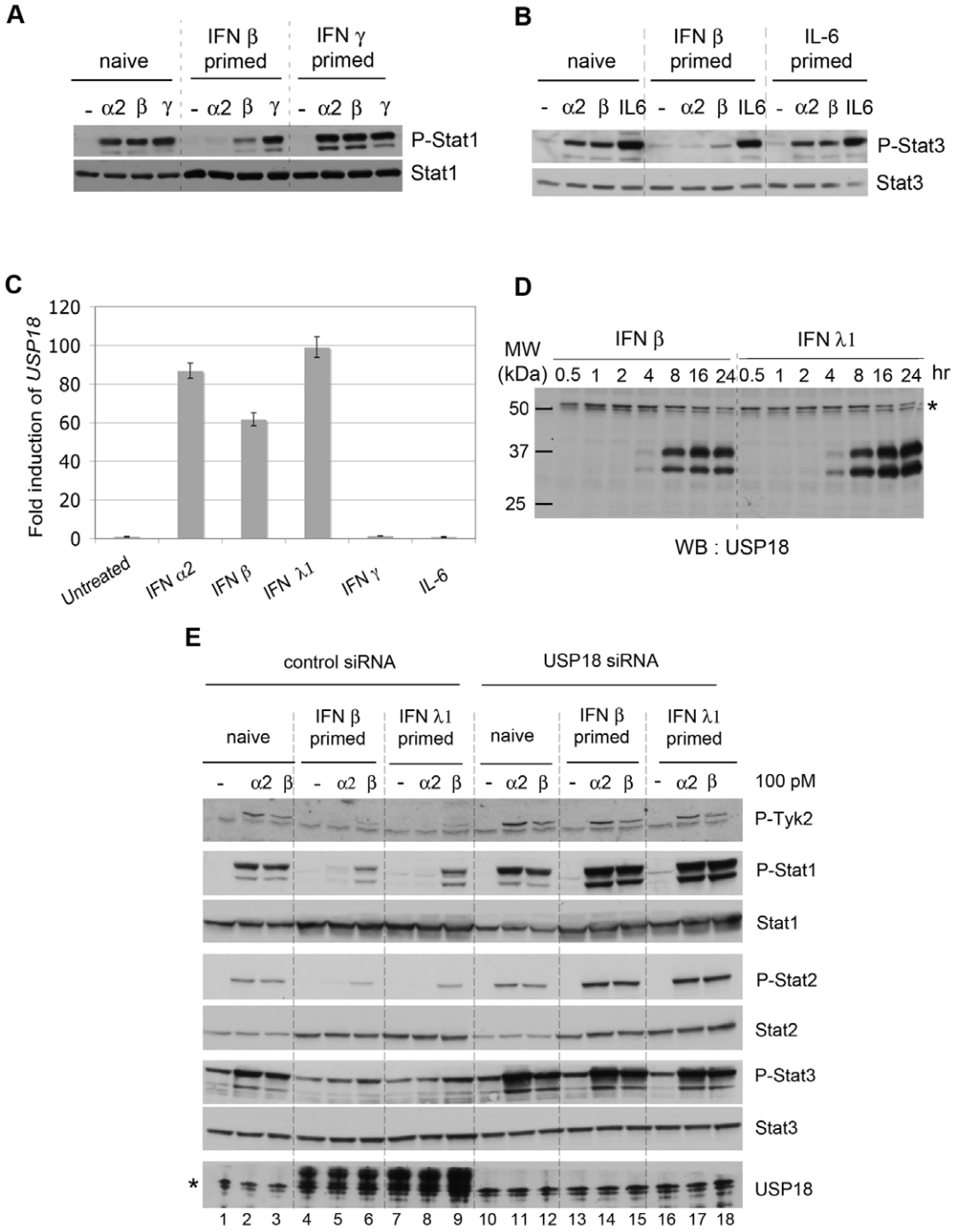

Figure 5. USP18 is necessary for differential desensitization. (A) Stat1 phosphorylation induced in HLLR1-1.4 cells stimulated for 30 min with IFN α2 (100 pM), IFN β (100 pM) or IFN γ (1 ng/ml) in naïve cells and in cells primed with either IFN β (500 pM) or IFN γ (10 ng/ml). Cells were primed for 8 hr and maintained without IFN for 16 hr. (B) Stat3 phosphorylation induced in HLLR1-1.4 stimulated for 30 min with with IFN α2 (100 pM), IFN β (100 pM) or hIL-6 (10 ng/ml) in naïve cells and in cells primed with IFN β (500 pM) or hIL-6 (100 ng/ml). Cells were primed for 8 hr and maintained without IFN for 16 hr. Lysates (30 μg) were immunoblotted with the indicated Abs. (C) Level of *USP18* mRNA in HLLR1-1.4 cells stimulated for 6 hr with IFN α2, IFN β (500 pM), IFN λ1 (50 pM), IFN γ (1 ng/ml) or hIL-6 (100 ng/ml) as determined by qRT-PCR. Each sample was run in triplicate. Transcripts were normalized to the level of 18S transcripts. The ratios between treated and untreated samples in each subset are shown, taking as 1 the ratio in untreated samples. (D) Kinetic profile of USP18 induction in HLLR1-1.4 cells stimulated with 100 pM of IFN β or IFN λ1 for the indicated times. Cell lysates (30 μg) were immunoblotted with the indicated Abs. The asterisk points to a nonspecific band. (E) USP18 is necessary for differential desensitization. HLLR1-1.4 cells were transfected with a control pool of siRNA (Control siRNA) or a pool of four USP18 targeting siRNA (USP18 siRNA). Twenty four hr after transfection, cells were either left untreated (naïve) or primed for 8 hr with the indicated IFN. After 16 hr of resting, cells were stimulated for 30 min with 100 pM of IFN α2 or IFN β. Cell lysates (30 μg) were analysed with the indicated antibodies. The asterisk in the bottom panel points to a band cross-reacting with anti-USP18 Abs (see also USP18 blot in Fig. 3C). Individual USP18 targeting siRNA were also used with similar results (data not shown).
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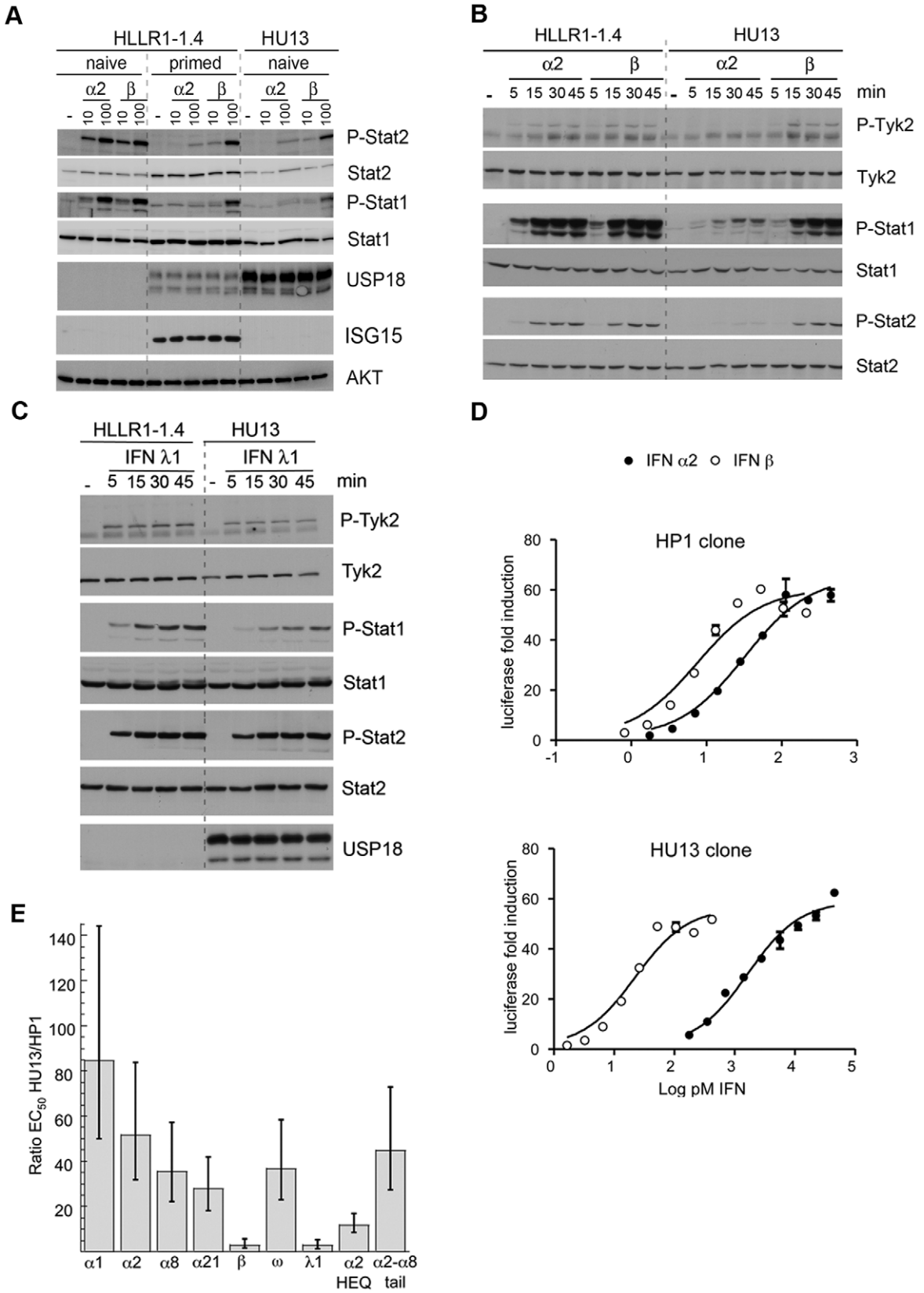

Figure 6. USP18 is sufficient to induce differential desensitization. (A) Level of Stat2 and Stat1 phosphorylation induced by 30 min stimulation with IFN α2 or IFN β in naïve and primed HLLR1-1.4 cells and in clone HU13 stably expressing USP18. Level of USP18 in naïve and primed HLLR1-1.4 cells (endogenous USP18) and in HU13 cells (ectopic USP18). Level of ISG15, a typical ISG, in naïve and primed HLLR1-1.4 and in HU13 cells. Loading was evaluated by measuring AKT. Lysates (30 μg) were immunoblotted with the indicated Abs. (B) Kinetics of Tyk2, Stat1 and Stat2

phosphorylation in the USP18-expressing clone HU13 and in the parental HLLR1-1.4 cells. Cells were stimulated as indicated with 100 pM of IFN α2 or IFN β. Lysates (30 μg) were immunoblotted with the indicated Abs. (C) Kinetics of Tyk2 and Stat1/2 phosphorylation in parental HLLR1-1.4 cells and USP18-expressing HU13 cells. Cells were stimulated as indicated with 30 pM of IFN λ1. (D) Luciferase activity induced by IFN α2 (closed circles) or IFN β (open circles) in HP1 control clone and in HU13 clone constitutively expressing USP18. (E) Ratio of the EC_{50} values determined for luciferase activity on the control clone HP1 and clone HU13. Cells were stimulated with the indicated IFN subtypes for 6 hr. Bars represent support limits of the ratio from 95% confidence intervals of the individual EC_{50}.
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have obtained preliminary evidence that, indeed, a catalytically inactive USP18 impairs IFN signaling when highly and stably expressed in naïve cells. In this context, however, desensitization is severe and not differential, as cells become refractory to IFN α and β subtypes. At present, we cannot exclude the possibility that the isopeptidase activity of USP18 could be required in certain physiological contexts, for instance when USP18 is below a given threshold or when the level of ISG15 conjugates is maximal. In

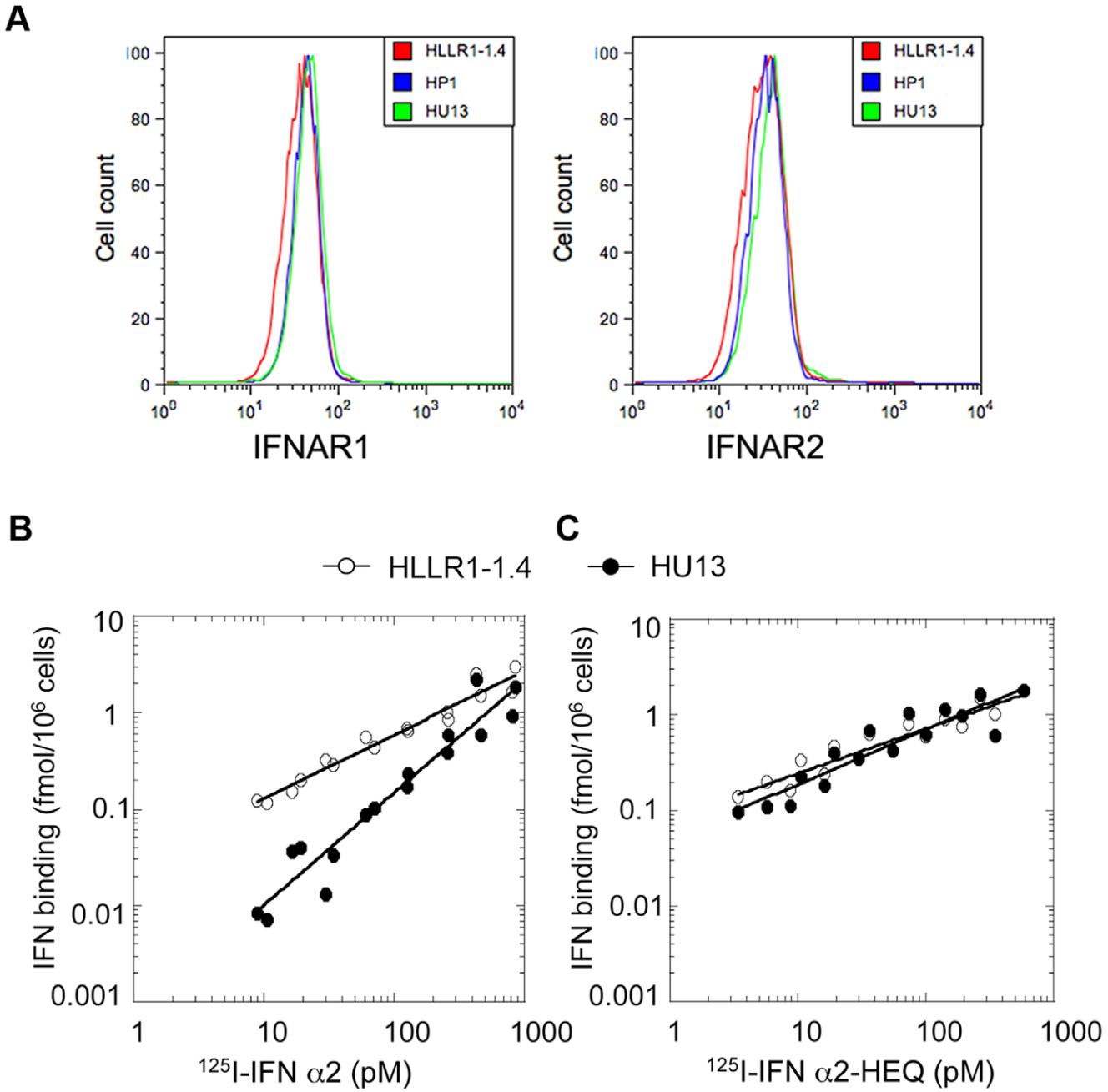

Figure 7. Cells expressing USP18 are defective in IFN α2 binding. (A) Cell surface levels of IFNAR1 (left) and IFNAR2 (right) in parental HLLR1-1.4 cells, USP18-expressing clone HU13 and control clone HP1was determined by FACS. Binding of ^{125}I labelled IFN α2 (B), or IFN α2-HEQ (C) at 37°C for 1 hr on HLLR1-1.4 cells (closed circles) and clone HU13 (triangles).
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fact, while it is remarkable that desensitization can be achieved by constitutive USP18 expression in naïve cells, it is also conceivable that the native protein acquires distinctive biochemical properties - in terms of stability, partners and/or substrates - when it gradually accumulates in IFN-stimulated cells, along with the ISGylation manchinery. On that line, additional work is required to understand the functional link between USP18, ISG15 and the ISGylation machinery in desensitization. Interestingly, several features of the ISG15 system are closely related to the ubiquitin system [20]. Notably, ISG15 is the only ubiquitin-like molecule whose C-terminal residues (LRLRGG) are identical to those of ubiquitin. These similarities suggest functional or regulatory overlap between the two pathways and indeed, *in vitro*, murine USP18 can remove ubiquitin from substrates [21,22].

From *in vitro* studies, it is known that the assembly of the IFN α2-receptor complex on artificial membranes is conditioned by the IFNAR1 concentration, whereas IFN β recruits IFNAR1 even if present at very low concentration [6]. Indeed, in cells the surface level of IFNAR1 is critical for the intensity of IFN α signaling [5,23,24]. Importantly and in accordance with other reports [13,25], we found that the presence of USP18 has no effect on the global cell surface level of IFNAR2 and IFNAR1. Nonetheless, our study shows that binding of IFN α is impaired in cells expressing USP18, whether IFN-primed or USP18-transfected. Overall, we favor a model whereby the interaction of USP18 with IFNAR2 ([13] and our data) may lead to a re-organization of the architecture of the type I IFN receptor. A change in lateral mobility of the receptor chains, in their localisation in membrane microdomains or their physical preassociation could weaken assembling and signaling of IFN α2. Conversely, owing to its higher affinity for the receptor, IFN β would retain activity on USP18-expressing cells.

It is remarkable that, at least in humans, the 13 α IFNs exhibit non-optimal affinity to the receptor chains and it is precisely this weakness that allows α/β differential bioactivities and desensitization [6]. Thus, in a viral infection, abundant IFN α is likely to be induced from the multiple genes and limits the spread of the virus by exerting potent antiviral action in a timely regulated mode on cells that will then be desensitized. On the other hand, the single IFN β - that is induced alone or, in response to viral infection, co-induced with IFN α [26] - is optimized to bind the receptor chains with high affinity and retains activity on cells desensitized for IFN α. This exclusive property of IFN β may be critical for the stimulation of adaptive immune responses necessary to eradicate the virus.

Type I IFNs and type III IFNs are induced by similar stimuli, exhibit common bioactivities and synergyze in antiviral activity towards several viruses, including HCV [27,28,29]. Their functional overlap was somehow expected given the activation, through different receptors, of the same transcriptional factor ISGF3 [30]. This is the first report of an inhibitory effect exerted by IFN λ upon IFN α activities. One particular context where cellular desensitization to IFN α could be relevant is the therapeutical control of chronic HCV infection. The current standard therapy is pegylated-IFN α2 and ribavirin, whose success is influenced by the virus genotype and multiple host factors. Among the strongest predictive factors of treatment outcome is the expression level of ISGs in liver tissue. Indeed, high baseline ISG expression in hepatocytes has been consistently associated with poor response to therapy [14,15,31,32,33]. Intrahepatic differences in ISG expression may reflect differences in host innate antiviral responses before and/or during the chronic phase. The ISG «signature» is likely to be driven and maintained by local innate cytokines, such as IFNs, and may ultimately result in failure to respond to therapeutic IFN.

Interestingly, USP18 is a component of the gene signature predictive of poor treatment response [15,32,33]. Moreover, the knockdown of USP18 in hepatoma cells was shown to potentiate the anti-HCV effect of IFN α [11]. Here, we provide evidence that primary human hepatocytes respond to IFN λ and, when primed with it, they express USP18 and become desensitized to IFN α2. Thus, it is tempting to speculate that this USP18-mediated refractoriness to IFN α could contribute, at least in part, to lower the effectiveness of an IFN α-based therapy. In that event, IFN β or λ would represent alternative therapeutic approaches.

Another strong predictive factor of successful treatment of chronically HCV infected patients (and spontaneous viral clearance) is the IFN λ3 (IL28B) genotype. Paradoxically, the good response IFN λ variant, *ie* predicting higher success rate of IFN α-based therapy, was found to be associated with higher viral load [34,35,36,37]. These consistent observations have spurred intensive studies to try to relate the IFN λ3 (IL28B) genotype with the level of hepatic ISG[33,38,39,40]. To date contradictory conclusions have been reported that do not yet provide a clear picture. Likewise, we are still missing consistent analyses of which of the variants, if any, alters expression level and/or potency of IFN λ.

In view of these and our present data, one can speculate that a patient with the hapless genotype may induce IFN λ inappropriately (e.g. altered level, potency or timing) upon HCV infection. On the one hand, this will lower the viral load without however clearing the virus and, on the other hand, will maintain a high level of ISGs, including USP18. Sustained level of USP18 may contribute, at least in part, to desensitize liver cells to administered IFN α.

Materials and Methods

Cells

HLLR1-1.4 cells are described in [16]. HLLR1-1.4 and derived clones were cultured in DMEM with 10% fetal calf serum (FCS), hypoxanthine, thymidine and aminopterin (HAT) and 400 μg/ml G418. To obtain HU and HUS clones, HLLR1-1.4 cells were co-transfected with pSVpuro and pMet7 empty vector or pMet7 encoding USP18 using FuGENE6 (Roche Applied Science). Colonies selected in 0.4 μg/ml puromycin were analysed by immunoblot for USP18 level as compared to primed HLLR1-1.4 cells. Primary hepatocytes were isolated as described [41] from a human liver sample obtained from a 51 y-o female with intrahepatic lithiasis. The French National Ethics Committee has authorized the use of these samples for research. The patient was free of any HCV, HBV and HIV markers at the time of surgery. Hepatocytes were plated at confluence in a 12-well plates at 10^6 cell/well precoated with collagen in culture medium consisting of Williams' E and Ham's F-12 (Sigma) (1/1 in volume). For the first 24 h, 5% FCS (Gibco) was added to favor cell attachment. The standard medium was then replaced with 1 ml of serum-free medium as described [41]. Cultures were incubated at 37°C and 5% CO2.

Plasmids and reagents

USP18 cDNA was cloned by PCR using as template the cDNA prepared from HLLR1-1.4 cells stimulated with IFN β-treated for 6 hr and as primers: forward 5′TTTGATATCCTGGGGGTT-TTGGAGTGA3′ and reverse 5′TAGACCGGTCTGAAGG-TTTTGGGCATTTC 3′. The PCR product was subcloned in pMET7 vector. The catalytic activity of USP18 was assessed by comparing the global protein ISGylation level in 293T cells transiently transfected with ISG15, E1, E2 and E3 enzymes of the

ISGylation machinery in the presence or absence of USP18. Rec IFN α2b was a gift of D. Gewert (Wellcome, UK) and IFN β was from Biogen Idec (Boston, MA). Mutants IFN α2-HEQ and IFN α2-α8tail were described in [6,42]. IFN α1, α8 and α21 were produced as in [43]. IFN ω was from G.R. Adolf (Bender, Wien). All IFNs were purified to homogeneity. Hyper-IL-6, chimeric fusion of human IL-6 and IL-6Rα, was a gift of Merck Serono S.A. Human IFN γ was from PBL, Biochemical Laboratories and IFN λ1 from Peprotech.

Luciferase reporter assay

To measure luciferase activity, cells were plated in 96-well plate and treated in triplicate for 6 hr with 9 dilutions of IFN in a concentration range covering 2.4 log. Cells were lysed and luciferase activity was quantified in a luminometer (LB960 Berthold). Non-linear regression fits and determination of EC_{50} were done using Prism 5 (GraphPad software).

Quantitative real-time PCR

Cells were treated with IFN for 4 hr. Total RNA was purified with RNeasy columns (Qiagen). Reverse transcriptions were primed with random primers and performed using Moloney murine leukemia virus reverse transcriptase (Invitrogen). Quantitative real-time PCR (qRT-PCR) was performed using the TaqMan gene expression assay technology (Applied Biosystems) for *USP18* (catalog no. Hs00276441). Each sample was run in triplicate, normalized to the 18S RNA amplification level in the same sample, and calculated relative to the expression of the target gene in unstimulated cells. For measuring *OAS 69K* mRNA, qRT-PCR assays were performed as in [26]. Quantification data are presented as the 95% confidence limits of ratio to the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) level.

Protein analysis

Cells were processed as in [44]. Polyclonal antibodies (Abs) used were: anti-phospho-Tyr1054/Tyr1055 Tyk2 (Calbiochem); anti-phospho-Tyr689 Stat2; anti-phospho-Tyr701 Stat1, anti-phospho-Tyr705 Stat3, and anti-USP18 (a gift from D.E. Zhang, The Scripps Research Institute, La Jolla, CA), anti-Jak1 (UBI, Lake Placid, NY), anti-Jak1-phospho-YY1022/23 (Biosource, CA) and anti-pan Akt (Cell Signaling Technology, Beverly, MA). Mouse mAbs were anti-Tyk2 T10-2 (Hybridolab, Institut Pasteur, France); anti-phosphotyrosine 4G10 (UBI, Lake Placid, NY) and anti-ISG15 clone 2.1 (a gift from E.C Borden, Cleveland Clinic, Cleveland, Ohio). Signal was revealed with the ECL enhanced chemiluminescence Western blotting reagent (Pierce) or the more sensitive Western Lightning Chemiluminescence Reagent Plus (PerkinElmer). Signals were acquired and quantified with a Kodak Image Station 440 cf. For flow cytometric analyses we used mAbs anti-IFNAR1 AA3 (BiogenIdec, Boston) and anti-IFNAR2 CD118 (P,BL, Piscataway, NJ) or D5 (BiogenIdec, Boston) as in [44]. Samples were analysed with Becton Dickinson FACScan or Canto flow cytometers.

IFN binding assays

IFN α2 and IFN α2-HEQ (30 μg) were labelled with Iodine^{125} (PerkinElmer, NEZ033A) by using a modified chloramine T method [45]. The labelled IFN preparations were titrated using a luciferase reporter assay relative to IFN α2 and IFN α2-HEQ references of known molar concentrations. The actual incorporations and monomer concentrations were as follows: α2: 75 nM and 54 Bq/fmol; α2-HEQ: 25 nM and 87 Bq/fmol.

For binding assays (Fig. 3C and D), naïve and 8 hr-primed HLLR1-1.4 cells were seeded on 6-well plates (8×10^5 cells/well) and 16 hr later incubated for 1 hr at 37°C with different concentrations of either ^{125}I-IFN α2 or ^{125}I-IFN α2-HEQ only or in the presence of a 100 fold excess of unlabeled cold IFN α2-HEQ competitor. Cells were washed three times in DMEM and 5% serum to eliminate unbound IFN, trypsinized, and counted for ^{125}I using a γ counter (Berthold). For binding assays on clones (Fig. 6B and C), cells were seeded on 6-well plates (8×10^5 cells/well) and treated as above.

USP18 silencing

USP18 ON-TARGETplus SMARTpool and a control siRNA (ON-TARGETplus non-targeting pool) were from Dharmacon. Cells were transfected with 25 nM of siRNA using Lipofectamine RNAi max reagent (Invitrogen), according to manufacturer's instructions. Twenty-four hr later, cells were either left untreated or primed, washed and challenged with IFN α2 or IFN β for 30 min to measure activation of Stats (scheme in Fig. 1A).

The siRNAs constituting the USP18 ON-TARGETplus SMARTpool were also tested individually.

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Author Contributions

Conceived and designed the experiments: VF-N SP GU. Performed the experiments: VF-N GMdFA BP-B DM LP-G. Analyzed the data: VF-N SP GU. Contributed reagents/materials/analysis tools: JP. Wrote the paper: SP GU.

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Part II: USP18 establishes the transcriptional and anti-proliferative interferon α/β differential

USP18 establishes the transcriptional and anti-proliferative interferon α/β differential

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ABSTRACT

Type I interferons (IFNs) are pathogen-induced immunoregulatory cytokines that exert antiviral and anti-proliferative activities through binding to a common cell surface receptor. Among the 17 human IFN subtypes, IFN β binds the IFNAR1/IFNAR2 receptor chains with particularly high affinity and is especially potent in select bioactivities (*e.g.* anti-proliferative and pro-apoptotic) when compared to IFN α2, However, no molecular basis has been ascribed to this differential action, specially since the two ligands are equipotent in immediate early signaling events. Here we report that IFN β induces Stat phosphorylation and transcriptional activation of interferon stimulated genes (ISGs), including two genes with pro-apoptotic functions, for a considerably longer time frame than IFN α2. We show that the diversification of α2/β responses progressively builds up at the receptor level as a result of accumulating USP18, itself an ISG, which exerts its negative feeback action by taking advantage of the weakness of IFN α2 binding to the receptor. This represents a novel type of signaling regulation that diversifies the biological potential of IFNs α an β.

Introduction

Type I interferons (IFNs) form a family of secreted cytokines that regulate cellular functions as diverse as resistance to viral infection, innate and acquired immune responses, normal and tumor cell survival and death (1). One unique feature of this IFN family is its high level of complexity in all mammals. In humans 13 IFNs α and one each of IFNs β, κ, ω and ε bind the same receptor and operate through the same Jak/Stat pathway. The type I IFN receptor is made of IFNAR1 and IFNAR2, single membrane-spanning proteins belonging to the class 2 cytokine receptor superfamily (2). Upon IFN binding, the receptor-associated tyrosine kinases Jak1 and Tyk2 are immediately activated and phosphorylate IFNAR2 on tyrosine residues, which serves as docking sites for Stat transcription factors. Once phosphorylated by the Jaks, activated Stat1/2 associate to IRF9 forming the prominent transcriptional ISGF3 complex that induces expression of ISRE-containing ISGs (3). Non canonical Stat complexes and IFN response factors (IRFs) can bind to ISRE-related sequences and reinforce ISG induction by type I IFN (4).

It is an open question as to the reason of the existence of multiple type I IFN genes. Population geneticists have recently addressed this question by investigating how natural selection acted upon these genes (5). Some IFN subtypes (α6, α8, α13, α14) were found to have evolved under strong selective constraints, others (α2, α5, α21, β, κ, ω) were shown to have accumulated some diversity and a third group of IFNs (notably α10, α16, α17, ε) display high frequencies of amino acid changes within the population. Thus, different degrees of constraint and redundancy characterize the human type I IFN family members. In that respect, it is relevant that all living mammalian orders possess a single or a small number of IFN β genes and a larger number of IFN α-related genes and that the α and β genes are differently regulated (6, 7). These and additional observations point to unique physiological roles of IFN β (8).

Several studies have reported on differential activities of type I IFNs, but no molecular mechanism has ever been elucidated. A differential is defined as a lack of correlation between two specific activities. Among the human subtypes, IFN β is

especially potent in bioactivities requiring long-term stimulation, such as proliferation inhibition, apoptosis and cell differentiation wherein IFN β can be over 50 fold more potent than IFN α2, but exhibits near equipotency with IFN α2 in antiviral activity (2). Substantial differences exist with respect to the binding of these two IFNs to the receptor. Hence, *in vitro* IFN β binds tighter to IFNAR1 and IFNAR2 than IFN α2, and forms a more stable ternary complex (2). Recently, IFNs of differing affinities and potencies have been co-crystallized with IFNAR1/2 ectodomains (9). The overall architecture of these solved ternary complexes is similar, confirming that the respective stabilities are relevant to differential potencies.

In cells the stability of ligand:receptor complexes appears to impact signals regulating receptor traffic. Within minutes of stimulation differential IFNAR2 routing can be appreciated: IFN β induces the down-regulation and degradation of cell surface IFNAR2, while IFN α2 induces its recycling (10). Related to this, mutants of IFN α2 that were designed to form a stable ternary complex are able to down-regulate surface IFNAR2 (11). Signaling feedback controls operating at immediate-early times include Ser/Thr kinase(s) and ubiquitin ligase(s) targeting the IFNAR1 subunit (12, 13) as well as SOCS-mediated action on receptor/Jaks and Stats. Another negative feedback control involves USP18, an IFN-induced isopeptidase able to cleave ubiquitin-like ISG15 from conjugates (14, 15).

In our previous analyses of IFN α2 vs. IFN β signaling in human transformed cells, we reported that the two subtypes activate the canonical Jak/Stat pathway, early ISG induction and cell cycle arrest with similar magnitude, but that IFN β induces more robust apoptosis than IFN α2 (10, 16). Our data suggested that the higher apoptotic potency of IFN β requires the activation of signals additional to the early acting Jak/Stat signaling events. In continuation of these studies, we have assessed Stat phosphorylation and ISG expression at later phases (> 8 hrs) of the response to IFNs. We show that the α2/β differential is progressively established at the level of Stat activation and gene induction through the expression of the negative feedback regulator USP18. Analyses of USP18 silenced cells demonstrate that USP18 is largely, if not entirely, responsible for establishing α/β differential bioactivities which require long-lasting stimulation.

Materials and Methods

Cells and reagents

The human amnion-derived WISH cells were cultured in DMEM and 10% heat-inactivated fetal calf serum and HLLR1-1.4 cells were cultured as previously described (17). Rec IFN α2 was from D. Gewert (Wellcome, UK); IFN β was from Biogen Idec (Boston, MA). IFN were purified to specific activities > 10^8 IU/mg of protein. Jak inhibitor 1 (Calbiochem) was used at 800 nM.

Protein analysis

Cells were lysed in RIPA buffer and lysates (40 μg) were analyzed as in (18). Antibodies used were against Phos-Tyr701 Stat1, TRAIL, USP18, Caspase-8, cleaved Caspase-3, cleaved Caspase-9, Akt (Cell Signaling Technology, Danvers, MA), Phos-Tyr689 Stat2 (Millipore), Stat2 (UBI), actin (Sigma), ISG15 (a gift of E. Borden), IFIT1, IFIT2, IFIT3 (gifts of Ganes C. Sen) (19), OAS2 p69 (a gift of A.G. Hovanessian) (20), and MxA (a gift of O. Haller) (21). Immunoblots were revealed using enhanced chemiluminescence detection reagents (Western Lightning, PerkinElmer) and bands were quantified with Fuji LAS-4000.

siRNA silencing lysates

USP18 ON-TARGETplus SMARTpool and a control siRNA (ON-TARGETplus non-targeting pool) were from Dharmacon. Cells were transfected with 25 nM of siRNA using Lipofectamine RNAi Max Reagent (Invitrogen), according to manufacturer's instructions. Twenty-four hours later, cells were either left untreated or stimulated with IFN α2 or IFN β.

Quantitative Real Time PCR

Cells were harvested using RNeasy Mini Kit (Qiagen #74104) according to manufacturer's instructions including RNase-Free DNase Set (Qiagen #79254) on column DNase digestion. Reverse Transcription was performed using Moloney murine leukemia virus (M-MLV) Reverse Transcriptase (Invitrogen #28025-013) according to manufacturer's instructions with Random Primers (Invitrogen 58875) and rRNasin (Promega 29457913). cDNA was purified with QIAquick PCR Purification Kit (Qiagen 28104). qPCR was performed with Fast Start Universal SYBR Green Master Mix (Roche 4913850001) and StepOne Plus Machine (Applied

Biosystems) using standard curve-based quantification with 60°C annealing temperature. PCR product standards were produced as above, purified with QIAquick PCR Purification Kit and diluted in Tris-EDTA (TE) Buffer with 10 μg/ml sheared salmon testes DNA (Sigma D-9156). PCR products for each primer pair were analyzed by agarose gel to confirm proper molecular weight and subjected to sequencing. Quantification data are presented as the 95% confidence limits of ratio to the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) level (n=4; Student's *t*-test).

Primers

The following forward (F) and reverse (R) primers were used for qPCR analyses of gene expression.
GAPDH-F: GAAGGTGAAGGTCGGAGTC, GAPDH-R: GAAGATGGTGATGGGATTTC, FAS-F: ATAAGCCCTGTCCTCCAGGT, FAS-R: TGGAAGAAAAATGGGCTTTG, STAT1-F: TTGGCAGTTTTCTTCTGTCA, STAT1-R: CACGCTCTCGCTCCTT, USP18-F: ACTCCTTGATTTGCGTTGAC, USP18-R: TTTCCCACGGGTCTTCTT, ISG15-F: GCGAACTCATCTTTGCCAGT, ISG15-R: CTTCAGCTCTGACACCGACA, IFIT2-F: CAGCTGCCTGAACCGAGCCC, IFIT2-R: GCATTCCAGGGCTGCCTCGT; MxA-F: GTGCATTGCAGAAGGTCAGA; MxA-R: TTCAGGAGCCAGCTGTAGGT; TRAIL-F: ACCAACGAGCTGAAGCAGAT; TRAIL-R: ACGGAGTTGCCACTTGACTT; TRIM22-F: GGTTGAGGGGATCGTCAGTA; TRIM22R: AGAACTTGCAGCATCCCACT; CXCL11-F: CGCTGTCTTTGCATAGGCCCTGG; CXCL11-R: GCCTTGCTTGCTTCGATTTGGGA; IFITM1-F: CAAAGCCAGAAGATGCACAA; IFITM1-R: ATGAGGATGCCCAGAATCAG; IFIT1-F: TCTCAGAGGAGCCTGGCTAA; IFIT1-R: TCAGGCATTTCATCGTCATC; OAS1-F: TTGACTGGCGGCTATAAACC; OAS1-R: TGGGCTGTGTTGAAATGTGT.

Flow cytometry

For flow cytometry intracellular staining phospho-Tyr701 Stat1 Abs (1:100, Cell Signaling Technology) and Alexa488-conjugated anti-rabbit secondary Abs were used according to manufacturer's instructions. Briefly, cells were trypsinized and washed with PBS prior to fixation in paraformadehyde (3.7%) 10 min at 37°C and permeabilization in methanol (90 %), 30 min at -20°C. After blocking in 2% BSA in PBS, cells were subjected to staining with primary and secondary antibodies and

then washed. Samples were analysed with Becton Dickinson FACS Calibur flow cytometer.

Immunofluorescence/Confocal Microscopy

WISH cells plated on glass cover slips and treated with IFN α2 or IFN β (500 pM) for the indicated times were washed twice with cold PBS, prior to fixation (3.2% paraformaldehyde) at 37°C for 10 min followed by permeabilization in methanol (100%) at -20°C for 24 hrs. Cells were washed with PBS, blocked with 2% BSA in PBS, and subjected to staining with phospho-Tyr701 Stat1 Abs (1:100) in blocking solution. After washing with PBS, cells were subjected to staining with Alexa488-conjugated anti-rabbit secondary Abs (1:500, Invitrogen) and DAPI (Invitrogen, 200 ng/ml) in blocking solution. Cells were washed, and coverslips were mounted using Fluoromount-G (SouthernBiotech), prior to confocal image acquisition with an LSM510 Meta inverted confocal microscope (Zeiss).

Anti-proliferative/Apoptosis Assays

Anti-proliferative activity of IFNs was assessed as previously described (16). Briefly, cells were seeded at $5x10^5$/60 mm dish and left to attach. Cells were transfected with control or USP18 siRNA for 24 hrs and then seeded in 96-well plates. 16 hrs later, cells were treated with varying doses of IFN α2 or β for 72 hrs, prior to crystal violet assessment of cell density. Apoptosis was assessed by 7-aminoactinomycin D assay as previously described (22).

RESULTS

IFN β more potently induces ISGs at late time points than does IFN α2

The extent of activation of the canonical Jak/Stat pathway after brief stimulation with IFN α2 and IFN β is comparable, as seen in several human cell types (10). Consistent with this, the accumulation of ISG transcripts in response to the two IFN subtypes is nearly equivalent within 2-8 hrs of stimulation [(16) and data not shown]. Nonetheless, by virtue of its higher affinity for the receptor, IFN β is a more potent inducer of apoptosis than IFN α2 (23, 24), particularly in cells with low density receptors (16). To explain this conundrum, we monitored the steady-state mRNA levels of well-characterized ISGs in WISH cells after long periods of stimulation. As shown in Fig. 1, at 8 hrs of IFN α2 and IFN β stimulation, no consistent differences in *IFIT1, MxA, USP18, CXCL11, OAS1 or ISG15* induction could be observed. A similar trend was seen for the induction profiles of pro-apoptotic *TRAIL* and *FAS*. However, from 16 to 36 hrs of continuous IFN stimulation, all of these ISGs were more highly expressed in IFN β-stimulated cells. Some transcripts, such as *IFIT1*, *USP18, FAS* and *TRAIL*, had remarkably diminished between 8 and 16 hrs of IFN α2 stimulation. Other transcripts, like *OAS1* and *ISG15,* had leveled off by 8-16 hrs of IFN α2 but continued accumulating in response to IFN β.

In agreement with the above data, western blot analyses of WISH cells stimulated from 8 hrs to 36 hrs revealed a progressive α2/β differential accumulation of ISG-encoded proteins (IFIT1, IFIT3, MxA, USP18, free and conjugated ISG15) starting at 16 hrs of treatment (Fig. 2A). This was the case also in fibrosarcoma HLLR1-1.4 cells (Fig. 2B, see also Fig. 4B) that exhibit a rather poor anti-proliferative response to IFNs (VFN, unpublished).

IFN β induces more persistent Stat1/2 phosphorylation than does IFN α2

In light of the above findings, we asked whether the activation levels of Stat1 and Stat2 could account for the delayed α2/β differential accumulation of ISG mRNAs. For this, we monitored tyrosine phosphorylated Stats 1 and 2 in WISH cells stimulated with either IFN α2 or IFN β (250 pM) from 1 to 12 hrs (Fig. 3A). No difference in Stat activation levels induced by the two IFNs could be observed at

early time points (0-4 hrs) as reported (10). However, at later time points (8 and 12 hrs), phospho-Stat1 and phospho-Stat2 levels were indeed higher in IFN β-stimulated cells. This was confirmed by monitoring the level of phospho-Stat1 by intracellular staining and flow cytometry (Fig. 3B) as well as by confocal microscopy (Fig. 3C). Both analyses revealed, at early times of stimulation an equivalent Stat1 activation level in response to IFN α2 and IFN β. Conversely, at 12 hrs of stimulation phospho-Stat1 (total or nuclear) was detectable only in IFN β stimulated cells. In conclusion, activated Stats are more persistent and appear to correlate with higher levels of ISG transcripts in IFN β stimulated cells. To assess whether the persistent Stat1/2 activation requires continuous receptor activation, we tested the effect of the potent Jak inhibitor 1. The robust Stats phosphorylation detected at 15 min of IFN β treatment was abrogated by a 15 min pre-incubation of the cells with the Jak inhibitor (Fig. 3D, lanes 2-3). In cells stimulated 9 hrs with IFN β, phosphorylated Stats level progressively decreased with increasing times incubation with the inhibitor. Nearly complete abrogation of the phosphorylated bands required 2 hrs of inhibitor, indicating a half life of 1 hr for both phospho Stats. These data suggest that the protracted Stat activation observed only in response to IFN β is not due to slower deactivation mechanisms but rather requires continuous activation of the receptor/Janus kinase complex.

USP18 is responsible for the IFN α2/β differential signaling, transcriptional and anti-proliferative activities

As shown above (Fig. 3A), the α2/β differential in phospho-Stats evident at 8 and 12 hrs parallels the accumulation of ISG-encoded proteins, including USP18, a negative regulator of type I IFN responses (15). Therefore, we assessed the effect of silencing USP18 on Stat1/2 activation at various stages of stimulation (from 1 hr to 36 hrs). Efficient silencing of USP18 totally abrogated the α2/β differential and resulted in a long lasting Stat1 and Stat2 phosphorylation which, importantly, was equivalent for the two IFN subtypes (Fig. 3E).

In order to assess the extent to which the control of Stat phosphorylation by USP18 regulates the α/β differential gene induction, we monitored ISG transcripts in cells silenced for USP18. Interestingly, cells lacking USP18 not only accumulated higher levels of ISGs (mRNA and protein) at late stimulation times (> 8 hrs), but also

responded similarly to the two IFN subtypes (Fig. 4A and 4B). This demonstrates a major role of USP18 in the IFN α2/β differential induction of ISGs.

The biological consequence of the USP18-dependent establishment of differential ISG induction was first assessed by measuring the percentage of apoptotic cells after 72 hrs of stimulation. As shown by 7-AAD staining of control cells, IFN β was about two fold more potent than IFN α2 (Fig. 5A, top panels). USP18 silencing augmented considerably both IFN α2 and IFN β-induced apoptosis and abolished the differential (Fig. 5A, bottom panels). Accordingly, the IFN α2 and IFN β-induced levels of pro-apoptotic TRAIL and of cleaved caspases 3, 8, and 9 were equalized in USP18 silenced cells (Fig. 5B). Next, we measured the anti-proliferative activity of IFN α2 and IFN β in control and USP18 silenced cells. In cells transfected with control siRNA the anti-proliferative potencies of IFN α and IFN β were profoundly different (EC_{50}s of 780 pM and 26 pM, respectively; EC_{50} α2/EC_{50} β: 30). On the other hand, in USP18 silenced cells the differential was muted (EC_{50}s of 37 pM and 7 pM, respectively; EC_{50} α2/EC_{50} β: 5) (Fig. 5C).

DISCUSSION

We have investigated the molecular mechanism underlying the differential action of two human type I IFN subtypes, IFN α2 and IFN β, towards apoptosis and proliferation control. We found that, in human transformed fibroblasts and epithelial cells, a low level of activated Stat1 and Stat2 is maintained upon stimulation with IFN β, but not IFN α2, through continuous low level activation of the receptor/Jak complex. Moreover, the transcriptional potential of IFN β persists for longer times. Importantly, we demonstrate that the α2/β differential Stat activation and ISG induction are dependent on the presence of USP18, since these differentials are abrogated upon silencing USP18. Furthermore, silenced cells exhibit a remarkably reduced IFN α2/β differential in long-term (72-hr) apoptotic and anti-proliferative responses. These data illustrate that the IFN-regulated accumulation of USP18, a canonical ISG, progressively restrains IFN α2-induced signaling more so than IFN β signaling. Based on our previous study (18), USP18 is expected to restrain signaling by all IFN α/ω subtypes.

USP18 associates to IFNAR2 (15) and does not modify the level of IFNARs at the cell surface but rather affects the assembly and/or stability of the receptor:ligand ternary complex (18). This was shown in non stimulated cells expressing exogenous USP18 as well as in cells primed for 8 hrs with type I IFN and then washed to secure full recovery of surface receptors. In cells under continuous IFN stimulation, as in the present work, the rising of USP18 may alter the properties of one or both receptors to the extent that pre-existing binding differences are magnified. In fact, USP18 appears to specifically lower IFN α2 activity below threshold levels by hindering the already weak association of receptor and ligand. Binding affinities of IFN α2 for IFNAR1 and IFNAR2 have been demonstrated to be 100 and 50 fold lower than those of IFN β, respectively (25). Thus, the tighter ternary complex formed by IFN β retains a moderate Jak/Stat signaling potential even in the presence of USP18. In support of this model, designer mutants of IFN α2 that form a tighter ternary complex with IFNAR1 and IFNAR2 have been shown to more potently induce ISG mRNAs, to exhibit IFN β-like anti-proliferative activities and to be less sensitive to USP18 action (11) (18). Thus, we propose that the USP18-driven negative feedback loop is an

integral part of the delayed IFN response, decoding ligand input specificity and setting the threshold of duration and amplitude of receptor activation induced by different ligands. It is likely that the dynamic range of the system may be very sensitive to the varying concentrations of USP18 in the cell.

The mechanisms by which IFN induces bioactivities requiring long term stimulation are complex, as they involve the actions of multiple ISGs and can be very much cell-context specific (26). While subtle differences may exist and go undetected in the early phase of robust Stat phosphorylation and ISG transcription, these do not appear sufficient to explain the α2 vs. β anti-proliferative differential. Indeed, we consistently observed greater induction of IFIT2 (ISG54) after 8 hrs of IFN β treatment than after IFN α2 treatment (Fig. 2B), and IFIT2 (ISG54) has been shown to induce apoptosis (27). However, our data and previous work by our laboratory show clearly that IFN β even at low doses (30 pM) exhibits more pronounced anti-proliferative activity than does IFN α2 at high doses (3 nM), eventhough at these respective doses IFN α2 induces much greater Jak/Stat phosphorylation than IFN β (10), demonstrating that early Jak/Stat phosphorylation can not explain the anti-proliferative differential.

Previous work has shown that the phosphoinositide 3-kinase (PI3K)/mammalian target of rapamycin (mTOR) signaling is critical for the induction of apoptosis by high dose (around 1.5 nM) IFN α2 in two cancer cell lines (28), and yet ISG mRNA induction has been shown to be largely independent of PI3K signaling (29). We cannot exclude that the difference in anti-proliferative potencies of IFNs α2 and β results from differential modulation of the PI3K pathway, however our observation that silencing USP18 reduces considerably the antiproliferative differential of the two cytokines suggests that USP18-dependent control of ISG mRNA induction is the key determinant. Furthermore, we were not able to detect consistent PI3K/mTOR activation following IFN addition (data not shown). At late stages of stimulation (> 8 hrs), modification of this pathway - and of other non Stat pathways - may likely result from autocrine acting factors (*e.g.* pro-apoptotic TRAIL) that are themselves ISG products.

In our study, silencing of USP18 increased and also equalized (α2 vs. β) the induction of pro-apoptotic genes (*e.g. TRAIL* and *FAS*) and the percentage of apoptotic cells in the two stimulated populations. Interestingly, USP18 was recently identified in a screen as the most powerful isopeptidase capable to protect E1A-transformed embryonic fibroblasts from apoptosis induced by anti-cancer drugs and relying on basal IFN (30). In these cells, IFN α - used at a 250 pM single dose - failed to induce apoptosis unless combined with USP18 silencing, allowing a very robust up regulation of *TRAIL* transcripts. Induction of *TRAIL* by IFN has been recurrently associated with apoptosis in different cell types (23, 24). In human bladder cancer cells *TRAIL* knockdown was shown to reduce IFN α2-induced apoptosis, and similar effects were observed upon knockdown of *FADD*, *CASP8*, *Stat1*, *IRF1* and *CDKN1A* (31). These results would be consistent with the TRAIL-TRAILR1/2-FADD-CASP8 pro-apoptotic pathway (32) being of key importance. In this model Stat1 and IRF1 function as transcription factors for *TRAIL* and other pro-apoptotic ISGs (31, 33). Accordingly, IRF1 was shown to be involved in IFN β-specific apoptosis of Ewing's sarcoma-derived cell lines (34). An attractive model to explain how even low dose IFN β limits cell proliferation and induces robust apoptosis would invoke the continued formation of ISGF3 in order to secure critical levels of ISGs during the time period in which ISG-encoded transcription factors (*e.g.* IRF1, Stat1) exert their cooperative actions.

The present work brings together past observations regarding the relative potencies of IFN α2 and IFN β. For instance, in primary human umbilical vein endothelial cells IFN β, used at 2 to 5 pM doses, was found to be two fold more potent than IFN α in Stat1 activation, a small difference relative to the 2-3 log difference measured in long term antiviral and anti-proliferative activities (35). A basal USP18 level in these cells could account for the differential induction of ISGs measured as early as 4 hrs of IFN stimulation. A well studied ISG encoding the chemokine CXCL11 (β-R1/ITAC) was previously shown to be specifically induced by IFN β and to require NFκB activation (36). Accordingly, we did observe a greater induction of *CXCL11* in WISH cells stimulated with IFN β than with IFN α2, particularly at later time points when USP18-mediated attenuation of IFN α2 and autocrine acting factors may come into play. Interestingly, in a physiological differentiation process of human monocytes, the 100

fold higher inhibition of osteoclastogenesis by IFN β with respect to IFN α2 was proposed to be mediated, at least in part, by autocrine-acting CXCL11, whose expression in monocytes undergoing osteoclastic differentiation was more efficiently up-regulated by IFN β (37).

In conclusion, USP18 is able to shift to a different extent the dose dependence of late responses to IFN α2 and β. In that respect, it is conceivable that, in any given cell type, a physiological level of USP18 - constitutively expressed or maintained by low level autocrine/paracrine IFN β or IFN λ (18) - may set the sensitivity threshold to pathogen- or stress-induced high level IFN. While USP18-mediated attenuation of IFN α signaling may protect infected cells from apoptotic death, the exclusive property of IFN β to signal more persistently may, in defined cellular contexts, allow the establishment of an adaptive immune response. A recent study in a murine infection model showed that USP18 can be critical to the establishment of antiviral immune responses (38). By restraining IFN responses in macrophages resident in the splenic marginal zone, USP18 allows local permissive VSV infection that is necessary to secure sufficient antigen production and activation of the adaptive immune response. On the other hand, in clinical settings, USP18 may counteract the efficacy of therapeutic IFN α as for example in chronically HCV infected patients, where high USP18 in pre-treatment livers has been associated with poor response to treatment (39, 40).

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FIGURE LEGENDS

Figure 1

IFN β exhibits prolonged expression (mRNA) of all assessed ISGs relative to IFN α2. mRNA levels relative to GAPDH are shown for *IFIT1, MxA, USP18, CXCL11, OAS1, ISG15, TRAIL* and *FAS* for WISH cells treated with 500 pM IFN α2 or IFN β for the indicated times. Error bars indicate 95% confidence intervals (Student's t-test).

Figure 2

IFN β exhibits prolonged expression (protein) of all assessed ISGs relative to IFN α2. Western blot analysis of USP18, ISG15, MxA, IFIT2 (HLLR1-1.4 only), IFIT1, IFIT3, OAS2 (HLLR1-1.4 only), and β-actin levels for WISH (A) and HLLR1-1.4 (B) cells treated with 100 pM of IFN α2 or IFN β for the indicated times.

Figure 3

Sustained phosphorylation of Stat1/2 upon IFN β treatment.

A) Western blot analysis of phosphorylated and total Stat1 and Stat2 and USP18 in WISH cells stimulated with 250 pM IFN α2 or IFN β for the indicated times.

B) Flow cytometric analysis of intracellular phospho-Tyr701 Stat1 in WISH cells unstimulated or stimulated for 1 hr or 12 hrs with 500 pM IFN α2 or IFN β. Mean fluorescence intensitites (MFI) are indicated for IFN stimulated cells (white histograms), and shaded histograms represent unstimulated cells (MFI: 2.9).

C) Immunofluorescence staining with phospho-Tyr701 Stat1 (Alexa488) in nuclei of WISH cells unstimulated (0 hr) or stimulated with IFN α2 and IFN β (500 pM) for 1 h, 4 hrs and 12 hrs. Nuclei are stained with DAPI (DNA). Sec Ab, cells were stained with secondary Ab only.

D) Western blot analysis of phosphorylated Stat1 and Stat2 in IFN stimulated cells treated with Jak inhibitor 1. WISH cells were stimulated for 9 hrs with IFN β (250 pM) and treated or not with Jak inhibitor 1 (800 nM) for the indicated times, before the end of the IFN stimulation. The efficiency of the inhibitor was controlled by pretreating cells for 15 min with the inhibitor and then adding IFN β for 15 min (lanes 2-3). Nearly

complete abrogation of the phosphorylated bands was obtained (98% and 80% for Stat1 and Stat2 respectively).
E) Western blot analysis of phosphorylated Stat1 and phosphorylated and total Stat2 for control and USP18 silenced HLLR1-1.4 cells treated with 100 pM of IFN α2 or IFN β for the indicated times reveals that USP18 silencing results in persistent phosphorylation of Stats.

Figure 4

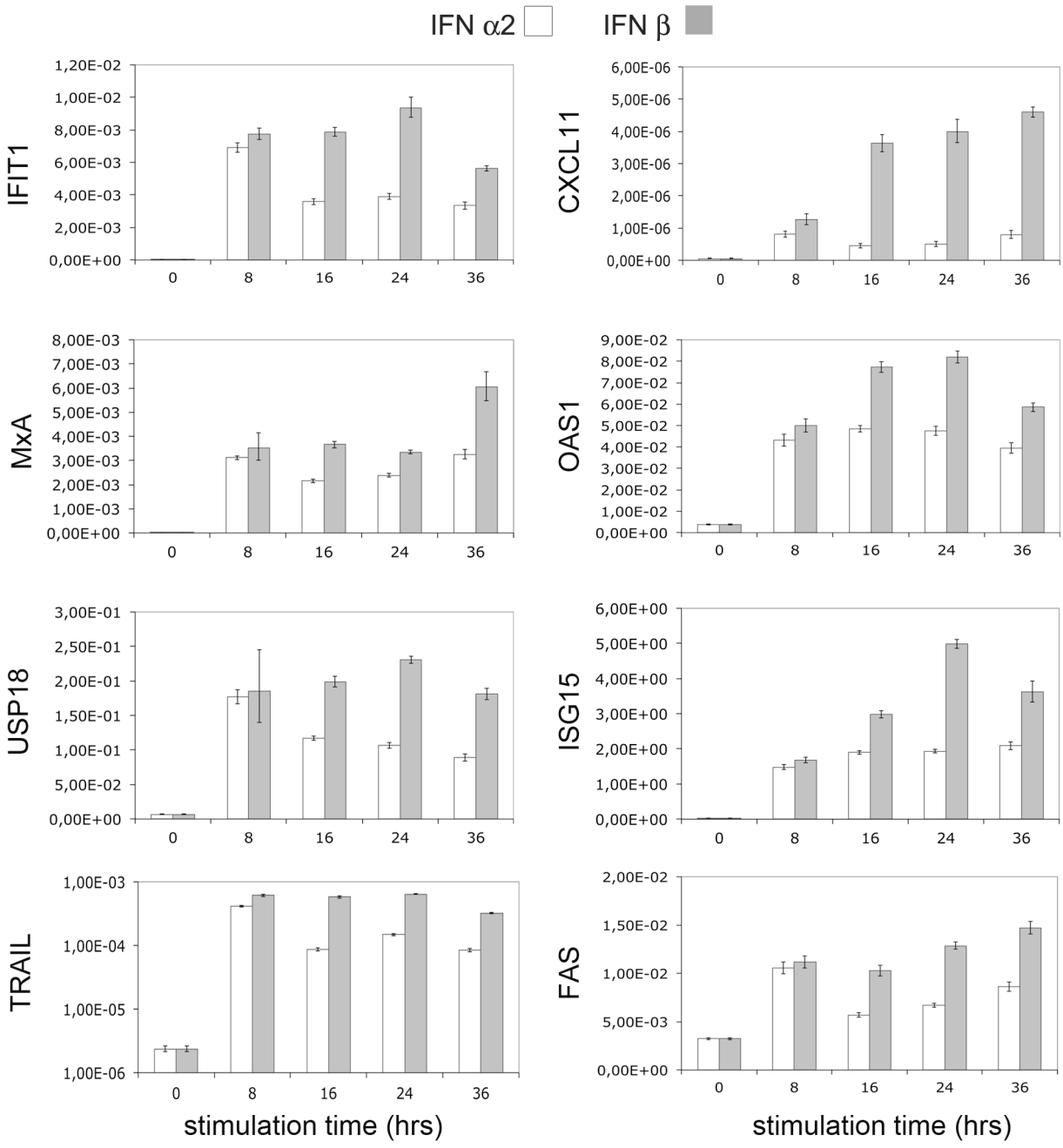
USP18 is responsible for differential late (8-72 hrs) ISG expression by IFN α2 and IFN β.

A) mRNA levels relative to GAPDH are shown for IFIT2, MxA, TRIM22, ISG15, IFITM1 and Stat1 for HLLR1-1.4 cells treated with 100 pM of IFN α2 or IFN β for the indicated times.
B) Western blot analysis of USP18, MxA, OAS2, IFIT1, IFIT3, ISG15, Stat1 and β-actin for control and USP18 silenced HLLR1-1.4 cells treated with 100 pM of IFN α2 or IFN β for the indicated times.

Figure 5

USP18 is responsible for differential apoptotic and anti-proliferative effects induced by IFN α2 and IFN β.

A) Flow cytometric analysis of 7-AAD incorporation in control and USP18 silenced WISH cells untreated or treated with IFN α2 or IFN β (500 pM) for 72 hrs.
B) Western blot analysis of full length and cleaved caspase-8 and cleaved caspases 9 and 3, TRAIL, ISG15 and MxA in WISH cells treated with with IFN α2 or IFN β (500 pM) for 72 hrs.
C) Cell density (crystal violet staining) was assessed after 72 hrs of IFN α2 (black circle) or IFN β (open circle) treatment at varying doses (between 0.1 pM and 3 nM) in control or USP18 silenced WISH cells.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IFN β
IFIT1
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1,00E-02
8,00E-03
6,00E-03
4,00E-03
2,00E-03
0,00E+00
0
8
16
24
36
CXCL11
6,00E-06
5,00E-06
4,00E-06
3,00E-06
2,00E-06
1,00E-06
0,00E+00
MxA
8,00E-03
7,00E-03
6,00E-03
5,00E-03
4,00E-03
3,00E-03
2,00E-03
1,00E-03
0,00E+00
OAS1
9,00E-02
8,00E-02
7,00E-02
6,00E-02
5,00E-02
4,00E-02
3,00E-02
2,00E-02
1,00E-02
0,00E+00
USP18
3,00E-01
2,50E-01
2,00E-01
1,50E-01
1,00E-01
5,00E-02
0,00E+00
ISG15
6,00E+00
5,00E+00
4,00E+00
3,00E+00
2,00E+00
1,00E+00
0,00E+00
TRAIL
1,00E-03
1,00E-04
1,00E-05
1,00E-06
FAS
2,00E-02
1,50E-02
1,00E-02
5,00E-03
0,00E+00
stimulation time (hrs)
stimulation time (hrs)

Figure 1

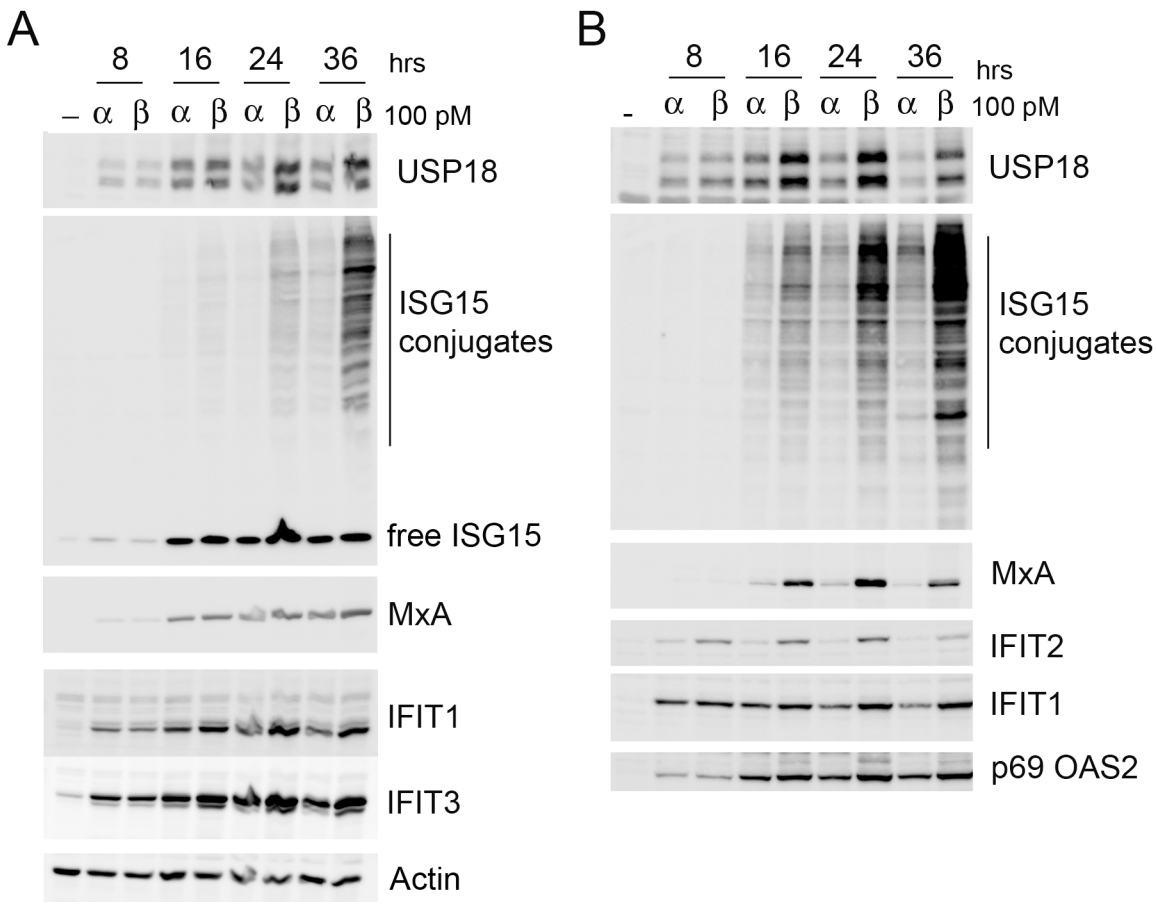
A
8
16
24
36
hrs
– α β α β α β α β 100 pM
USP18
ISG15
conjugates
free ISG15
MxA
IFIT1
IFIT3
Actin
B
8
16
24
36
hrs
- α β α β α β α β 100 pM
USP18
ISG15
conjugates
MxA
IFIT2
IFIT1
p69 OAS2

Figure 2

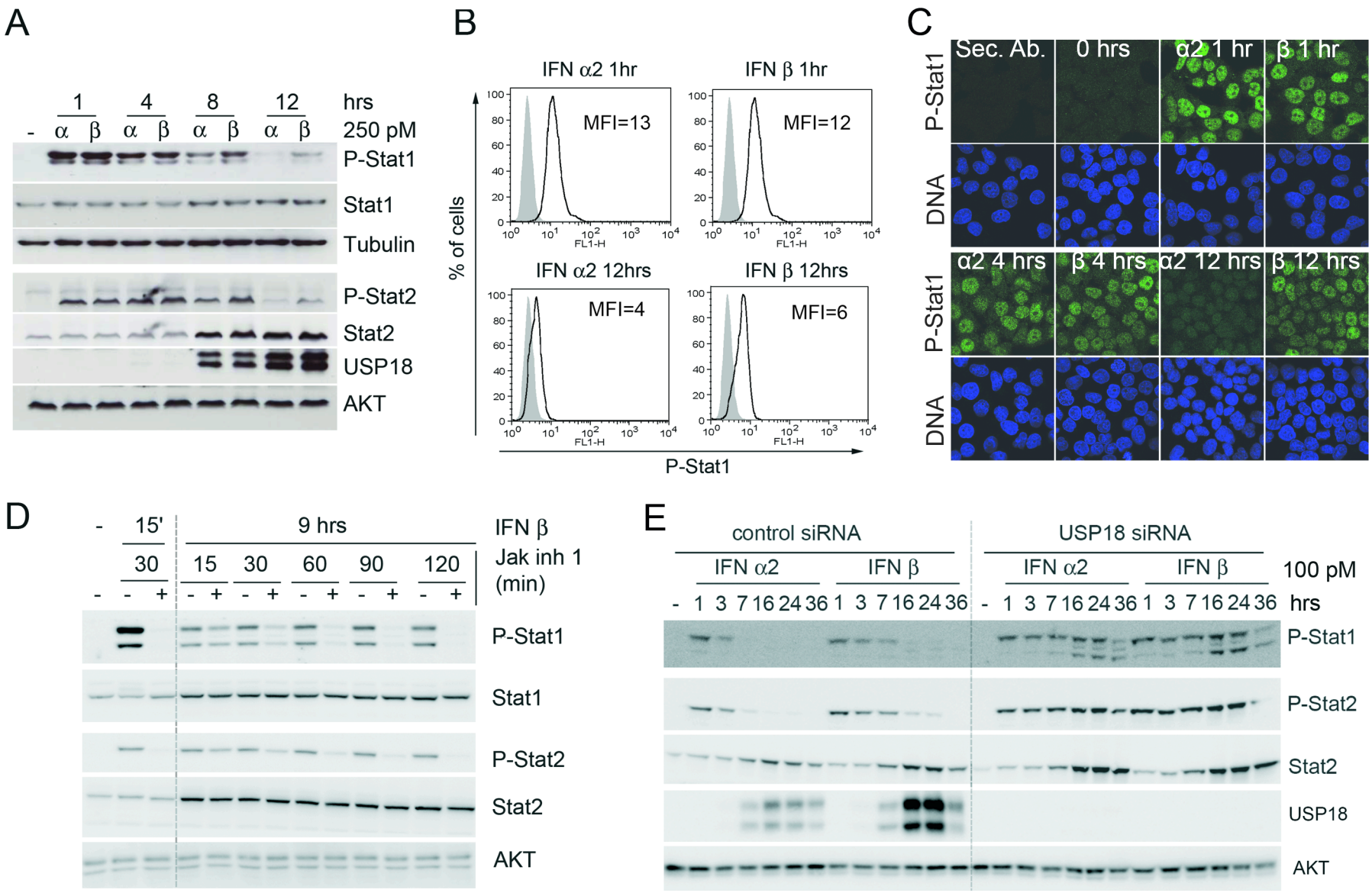
A
1
4
8
12
hrs
-
α
β
α
β
α
β
α
β
250 pM
P-Stat1
Stat1
Tubulin
P-Stat2
Stat2
USP18
AKT
B
IFN α2 1hr
IFN β 1hr
MFI=13
MFI=12
IFN α2 12hrs
IFN β 12hrs
MFI=4
MFI=6
% of cells
FL1-H
P-Stat1
C
Sec. Ab.
0 hrs
α2 1 hr
β 1 hr
P-Stat1
DNA
α2 4 hrs
β 4 hrs
α2 12 hrs
β 12 hrs
P-Stat1
DNA
D
-
15'
9 hrs
IFN β
30
15
30
60
90
120
Jak inh 1
(min)
-
-
+
-
+
-
+
-
+
-
+
-
+
P-Stat1
Stat1
P-Stat2
Stat2
AKT
E
control siRNA
USP18 siRNA
IFN α2
IFN β
IFN α2
IFN β
100 pM
-
1
3
7
16
24
36
1
3
7
16
24
36
-
1
3
7
16
24
36
1
3
7
16
24
36
hrs
P-Stat1
P-Stat2
Stat2
USP18
AKT

Figure 3

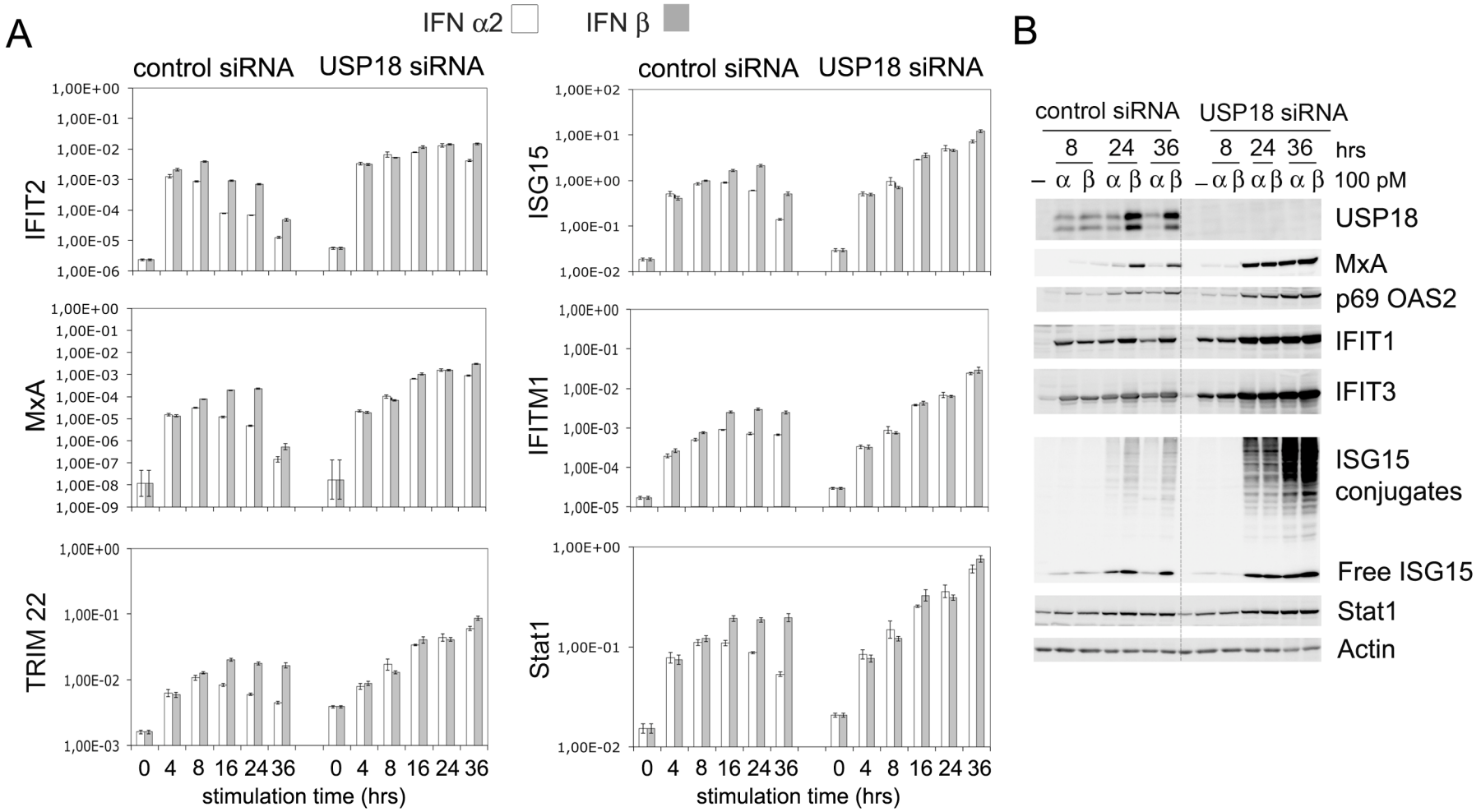
A
IFN α2
IFN β
control siRNA
USP18 siRNA
IFIT2
ISG15
MxA
IFITM1
TRIM 22
Stat1
stimulation time (hrs)
B
control siRNA
USP18 siRNA
hrs
100 pM
USP18
MxA
p69 OAS2
IFIT1
IFIT3
ISG15 conjugates
Free ISG15
Stat1
Actin

Figure 4

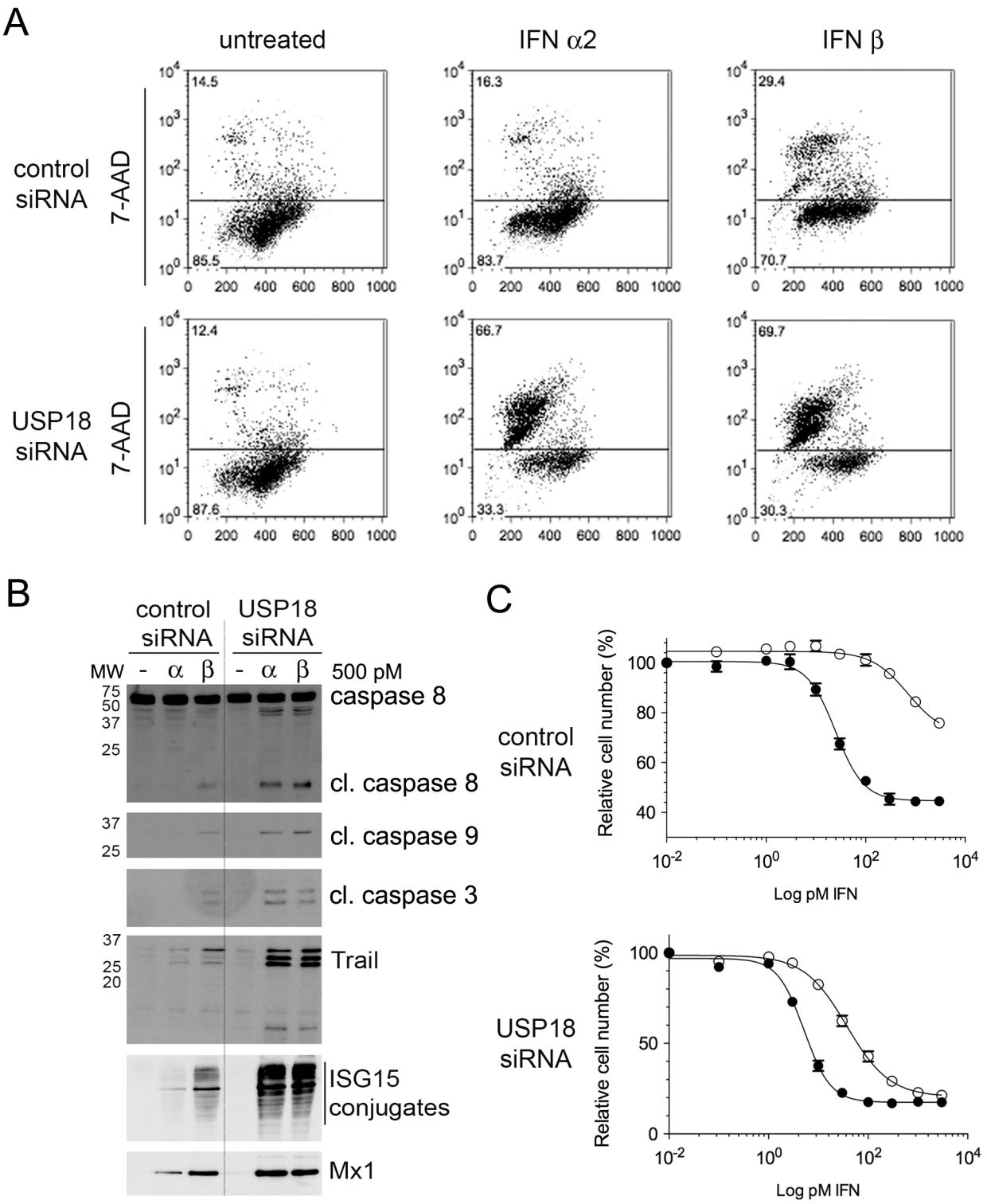
A
untreated
IFN α2
IFN β
control siRNA
USP18 siRNA
7-AAD
14.5
85.5
16.3
83.7
29.4
70.7
12.4
87.6
66.7
33.3
69.7
30.3
B
control siRNA
USP18 siRNA
MW
-
α
β
500 pM
caspase 8
cl. caspase 8
cl. caspase 9
cl. caspase 3
Trail
ISG15 conjugates
Mx1
C
control siRNA
USP18 siRNA
Relative cell number (%)
Log pM IFN

Figure 5

Part III: Additional results

Materials and Methods (Additional)

Cell lines

HLLR1-1.4 cells is a clone derived from human fibrosarcoma HT-1080 cells stably expressing the IFNLR1 receptor chain and the luciferase reporter gene controlled by an ISGF3-dependent promoter (uze and monneron). Thus, HLLR1-1.4 cells are responsive to type I IFNs as well as to type III IFNs. HLLR1-1.4 and derived clones were cultured in Dulbecco'modified Eagle medium (Gibco) supplied with 10% fetal calf serum (FCS), hypoxanthine, thymidine and aminopterin (HAT) and 400 µg/ml G418 (Gibco). To obtain HU, HUS, DS and HQ clones, HLLR1-1.4 cells were co-transfected with pSVpuro and pMet7 empty vector or pMet7 encoding USP18 using FuGENE6 (Roche Applied Science). Colonies were selected in 0.4 µg/ml of puromycin (Gibco) and 400 µg/ml G418.

Plasmids

USP18 cDNA was cloned by PCR using as template the cDNA prepared from HLLR1-1.4 cells stimulated with IFN b for 6 hrs and as primers (made by Dr. G.UZE)

Forward 5′TTTGATATCCTGGGGGTTTTGGAGTGA3′

Reverse 5′TAGACCGGTCTGAAGGTTTTGGGCATTTC 3′

The PCR product was subcloned in pMET7 vector.

All the USP18 mutants were generated by site-directed mutagenesis, using the quikchange site directed mutagenesis kit (Stratagene). The following primers were used: Forward (C64S) 5' CAACATTGGACAGACCTCCTGCCTTAACTCCTTG 3', reverse (C64S) 5' CAAGGAGTTAAGGCAGGAGGTCTGTCCAATGTTG 3'

Forward (H318Q) 5' GCAGACTCCGGTCAGTACTGTGTCTACATCC 3', reverse (H318Q) 5' GGATGTAGACACAGTACTGACCGGAGTCTGC 3'

Forward (D336S) 5' GGTTCTGCTTCAATTCCTCCAATATTTGCTTGGTGTCCTG 3', reverse (D336S) 5' CAGGACACCAAGCAAATATTGGAGGAATTGAAGCAGAACC 3'

Immunoprecipitation assay

Cells were lysed in 50 mM Tris, pH 6.8, 0.5% Nonidet P-40, 200 mM NaCl, 10% glycerol, 1 mM EDTA, imMsodium vanadate, 1 mM sodium fluoride, 10 mM phenylmethylsulfonyl fluoride, 3µg/ml aprotinin and 3µg/ml leupeptine. IFNAR2-v5 was immunoprecipitated from 1 mg of post-nuclear lysate for 2 hrs, using a monoclonal Ab directed against the v5 peptide (sigma). The lysates was then further incubated for 1 hr with 30 µl of a mix of

protein A and protein G agarose beads. The beads were washed three times with lysis buffer and resuspended in 30 µl of laemmli buffer. Immunoprecipitates were separated by SDS-PAGE and transferred to nitocellulose membrane. Immunoblots were analysed by enhanced chemiluminescence reagent plus (PerkinElmer).

siRNA

USP18, UBE1L, UbcH8, HERC5 and EFP ON-TARGETplus SMARTpool and a control siRNA (ON-TARGETplus non-targeting pool) were from Dharmacon. Cells were transfected with 25 nM of siRNA using Lipofectamine RNAi max reagent (Invitrogen), according to manufacturer's instructions. Twenty-four hr later, cells were either left untreated or primed, washed and challenged with IFN α2 or IFN β for 30 min to measure activation of Stats

The siRNAs constituting the USP18 ON-TARGETplus SMARTpool were also tested individually.

Three different siRNA targeting ISG15 (Sigma) were tested. Their sequences are; ISG15#9 siRNA: GGACAAAUGCGACGAACCU, ISG15#11siRNA: GCAGAUCACCCAGAAGAUU; ISG15 #12 siRNA : GCAACGAAUUCCAGGUGUC

Flow cytometry

Cells were detached in phosphate-buffered saline (PBS), 5mM EDTA and resuspended in PBS containing 3 % fetal calf serum. The mouse monoclonal antibodies (mAbs) AA3 (Biogen Idec, Boston) and CD118 (PBL Biomedical Laboratories), specific for human IFNAR1 and IFNAR2 respectively were used at 10 µg/ml. The signal was amplified with biotinylated rat anti-mouse IgG (Jackson Immunoresearch) and streptavidin-phycoerythrin (Jackson ImmunoResearch). Samples were analysed with a FACScalibur flow cytometer (Becton and Dickinson)

ADDITIONAL RESULTS

Is the isopeptidase activity of USP18 required for negative regulation of IFN signaling?

As described in the Introduction, USP18 is a member of the large USP family of cysteine proteases specialized in removing ubiquitin-type moieties from conjugated proteins. In particular, USP18 was described to be able to remove the ubiquitin-like ISG15. The *USP18* gene is a *bona fide* ISG and is transcriptionally induced by Type I IFN in parallel to ISG15 and the ISGylation machinery. We have shown that IFN-induced USP18 acts as a potent negative feed back regulator of IFN α signaling and as such is an important determinant of α/β differential activities and is responsible for the refractoriness of primed cells to IFN α ((Francois-Newton et al., 2011); Francois-Newton, submitted).

In continuation of the work described in part 1, we asked whether the catalytic activity of USP18 is required for this function. In so doing, we discovered a complex control on the USP18 protein by ISG15.

Functional studies of USP18 mutants

The most direct approach to the question above is to functionally analyze a catalytically inactive form of the enzyme. As for other cysteine proteases, a single substitution (Cys64 to Ser) in the catalytic site is predicted to abolish isopeptidase activity. Indeed, the murine Cys61 to Ser mutant (Usp18 C61S) was shown to be inactive in a transient global deISGylation assay (Malakhova et al., 2006). Therefore, we introduced the mutation in human USP18 and we stably transfected the vector into HLLR1-1.4 cells. Clone HUS19 expressed USP18 C64S to equivalent level as clone HU13 expressing WT USP18. Fig. 1A shows that HU13 cells are refractory to IFN α, but this is not the case for HUS19 cells that induce phospho-Stats almost to the same extent as parental HLLR1-1.4. This result suggests that the catalytic activity of USP18 is important. However, since Usp18 has been shown to interact with hu IFNAR2 (Francois-Newton et al.) (see Introduction), an alternative possibility was that the binding of the mutated C64S protein was altered. To test this, we monitored the interaction of V5 tagged IFNAR2 with USP18 WT and C64S expressed at decreasing levels in transiently transfected 293T cells. As shown in Fig. 1B, USP18 C64S

co-immunoprecipitated with IFNAR2-V5 as well as the WT protein. *This suggests that the C64S mutation does not affect the binding of USP18 to the receptor subunit.*

We have shown that IFN-primed cells are altered in their efficiency of IFN α2 binding (Figs. 4 and 7 in (Francois-Newton et al., 2011). Here, we analyzed the IFN binding property of HUS19 cells. We compared the uptake of ^{125}I-IFN α2 and of the high affinity ^{125}I-IFN α2-HEQ by HUS19, HU13 and naïve HL116-1.4 cells. The uptake of ^{125}I-IFN α2 by HU13 cells was clearly reduced compared to HLLR1-1.4 cells (Fig. 1C). Conversely, the uptake of ^{125}I-IFN α2 by HUS19 cells was indistinguishable from the one measured on HLLR1-1.4 cells (Fig. 1C). The two clones showed equal capacity to bind ^{125}I-IFN α2-HEQ (Fig. 1C). In conclusion, these data demonstrate that forced expression of the USP18 C64S in naïve cells does not recapitulate the IFN α2 binding alteration observed in primed cells where endogenous USP18 is expressed.

Altogether, the above results point to a catalytic function of USP18. In contrast, previous work performed by the group of Zhang in murine cells suggested that the negative action of Usp18 on the IFN response is independent of its isopeptidase activity (Malakhova et al., 2006) (see Introduction). We therefore attempted to reconcile these contrasting observations by using additional approaches, as described below.

First, we tested the possibility that the inactive USP18 C64S mutant can compensate catalytic impairment if it is expressed at high level. For this, we studied clones stably expressing low or high levels of the mutant protein. Clone HUS18 expresses 5 fold less and clone HUS10 expresses over 50 fold the amount of endogenous USP18 in 8 hr-primed cells (Table 1). These two clones were analyzed side by side with clone HUS19 for their ability to induce phospho-Stat1/2 (Fig. 2A) and luciferase activity (Fig. 2B-E) in response to IFNs α2 and β . Clones HUS18 and HUS19 were not or poorly desensitized, compared to the HP1 control clone expressing empty vector. Clone HUS10 was fully desensitized to both IFN subtypes.

These data corroborate the view that the catalytic activity is required for the regulatory function of USP18 and is critical for the differential effect, unless the protein is expressed at very high level.

As a second approach to investigate the catalytic involvement of USP18, we studied two additional point mutants. As described in the Introduction, the catalytic activity of cysteine proteases relies on three residues which constitute the catalytic triad. In human USP18 these residues are predicted to be Cys64, His318 and Asn335. In addition to the catalytic triad, an Asp residue (Asp336 in USP18) conserved in all USPs (section 5.2 and Fig. 19 in the Introduction) has been shown to be involved in stabilising the oxyanion hole. In the de-ubiquitinase HAUSP (USP7), the mutation of anyone of these 4 residues was reported to abrogate catalytic activity (Hu et al., 2002).

We therefore generated two USP18 mutants (H318Q and D336S) that were predicted to be catalytically inactive. To assess the catalytic potential of these mutants, we used a transient assay that measures global de-ISGylation. Briefly, a high level of ISG15 conjugates forms in 293T cells that are transfected with ISG15 and the conjugation enzymes, UBE1L (E1), UbcH8 (E2) and HERC5 (E3) (Fig. 3A). When wt USP18 was co-transfected with this machinery, the amount of conjugates was remarkably reduced. On the other hand, when C64S or H318Q mutants were co-transfected, the level of conjugates was as high as in cells devoid of USP18, this result indicating catalytic impairment. Surprisingly, co-transfection of the D336S led to a considerable reduction of conjugates, suggesting that this mutant form retains activity (Fig. 3A). Of note, the H318Q and D336S proteins consistently migrated slower and faster with respect to the WT and C64S proteins. We do not know whether the difference in migration is due to altered folding of the mutants.

The H318Q and D336S mutants were stably transfected in HLLR1-1.4 cells. Clones were chosen according to their level of USP18 expression. We verified by FACS that the cell surface levels of IFNAR1/2 were comparable in the chosen clones (Fig. 3C) and then monitored phospho-Stats in response to IFN stimulation. As shown in Fig. 3B, clone HQ14 expressing mutant H318Q was refractory to IFN α2 similar to the wt-expressing HU13 clone. Conversely, clone DS8, expressing mutant D336S, responded to IFN α2 as did cells lacking USP18 or HUS19 cells expressing the C64S mutant. These data can be tentatively summarized as follows: mutant H318Q functions as negative regulator, even if this mutant is inactive in global de-ISGylation; mutant D336S is unable to control IFN signaling, even if this mutant is active in global de-ISGylation.

In conclusion, the catalytic (de-ISGylase) activity of the mutants H318Q and D336S, measured in the transient assay, did not correlate with their ability to negatively regulate IFN signaling in naïve cells. As shown above, for the wt and the *bona fide* catalytic inactive C64S mutant the two activities correlated well.

ISGylation/de-ISGylation regulate late IFN signaling

To further dissect the action of USP18, we studied the effect of silencing ISGylation enzymes on the response of primed cells. We have shown that cells silenced for USP18 and primed are not desensitized and, expectedly, they also exhibit a high level of global ISGylation (Malakhova et al., 2003). We reasoned that, if USP18 needs to be catalytically active to restrain IFN signaling, it presumably acts on an ISGylated substrate(s). Thus, by silencing conjugation enzymes - thus preventing substrate formation - USP18-silenced cells should loose their unregulated response. As shown in Fig. 4A, cells silenced for UBE1L (E1) + USP18 or EFP (E3) + USP18 and then primed were less responsive (70% and 50% reduced phospho Stat1) than USP18 only-silenced cells. Cells silenced for the three enzymes (UBE1L+EFP+USP18) exhibited 80% decrease of phospho-Stat1. Interestingly, the combined silencing of USP18 + Herc5, another ISG15 E3 enzyme, did not result in decreased response to IFN α2, but rather the opposite (Fig. 4B).

Overall, these results suggest that the ISGylation machinery is essential for USP18 to exert its regulatory function. Moreover, it appears that the E3 enzyme EFP, rather than Herc5, is implicated in the ISGylation of a putative USP18 substrate(s).

ISGylated IFNAR2 is a potential substrate of USP18

The above findings raised the question of the substrate of USP18. IFNAR2 was considered a potential substrate, since it was previously shown to bind murine Usp18 (Malakhova et al., 2006) and to somehow affect receptor/ligand assembly (Francois-Newton et al., 2011). Since the proportion of endogenous ISGylated proteins is usually very small (Durfee et al.), to detect the putative ISGylated IFNAR2, we used the previously characterized U5-hi/hi cells (Moraga et al., 2009). These cells were derived from IFNAR2-minus U4 cells and express high levels of IFNAR1 and of V5-tagged IFNAR2, relative to the parental 2fTGH cells, and are very sensitive to IFN.

We first assessed the interaction between USP18 and IFNAR2. U5-hi/hi cells were primed for 8 hr to induce USP18. Following a resting period of 16 hr to ensure replenishment of receptors at the plasma membrane, IFNAR2 was immunoprecipitated with the V5 mAb. As shown in Fig. 5A, USP18 co-immunoprecipitated with IFNAR2 in primed U5-hi/hi cells. Interestingly, using a transient assay, Zhang and co-workers showed that Usp18 interacts with IFNAR2 and displaces Jak1 (Malakhova et al., 2006). In contrast, in our assay, Jak1 was not displaced as the level of Jak1 co-immunoprecipitating with IFNAR2 was slightly increased in cells expressing USP18 (Fig. 5A) and this did not correlate with an increase of Jak1 content (data not shown).

To test the possibility that IFNAR2 is a substrate of USP18, we first verified if the protein could be ISGylated in U5-hi/hi cells. To avoid de-ISGylation of conjugated forms and hence facilitate detection, cells were silenced for USP18, stimulated with IFN for 8 hr and allowed to rest. Cell lysates were made and analyzed for global ISGylation (Fig. 5B, left panel) and for the presence of modified IFNAR2 forms (Fig. 5B, right panel). In addition to the abundant IFNAR2 band, a minor ~150 kDa band was detected with the anti-V5 abs only in USP18-silenced/primed cells. If this band corresponds to ISGylated IFNAR2, its level should decrease in cells silenced for ISG15. As shown in Fig. 5C, the combined silencing of ISG15 + USP18 or of EFP + USP18 led to a decrease in the amount of this band. In contrast, co-silencing of Herc5 + USP18 augmented its level. Moreover, the amount of ISGylated IFNAR2 in cells silenced for each of the E3 enzymes (Fig. 5) appeared to correlate with the level of the IFN response (see Fig. 4). We also analyzed the kinetics of appearance of the ~150 kDa IFNAR2 species in USP18-silenced cells that were continuously stimulated with IFN (from 8 hr to 36 hr). As shown in Fig. 5D, the ~150 kDa band was detected only in silenced cells after prolonged IFN stimulation, in parallel to ISGylated conjugates. Interestingly, a 50 kDa band was detected with anti-V5 Abs in IFN-stimulated cells (control siRNA and USP18 siRNA) (Fig. 5B-D). The intensity of this band increased with time of stimulation in cells silenced for USP18 (Fig. 5D). This band may either correspond to an unglycosylated or a cleaved form of IFNAR2.

Altogether, these results indicate that a small fraction of total IFNAR2 in IFN-stimulated cells is ISGylated, probably in an EFP-dependent manner.

ISG15 sustains the level of the negative feed back regulator USP18

The data above suggested that, at late stages of the response to IFN - as in primed cells - type I IFN signaling becomes sensitive to a fine control by the ISGylation/deISGylation machinery. Interestingly, Chua *et al* reported that, in a human hepatoma HuH7 cell line harboring an HCV replicon, silencing of ISG15 potentiated the anti-HCV activity of IFN α (Chua et al., 2009). While this finding suggested a negative role of ISG15 on the IFN response, the authors could not detect differences in Stat activation between control and ISG15-silenced cells stimulated from 1 hr and 7 hr with IFN α (1 pM). Thus, at least within this time frame, IFN-induced Jak/Stat activation was not affected by ISG15. Nonetheless, Chua reported that at day 3 of stimulation a higher level of ISGs accumulated in ISG15-silenced cells.

Based on this report and our previous findings, we hypothesised that ISG15 might influence Stat signaling at later stages, when the ISG15 conjugation machinery and USP18 accumulate to high levels. On this basis, we set up to study the IFN response in HLLR1-1.4 cells silenced for ISG15 and primed for 8 hr with IFN and rested. In parallel, we also monitored cells silenced for USP18 or for both ISG15 + USP18. As shown in Fig. 6A, silencing of ISG15 was very efficient (>95%) and, unexpectedly, in ISG15-silenced cells USP18 did not accumulate. Consistent with the lack of expression of USP18, ISG15-silenced/primed cells responded perfectly well to both IFN subtypes. The combined ISG15 + USP18 silencing resulted in a small increase in phospho-Stat1/2 compared to cells individually silenced. The remarkable effect of ISG15 silencing on USP18 expression was confirmed in 2fTGH cells that were either primed or kept under continuous (24 hr) IFN stimulation (Fig. 6B).

To exclude the possibility of an off-target effect, two additional ISG15-targeting oligos (#9 and #11) were designed. As shown in Fig. 6C, all three oligos targeted ISG15, with oligo #12 being the most efficient (96% efficiency). Importantly, each of the three oligos led to a remarkable reduction in the level of USP18 and, as a consequence, silenced cells responded to IFN α2 as naïve cells. We verified that the three oligos efficiently targeted *ISG15* transcripts that were induced by an 8 hr-treatment with IFN β. In the same samples, we measured also the level of *USP18* transcripts, that were found to accumulate to comparable levels in control and ISG15-silenced cells (Fig. 6D).

In conclusion, these experiments suggest that ISG15 contributes to the accumulation and/or maintenance of the USP18 protein. In this scenario, ISG15 would restrain IFN signaling at late stimulation time by sustaining the level of the negative feed back regulator USP18. These data suggested a complex interplay between ISG15 and USP18 levels.

Free ISG15 controls USP18 levels

To study the mechanism by which ISG15 sustains USP18, we defined the time frame of this effect. For this, we monitored USP18 protein levels in control and ISG15-silenced cells stimulated with IFN β for increasing times (from 2 hr to 36 hr). At 8 hr, USP18 was similarly induced in control and silenced cells. Between 8 hr and 16 hr USP18 were remarkably different in the two cell populations, having increased 10 fold in control cells and only 2 fold in ISG15-silenced cells (Fig 7A and B). Of note, this difference was not observed for other ISGs (OAS2, MxA and IFIT1) that accumulated to a similar extent in the two cell populations (Fig. 7B).

We also measured the steady-state level of *ISG15*, *USP18* and *OAS1* transcripts in control and ISG15-silenced cells (Fig. 7C). Silencing of *ISG15* was efficient. *USP18* and *OAS1* mRNAs showed a similar profile, i.e. during the first half of the stimulation these transcripts accumulated to nearly equivalent levels in silenced and control cells. However, at later time points (> 18 hr), the level of transcripts declined in control cells but not in ISG15-silenced cells. This behaviour is reminiscent of that of USP18-silenced cells, where ISGs accumulation does not decline (Francois-Newton, submitted).

To further investigate the interplay between USP18 and ISG15, we asked whether free or conjugated ISG15 sustains USP18. For this, USP18 levels were monitored in cells where ISGylation was abrogated by silencing the conjugation enzyme UBE1L. We found that USP18 induction was comparable in UBE1L-silenced cells and control cells. Moreover, at 15 hr and 20 hr of IFN stimulation, USP18 was slightly more abundant in UBE1L-silenced cells (Fig. 8). As expected from the data shown in Fig. 2, the level of USP18 in ISG15-silenced cells was dramatically reduced.

These results strongly suggest that, at late time of the response (> 10 hr), free ISG15, rather than ISG15-conjugates, controls the negative feed back regulator USP18. Through this effect, ISG15 indirectly restrains induction of ISGs.

How does free ISG15 sustain USP18 protein ?

ISG15 could promote USP18 accumulation by either increasing the protein half life and/or boosting its translation. To test if ISG15 controls the stability of USP18, we measured the decay of induced USP18 in presence of the protein synthesis inhibitor cycloheximide (CHX). Briefly, control and ISG15-silenced cells were stimulated for 5 hr with high IFN β in order to induce comparable levels of USP18. CHX was then added for 30 min to 5 hr and the level of USP18 was measured by western blot. In control cells, USP18 was resistant to a long CHX treatment. Conversely, in ISG15-silenced cells the level of USP18 had considerably decreased (~50 %) after only 1 hr of CHX (Fig. 9A). This result suggests that ISG15 sustains the level of USP18, probably slowing down its degradation.

The effect of ISG15 on USP18 could be also due to a control on mRNA translation. The control of mRNA translation occurs principally through the 5' and 3' untranslated regions (UTR) of mRNA molecules. To test whether ISG15 regulates *USP18* translation through UTR sequences, we used HU13 cells in which USP18 is translated from a cDNA-encoded transcript devoid of UTR sequences and thus expected to be insensitive to ISG15 regulation. Silencing of ISG15 in HU13 cells did not affect the level of transfected USP18 (Fig. 9B, compare lane 2 and 6; Fig. 9C, compare lane 1 and 6). On the other hand, silencing of ISG15 affected the accumulation of the endogenous USP18 (Fig. 9B, compare lane 3-4 with lane 7-8). This suggests that silencing ISG15 affects endogenous USP18 but not transfected USP18.

Overall, these observations suggest that a USP18 transcript lacking endogenous 5' and 3' UTR sequences is resistant to the ISG15-based control. These preliminary analyses support the possibility that ISG15 may boost USP18 biosynthesis by controlling translation via 5' or 3' UTR sequences.

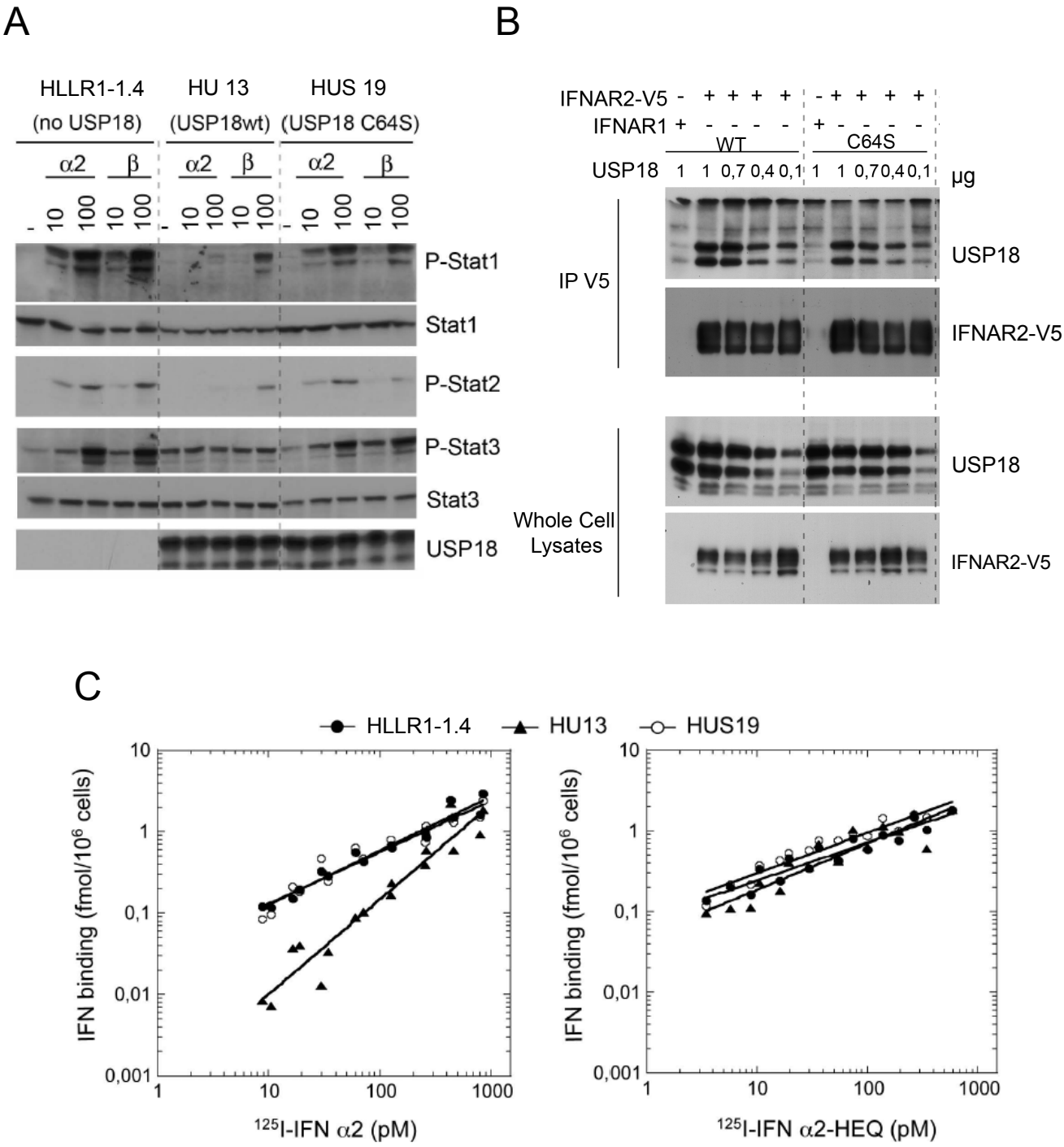

Figure 1 Enzymatic activity of USP18 is required to induce differential desensitization.
(A) Level of tyrosine phosphorylation of Stats in parental HLLR1-1.4 cells, in clone HU13 expressing USP18 and in clone HUS19 expressing USP18 C64S. Cells were stimulated for 30 min with the indicated doses of IFN α2 or IFN β. Cell lysates (30 µg) were analysed with the indicated Abs.
(B) IFNAR2-V5 (1 µg) and different concentrations of USP18 WT or USP18C64S were transfected in 293T cells. 24 hr after the transfection, IFNAR2 was immunoprecipitated with V5 mAb. Lysates were separated on 10% SDS-PAGE and immunoblotted with the indicated Abs.
(C) Binding of ^{125}I labelled IFN α2 (left) or IFN α2-HEQ (right) at 37°C for 1 hr on HLLR1-1.4 cells (closed circles), HU13 cells (triangles) and HUS19 cells (open circles).

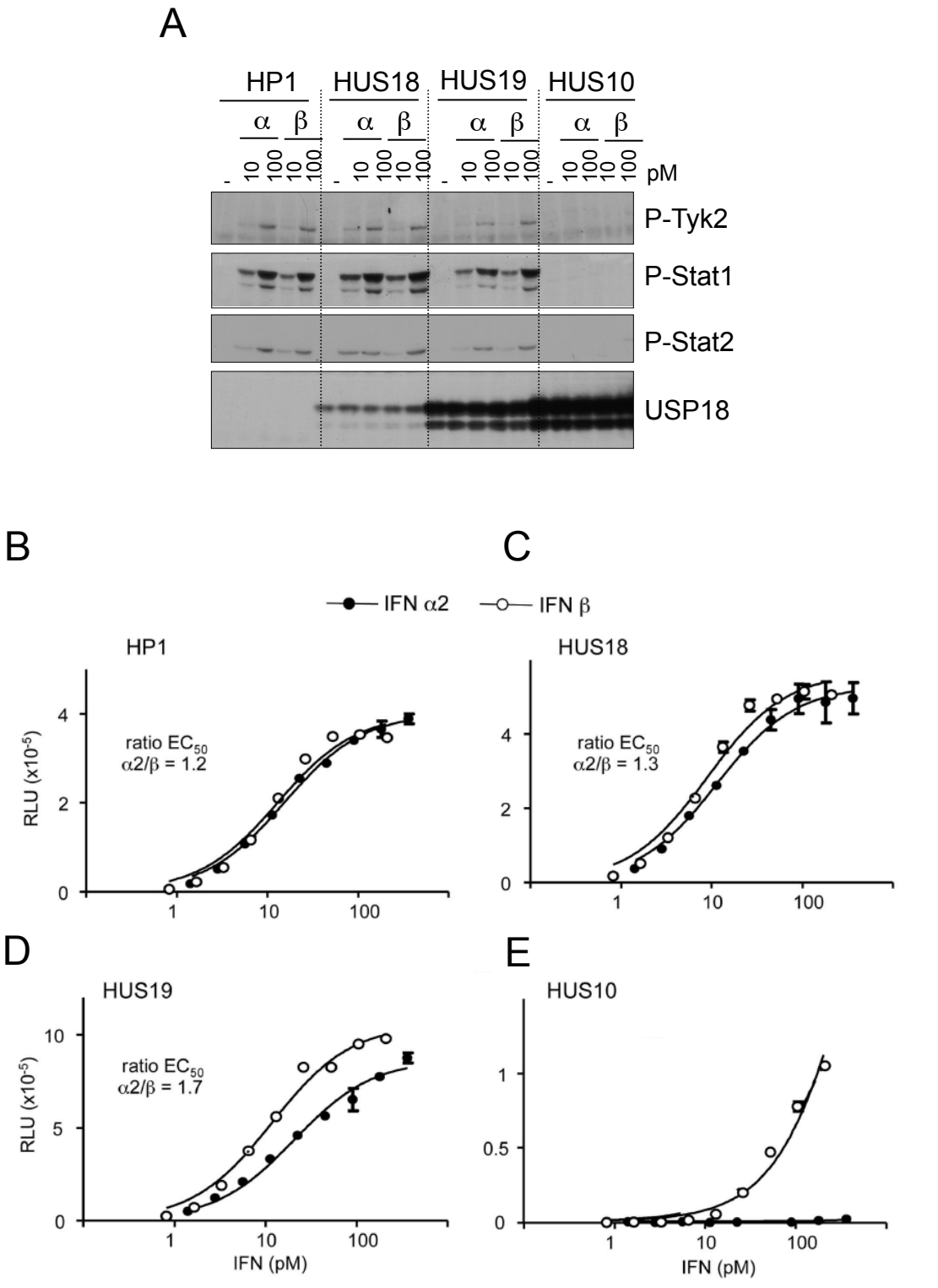

Figure 2 IFN response of clones expressing different levels of USP18 C64S.

(A) Level of tyrosine phosphorylation of Stats in clone HP1 transfected with the empty vector and in three HUS clones expressing increasing amount of USP18 C64S. Cells were stimulated for 30 min with the indicated doses of IFN α2 or IFN β. Cell lysates (30 µg) were analysed with the indicated Abs.

(B-E) Luciferase activity induced by IFN α2 (closed circles) or IFN β (open circles) measured in the following clones: (B) clone HP1; (C) clone HUS18; (D) HUS19 and (E) HUS10.

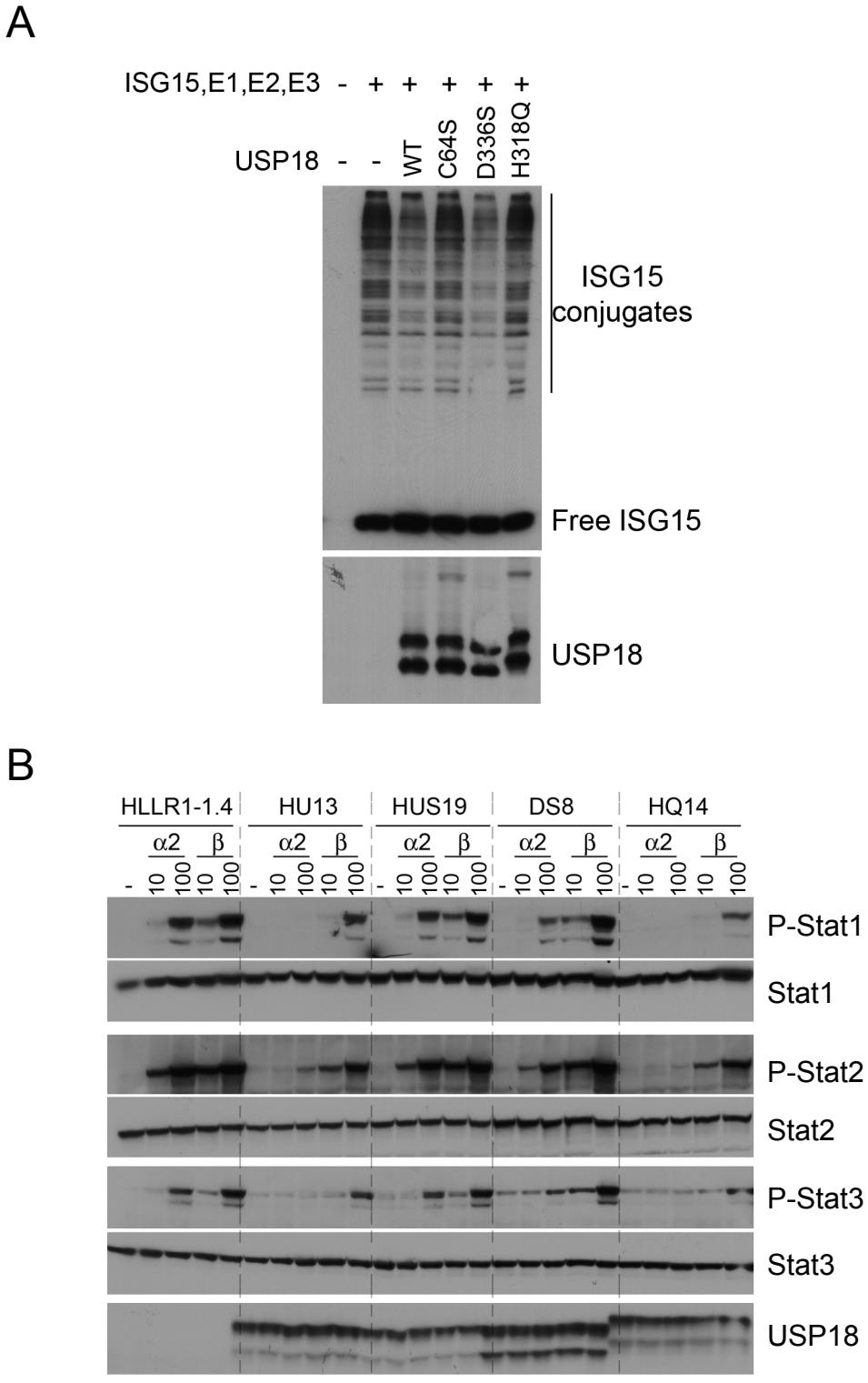

Figure 3 IFN response of clones expressing mutants of USP18.

(A) 293T cells were transfected with empty vector (-) or with ISG15 (1 μg), UBE1L (0.5 μg), UbcH8 (0.5 μg), Herc5 (1 μg) in the absence or presence of USP18 (1 μg). Forty hr after transfection, cell lysates (50 μg) were analysed with the indicated Abs.

(B) Level of tyrosine phosphorylation of Stats in parental HLLR1-1.4 cells, HU13 cells expressing USP18 WT), HUS19 cells expressing USP18 C64, in DS8 cells expressing USP18 D336S and in HQ14 cells expressing USP18 H318Q. Cell lysates (30 μg) were analysed with the indicated Abs.

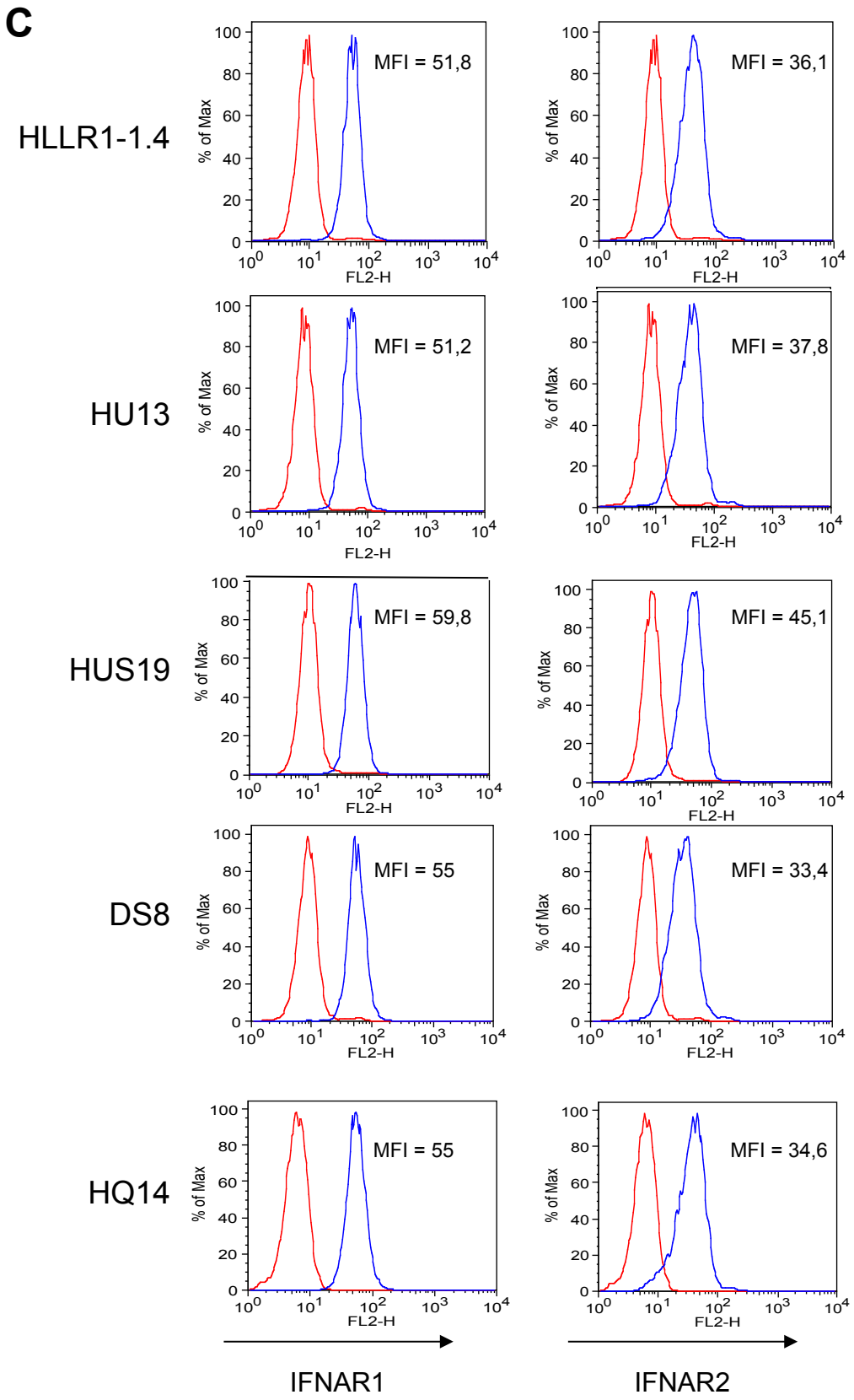

Figure 3C Level of IFNAR1 and IFNAR2 in HLLR1-1.4 cells and USP18-expressing clones. Surface IFNAR1 and IFNAR2 were quantified by FACS using AA3 and CD118 mAbs, respectively. Red, isotypic control Ab; blue, staining with IFNARs Abs.
Mean Fluorescence Intensity (MFI) of the isotypic control Ab varied from 8.3 (HQ14) to 10 (HUS19). MFI values indicate the level of IFNAR1 and IFNAR2.

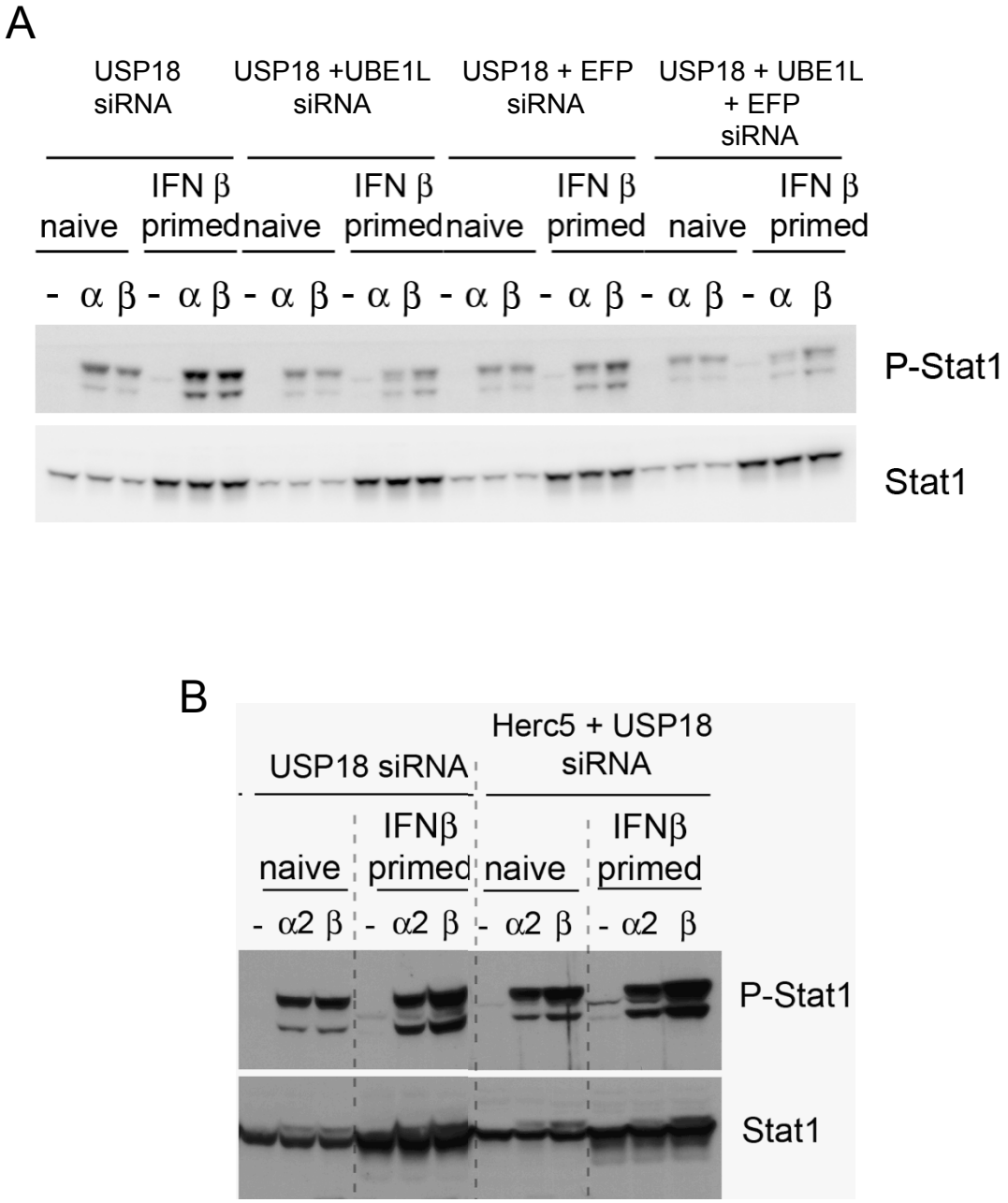

Figure 4 IFN response in cells depleted of the ISGylation machinery.

(A) HLLR1-1.4 cells were transfected with the indicated siRNA. Twenty four hr after, cells were left untreated (naïve) or primed for 8 hr with IFN β (500 pM). After 16 hr of resting, cells were stimulated for 30 min with 100 pM of IFN α2 or IFN β. Cell lysates (30 µg) were analysed with the indicated antibodies.

(B) HLLR1-1.4 cells were transfected with the 4 siRNA-pool targeting USP18 or with combined siRNA directed to USP18 and Herc5, and processed as in (A).

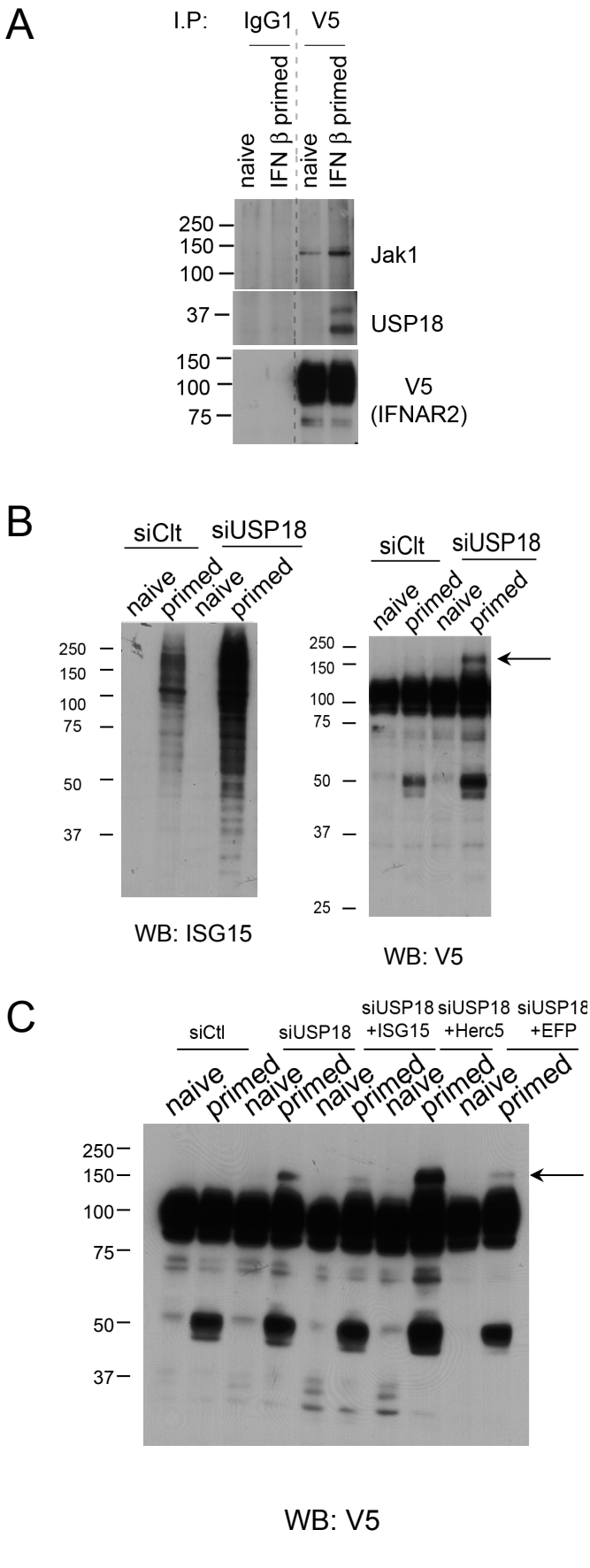

Figure 5 Analysis of ISGylated IFNAR2
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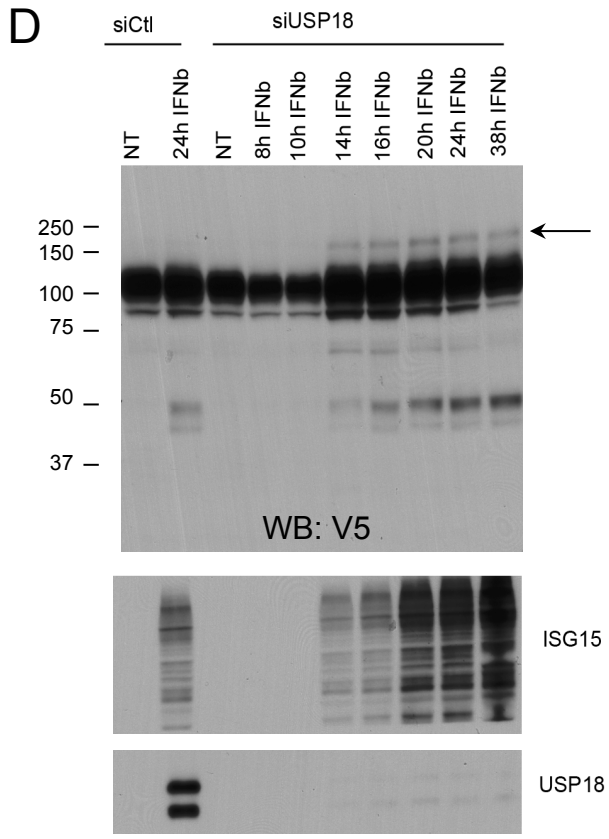

Figure 5 Analysis of ISGylated IFNAR2

(A) U5-hi/hi cells were left untreated (naïve) or primed for 8 hr with IFN β (500 pM). After 16 hr of resting, cells were lysed. One mg of proteins was immunoprecipitated with control IgG1 or anti-V5 Abs and subjected to immunoblotting as indicated.

(B) U5-hi/hi cells were transfected with control siRNA or a pool of 4 siRNA targeting USP18 (USP18 siRNA). 24 hr after transfection, cells were left untreated (naïve) or primed for 8 hr with IFN β (500 pM). After 16 hr of resting, cells were lysed and cell lysates (50 µg) were analysed with ISG15 Abs (left panel) or anti-V5 Abs (right panel).

(C) U5-hi/hi cells were transfected with the indicated siRNA. 24 hr after the transfection, cells were left untreated (naive) or primed for 8 hr with IFN β (500 pM). After 16 hr of resting, cells were lysed and cell lysates (50 µg) were analysed with anti-V5 Abs. The black arrows indicate the ISGylated form of IFNAR2.

(D) U5-hi/hi cells were transfected with control siRNA or USP18 siRNA. 24 hr after the transfection, cells were left untreated (NT) or treated for different times with 500 pM of IFN β. Cell lysates (50 µg) were analysed as indicated.

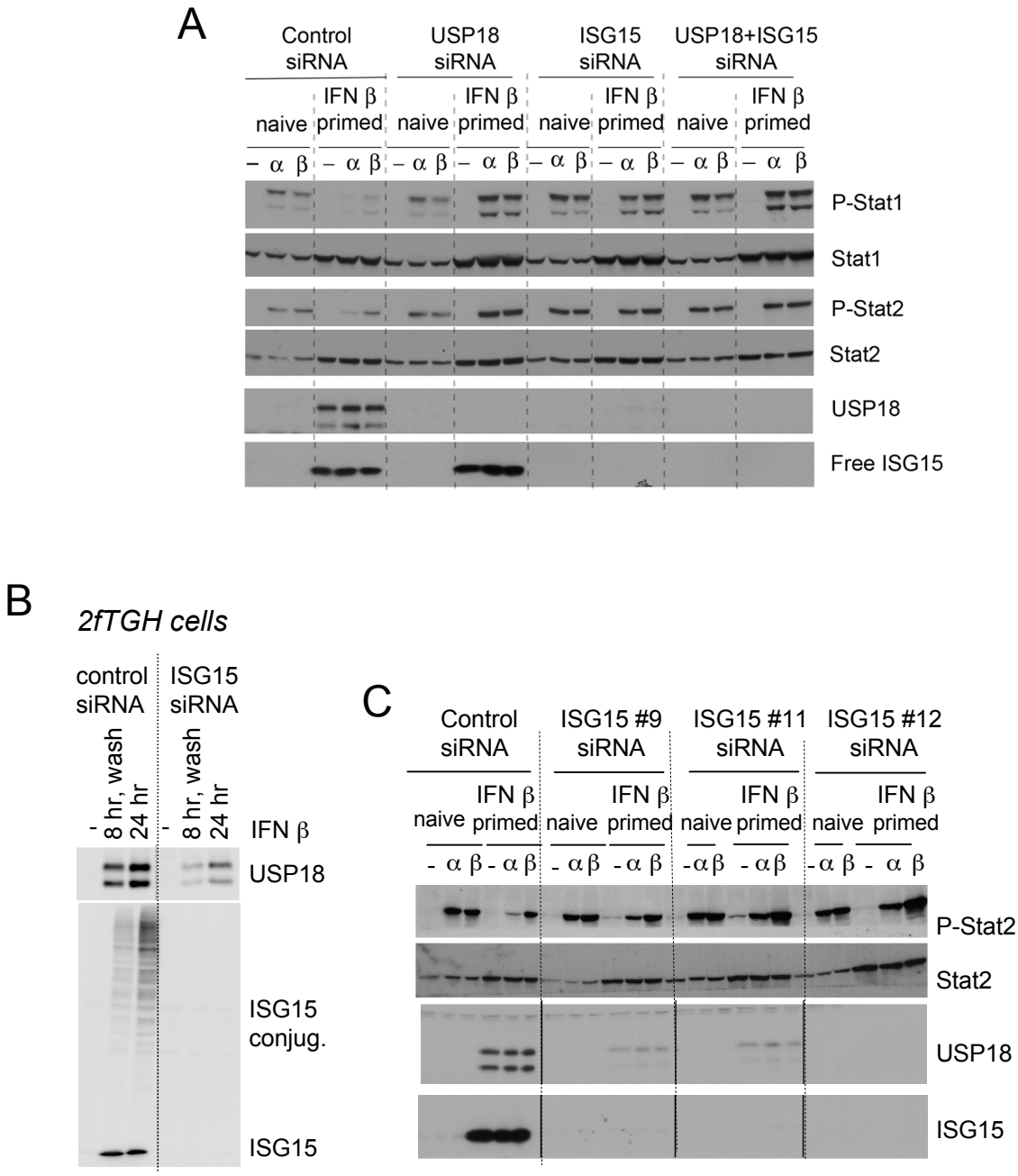

Figure 6 ISG15 sustains the level of USP18 protein.
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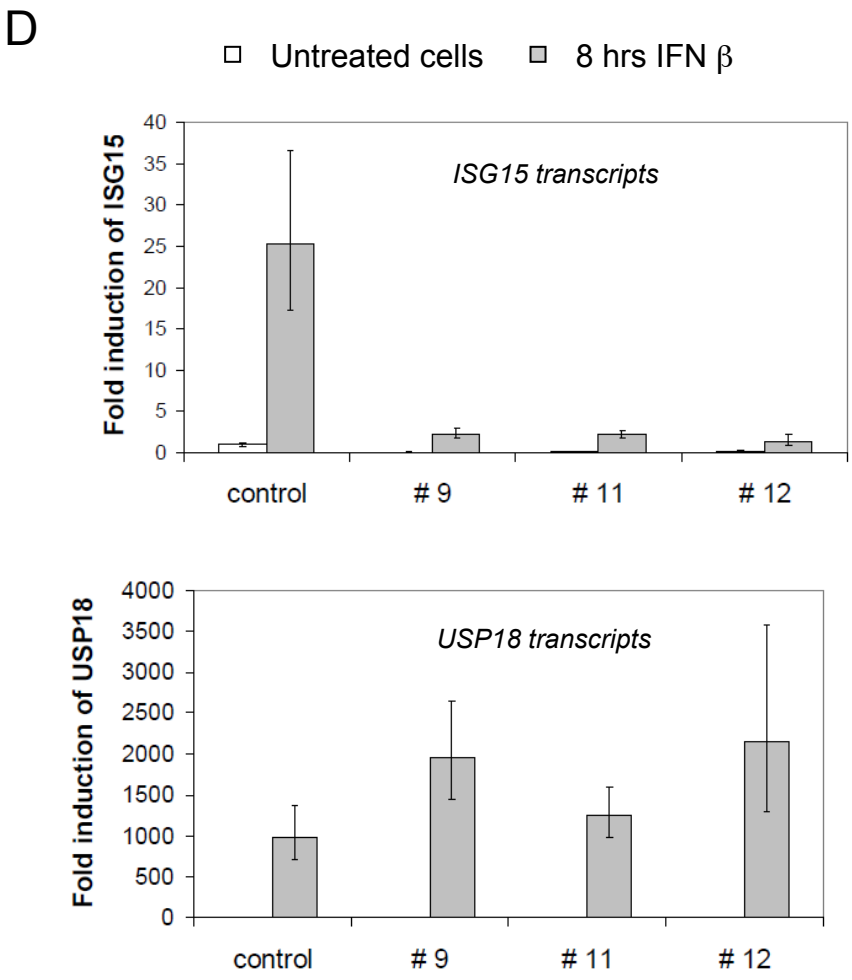

Figure 6 ISG15 sustains the level of USP18 protein.

A) HLLR1-1.4 cells were transfected with the indicated siRNA. Twenty four hr after transfection, cells were left untreated (naïve) or primed for 8 hr with IFN β (500 pM). After 16 hr of resting, cells were stimulated for 30 min with 100 pM of IFN α2 or IFN β. Cell lysates (30 µg) were analysed with the indicated antibodies.

B) 2fTGH cells were transfected with the indicated siRNA Cells were either primed and washed or continuously treated for 24 hr with 500 pM of IFN β. The levels of USP18 and ISG15 were then monitored.

C) HLLR1-1.4 cells were transfected with the indicated siRNA. Twenty four hr later, cells were left untreated (naïve) or primed for 8 hr with IFN b (500 pM). After 16 hr of resting, cells were stimulated for 30 min with 100 pM of IFN α2 or IFN β. Cell lysates (30 µg) were analysed with the indicated antibodies.

D) HLLR1-1.4 cells were transfected with control siRNA or ISG15 siRNA (oligo #9, #11 or #12) and treated for 8 hrs with IFN β (500 pM). Fold induction of *ISG15* (top) and *USP18* (bottom) transcripts were monitored by qRT-PCR. Each sample was run in triplicate. Transcripts were normalized to the level of 18S transcripts. Ratios between treated and untreated samples in each subset are shown, taking as 1 the ratio in untreated control siRNA samples.

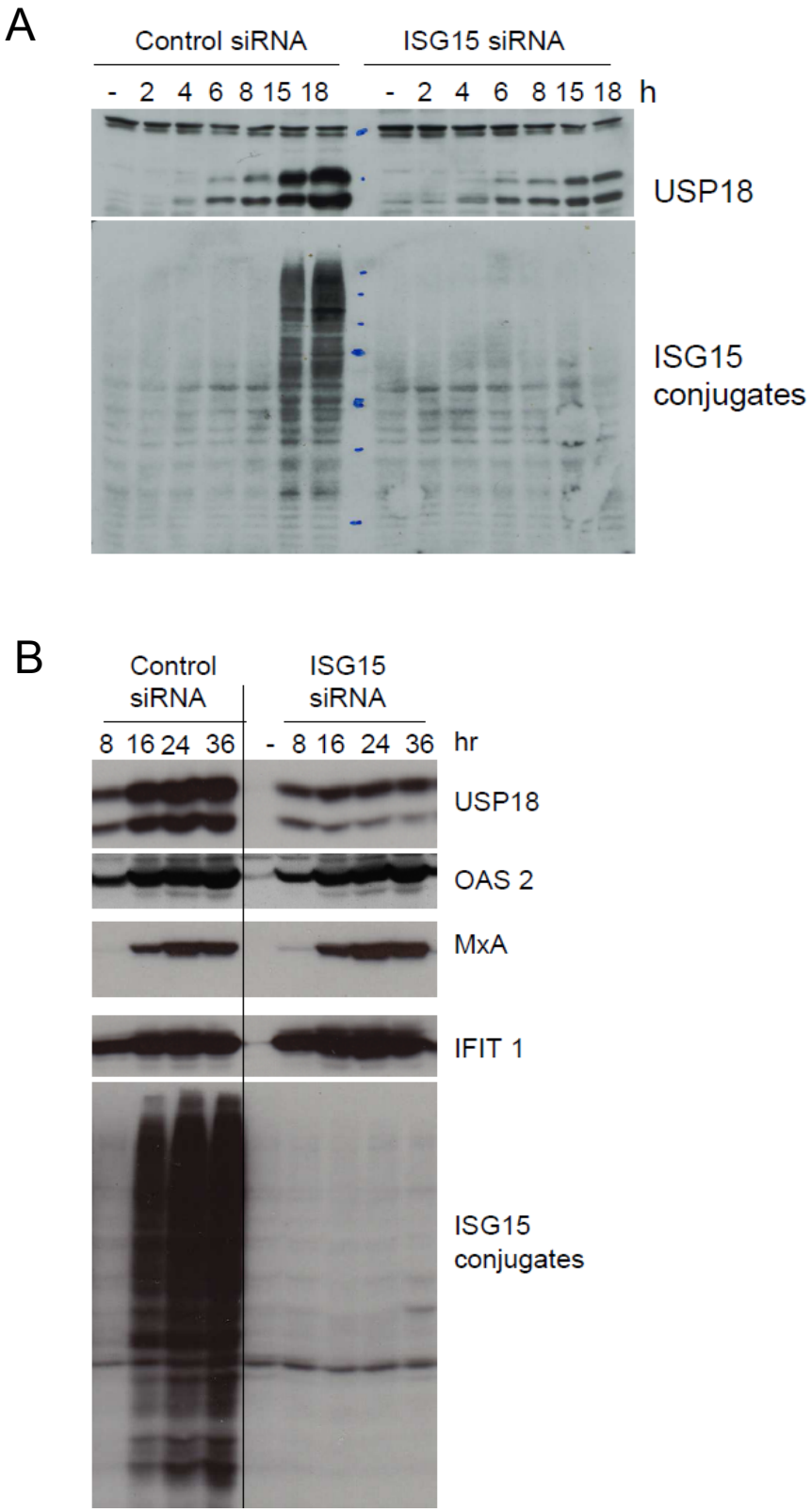

Figure 7 Accumulation of USP18 and other ISGs in ISG15-silenced cells.
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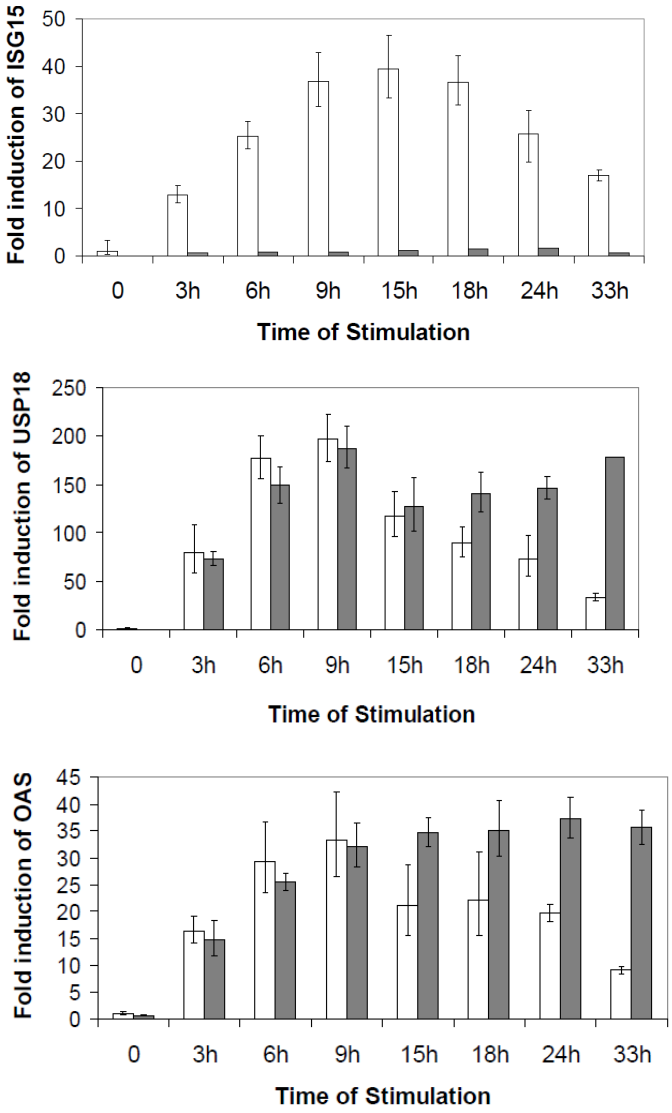

Figure 7 Accumulation of USP18 and other ISGs in ISG15-silenced cells.

(A) HLLR1-1.4 cells were transfected with control siRNA or ISG15 #12 siRNA. Twenty hr after transfection, cells were treated for different times with IFN β (500 pM). The levels of USP18 and ISG15 conjugates were monitored by Western (30 µg/lane).

(B) HLLR1-1.4 cells were transfected with the control siRNA or ISG15 #12 siRNA. 24 hrs after transfection, cells were treated for different times with IFN β (500 pM). Cell lysates (30 µg) were immunoblotted with the indicated antibodies.

(C) HLLR1-1.4 cells were transfected with control siRNA (white) or ISG15 #12 siRNA (grey). Twenty hr after transfection, cells were treated for different times with IFN β (500 pM). The fold induction of *ISG15* (top), *USP18* (middle) and *OAS* (bottom) mRNAs were monitored by qRT-PCR. Each sample was run in triplicate. Transcripts were normalized to the level of 18S transcripts. The ratios between treated and untreated samples in each subset are shown, taking as 1 the ratio in untreated control siRNA samples.

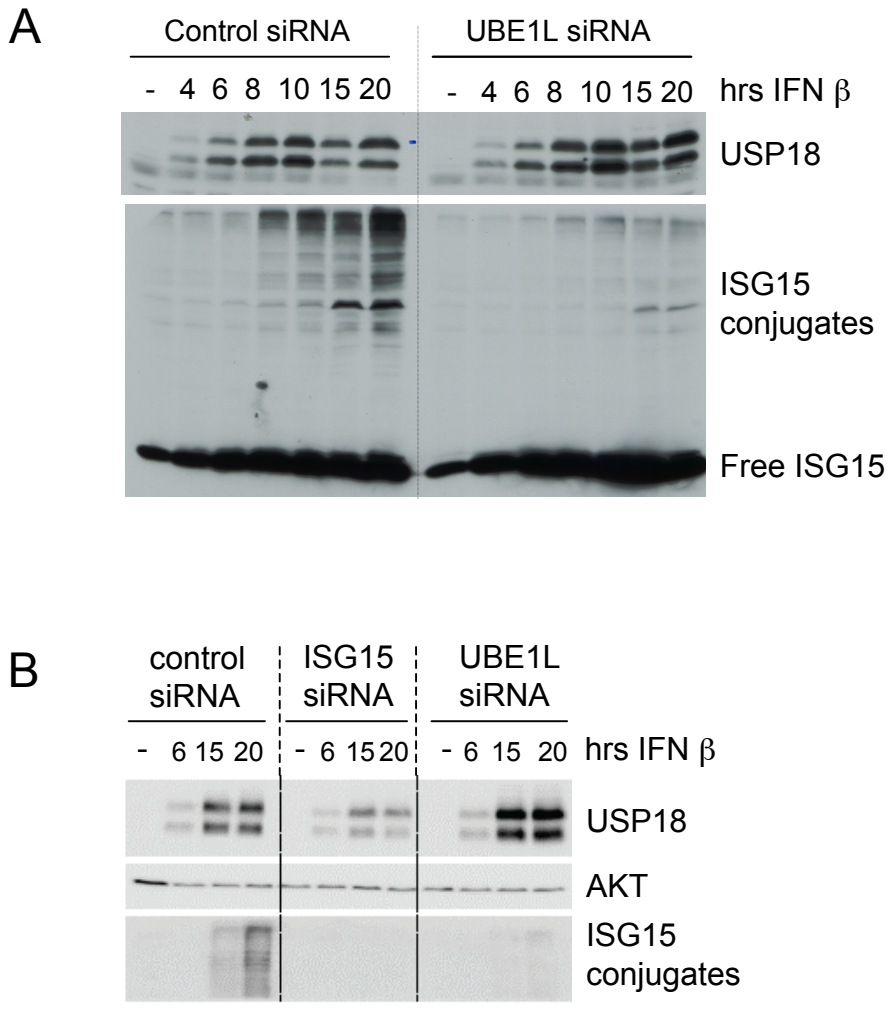

Figure 8 Accumulation of USP18 in UBE1L silenced cells.

(A) HLLR1-1.4 cells were transfected with control siRNA or a pool of 4 siRNA against UBE1L (UBE1L siRNA). Twenty hr after transfection, cells were treated for different times with IFN β (500 pM). The level of USP18 and ISG15 were monitored by Western blot (30 µg/lane).

(B) HLLR1-1.4 cells were transfected with control siRNA, ISG15 #12 siRNA or a pool of 4 siRNA against UBE1L. Twenty hr after transfection, cells were treated for different times with IFN β (500 pM). USP18, AKT and ISG15 levels were monitored by Western blot (30 µg/lane).

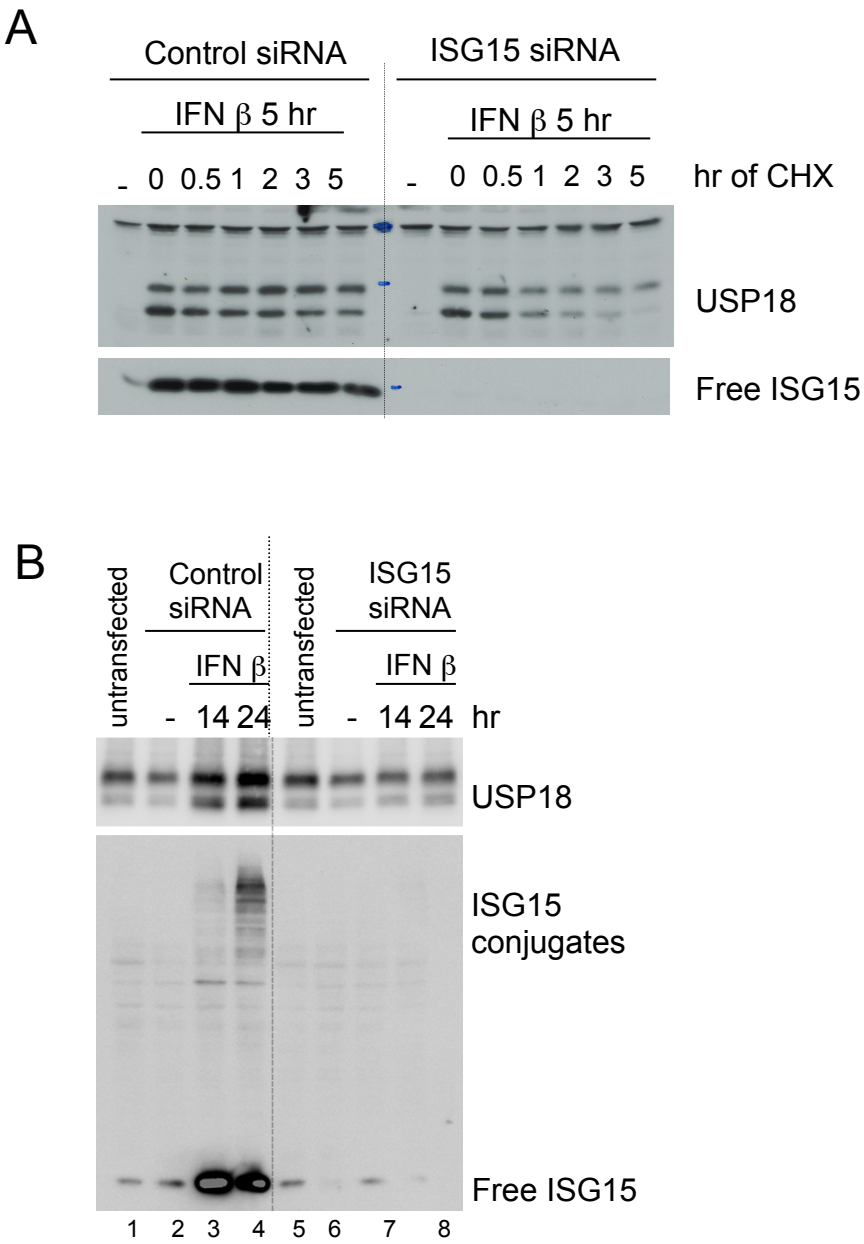

Figure 9 Stability of USP18 in ISG15 silenced cells.

(A) HLLR1-1.4 cells were transfected with control siRNA or ISG15 #12 siRNA. Twenty hr later, cells were treated for 5 hr with IFN β (500 pM) and then treated for different times with cycloheximide (CHX). Cell lysates (30 μg) were immunoblotted with the indicated Abs.

(B) HU13 cells were left untransfected or were transfected with control siRNA or ISG15 #12 siRNA. Twenty hr later, cells were treated for different times with IFN β (500 pM). Cell lysates (30 μg) were immunoblotted with the indicated Abs.

Table 1. Level of USP18 in the different cell lines

	Level of USP18
8hr-primed cells	1 X
8hr-treated cells	1 X
24hr-treated cells	5 X
HU13	5 X
HUS19	5 X
HUS18	0.2 X
HUS10	50 X

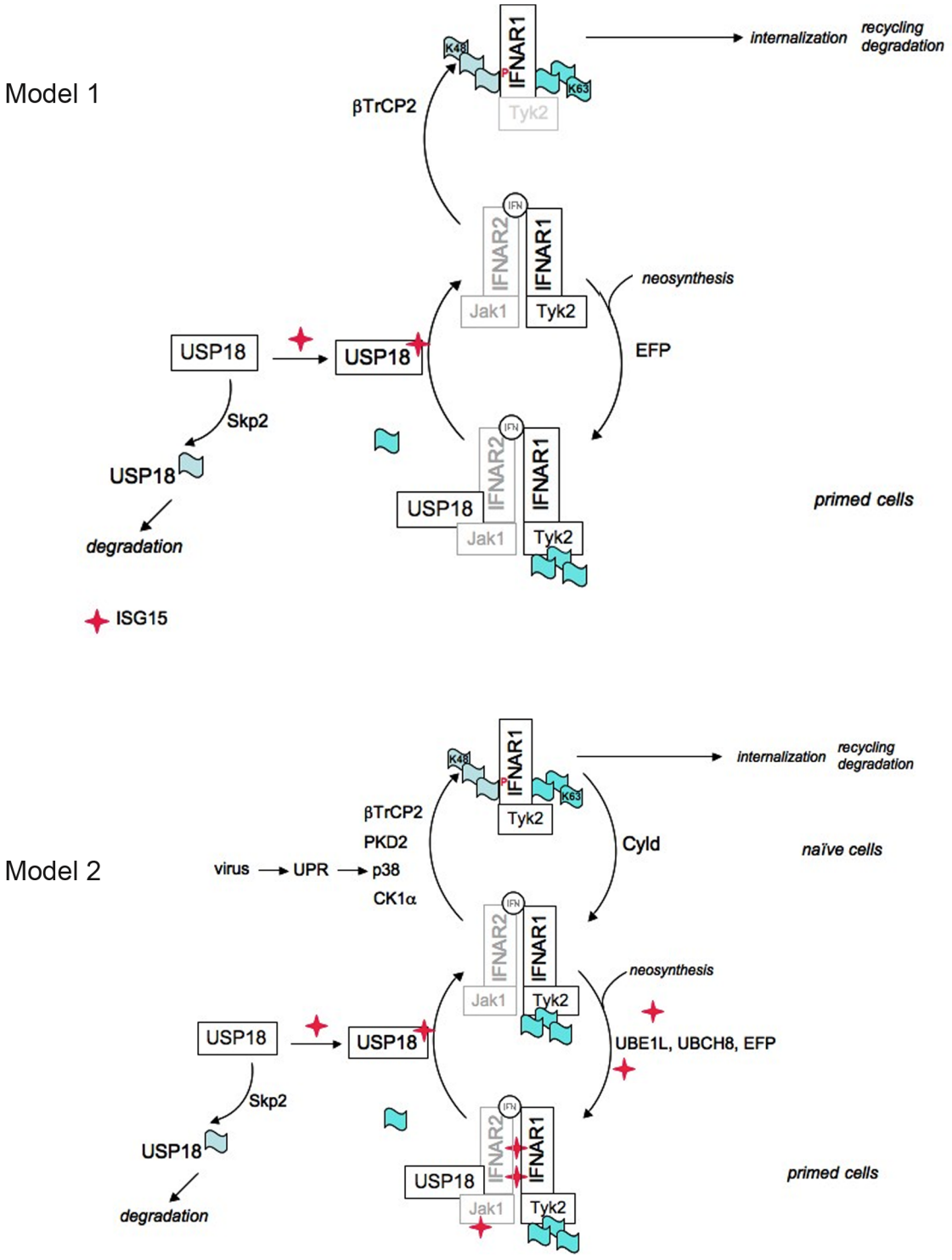

Figure 10: Model of the possible mode of action of USP18. Model 1: USP18 deUbiquitinates the K63-linked ubiquitin chains of Tyk2, destabilises Tyk2 and therefore promotes ubiquitination and internalisation of IFNAR1 Model 2: In naïve cells, CYLD deubiquitinates IFNAR1 and stabilises it at the cell surface. In primed cells, the deUbiquitinated IFNAR1 is ISGylated and therefore protected from ubiquitination and degradation. In the presence of USP18, the latter acts on the ISGylated IFNAR1 and promotes the ubiquitination and degradation of IFNAR1.

Discussion additional results

Is the catalytic activity of USP18 required for differential desensitization?

USP18 is a cysteine protease member of the USP family and is specialised in removing the ubiquitin-like moiety ISG15 from conjugated proteins. In the murine system Usp18 was also reported to be a negative feedback inhibitor of type I IFN signaling independently of its isopeptidase activity (Malakhova et al., 2006). Subsequent to this study, Potu et al reported that forced expression of USP18 - either the WT or the inactive C64S mutant - in E1A-transformed embryonic fibroblasts reduces IFN α-induced apoptosis. This led the authors to attribute to USP18 an anti-apoptotic function which would be independent of catalytic activity (Potu et al., 2010).

The various experiments that we have performed with cells expressing the C64S mutant suggest that USP18 catalytic activity is required for the negative regulation of the IFN response (Fig. 1A). The apparent discrepancy between our data and the published data could relate to the expression level of USP18. Plasmid-driven constitutive expression of a cDNA from an artificial promoter may lead to abnormal level of protein and this may overcome the need for catalytic activity, allowing it to exert its negative function In support of this hypothesis, we obtained evidence that, above a certain level, the C64S mutant makes cells refractory to both IFN α and β (Fig. 2A-E). Indeed, the level of exogenous USP18 (wt or C64S) in the transfected clones studied in Potu et al (Potu et al., 2010) was higher than the level of IFN-induced (endogenous) USP18.

The experiments that we have performed on two additional « catalytic mutants », USP18 H318Q and USP18 D336S, were not conclusive. In SDS-PAGE these two point mutants migrated differently than the WT and the C64S proteins (Fig. 3). This migration difference could reflect altered folding. The H318Q mutant lacks catalytic activity measured as global de-ISGylation in 293T cells (Fig; 2A). This result was expected based on the alignment with the HAUSP deubiquitinating enzyme, for which the crystal structure is available. On the other hand, the D336S was found to be active. This residue was chosen since it is highly conserved in the members of the USP family and the mutation of this residue in HAUSP abrogates catalytic activity (Hu et al., 2002).

The lack of correlation between the ability of the two mutants to negatively regulate IFN signaling in naïve cells and their ability to de-ISGylate in 293T cells is puzzling (Fig. 2B).

The group of Zhang mapped the IFNAR2-binding site at the carboxyl end of murine Usp18 (aa 312-368) (Malakhova et al, embo 2006), where key catalytic residues are present. By analogy, the H318 and D336 residues in the human protein may be part of the IFNAR2 binding surface (see Fig. 15 in Introduction). If this proves to be correct, the substitution of these residues may impair catalytic activity, as is the case of H318Q, but may also, or only, impair the binding of USP18 to IFNAR2.

In any extent, it should be noted that « naïve » cells engineered to express constitutive USP18 must be very different from IFN-stimulated or primed cells where the native USP18 is transcriptionally induced. In this latter context, the level of native USP18 rises from undetectable or low levels; the protein is co-induced with ISG15 and ISGylation enzymes and confronted with ISGylated proteins as possible substrates. Moreover, the catalytic activity may be exerted towards substrates that are absent in naive cells. Therefore, native USP18 exists and performs its function in an extremely dynamic context, as opposed to ectopic USP18.

Overall, these results highlight the drawbacks of studying ectopically expressed USP18 mutants. The study of the Usp18 C61S knock-in mouse cells may provide some answers to our questions. In this context, the protein will rise from undetectable or low levels and it will be co-induced with ISG15 and ISGylation enzymes. Experiments done in these mice will ascertain whether the catalytic activity of USP18 is required for the negative regulation of Type I IFN. This approach may however have a drawback if the regulation network differs between the human and murine system.

Does ISGylation of IFNAR2 affect its function ?

We obtained recent evidence that IFNAR2 is ISGylated in IFN-stimulated cells (Fig. 5B-D). Thus, ISGylated IFNAR2 could be a *bona fide* substrate of USP18. One appealing hypothesis would be that IFNAR2, once modified by ISGylation, recruits USP18 that in turn would exert its catalytic activity on IFNAR2 itself and/or on neighbouring components of the type I IFN complex (Jak1, IFNAR1 or Tyk2) resulting in attenuation of IFN α signaling.

The proportion of ISGylated IFNAR2 that we observe in primed U5-hi/hi cells (Moraga et al., 2009) is low (5-10% of total IFNAR2) and it is difficult to determine whether this

modification has functional consequences. In principle, ISGylation could lead to a gain-of-function, a loss-of-function, or cause a dominant-negative effect. A gain-of-function or dominant-negative effect may allow a small fraction of ISGylated proteins to exert a strong effect. On the other hand, a loss-of-function involving a small fraction of the total protein is unlikely to have a functional consequence, unless ISGylation occurs preferentially on the "active" pool of proteins. In some cases, ISGylation of a small fraction of the total protein can affect function. For example, a small fraction of total filamin B has been described to be ISGylated and to impair its ability to support IFN-induced Jun N-terminal kinase (JNK) activity and apoptosis (Jeon et al., 2009).

It has also been reported that the ubiquitin-like moiety SUMO (small ubiquitin-like modifier) is covalently linked to a variety of proteins and is deconjugated by SUMO-specific proteases. A characteristic of SUMO modification is that the biological consequences of conjugation do not appear proportionate to the small fraction of substrate that is modified. SUMO conjugation appears to alter the long-term fate of the modified protein even though the SUMO may be rapidly deconjugated. Thus an unmodified protein with a history of SUMO modification may have different properties from a protein that never has been modified. An exemple is the sumoylation of STAT1. A very small fraction of STAT1 is sumoylated and this small fraction was shown to interfere with STAT1 transcriptional activity (Begitt et al., 2011).

IFNAR2 possesses 5 lysines in its cytoplasmic tail (Tang et al., 2007). Based on the shift in size of modified IFNAR2 (100 kDa→150 kDa) (Fig. 5), three lysines in IFNAR2 maybe ISGylated. It would therefore be interesting to monitor which lysine residues are ISGylated in IFNAR2 and the consequence of these modifications in IFNAR2 function.

IFNAR2 can be acetylated on Lys399 and this modified residue was proposed to serve as docking site for IRF9 (Tang et al., 2007). It would be interesting to study whether there is a competition between ISGylation and acetylation and the resulting functional consequence.

ISG15 and USP18: a complex relationship

In 2009, Chua et al. reported that silencing ISG15 in HCV-infected HuH7.5 cells potentiated the activity of IFN α (Chua et al., 2009). This result is quite intriguing since all reported studies of ISG15 or Ube1L null mice have always pointed to ISG15 as an antiviral rather than a pro-viral molecule. Our analysis revealed that silencing of ISG15 results in the

dramatic down-regulation of USP18 (Fig. 6A-C). Thus, a decrease in the expression of USP18 may account for the phenotype observed by Chua and co-workers.

In agreement with Chua et al, we confirmed that the level of *USP18* mRNA is not affected by silencing of ISG15 (Fig. 6D). On the other hand, we showed that ISG15 silencing affects USP18 protein accumulation. This suggests that ISG15 sustains the protein level of USP18.

The effect of ISG15 on USP18 protein has not yet been directly analysed in the murine system. However, it does not seem to be observed in ISG15 null mice since the phenotype observed in these mice is quite distinct from the phenotype observed in Usp18-/- mice or Usp18-/- ISG15-/- DKO mice.
After at least 24 hr of IFN stimulation, the expression of two ISGs, IRF7 and OAS, was prolonged in MEF lacking either Usp18 or Usp18 and ISG15 when compared to the WT MEF (Knobeloch et al., 2005). However, no difference in the induction of the genes was reported between WT and ISG15-/- MEF. This suggests that the post-transcriptional regulation of USP18 may differ in the human and murine systems.

Our data suggest that, at least in the human system, free ISG15 rather than the conjugated form sustains the level of USP18 (Fig. 8). Free ISG15 could do so for example by increasing the protein half-life and/or boosting its translation.

Free ISG15 may protect USP18 from ubiquitination

USP18 has been identified in a yeast two-hybrid screen as a substrate of the SCF Skp2 ubiquitin E3 ligase complex. Skp2 belongs to the family of F-box proteins that function as substrate recognition factors for SCF (Skp1, Cullin, F-box protein) complex. Interestingly, SCF Skp2 was shown to promote USP18 ubiquitination and degradation (Tokarz et al., 2004).
An example of regulation of an enzyme by free ISG15 was proposed by the group of Zhang who reported that ISG15 in its free form was sufficient to inhibit the activity of the ubiquitin HECT E3 ligase Nedd4 (Malakhova and Zhang, 2008). The authors proposed that ISG15 bound Nedd4 specifically and prevented the transfer of ubiquitin from an E2 to Nedd4, thus inhibiting Nedd4 E3 ligase activity.

In Fig. 10 I have drawn an hypothetical model that tries to integrate my observations and data from others. It can be seen that free ISG15 may protect USP18 from degradation. Indeed, we could show that, in the presence of cycloheximide, USP18 decayed more rapidly in ISG15-silenced cells than in control cells (Fig. 9A). It is however surprising that the level of ectopic USP18 was not affected by ISG15-silencing in clones HU13 (Fig. 9B). The *USP18* transcript expressed in this clone differed from the endogenous *USP18* transcript, in that it lacks the native USP18 UTR sequences. This observation suggests that ISG15 might control protein translation of *USP18* mRNA via the 5′ or 3′ UTR sequences.

A possible mechanism by which ISG15 may regulate USP18 mRNA translation is by controlling translational regulator(s). Examples of an effect of ISG15 on translation can be found. 4EHP binds to the cap structure of mRNA and inhibits translation by competing with the translation initiation factor eIF4E. 4EHP can be ISGylated and in its modified form it can bind to the mRNA cap with greater affinity than the unmodified protein (Okumura et al., 2007). Furthermore, the translational repressor p56 can be ISGylated but the consequences on its function have not been studied (Zhao et al., 2005). In our case this possibility is unlikely since the abrogation of ISGylation by silencing of UBE1L does not affect the level of USP18, suggesting that free ISG15 rather than an ISGylated protein regulates USP18 expression.

Another possibility would be that, in the absence of ISG15, USP18 mRNA translation is inhibited by the up-regulation of one or more microRNAs (miRNAs). miRNAs are a class of small molecules and non-coding single strand RNAs that regulate gene expression at the post-transcriptional level by binding to specific sequences such as the 3′-UTR regions of mRNA targets. Indeed, it has recently been reported that USP18, *via* its isopeptidase activity, regulates positively the level of EGFR by downregulating the expression of microRNA-7 (Duex et al., 2011). The mechanism by which USP18 regulates microRNA-7 expression is still ill-defined.

In summary, the data presented here show that ISG15 is a potent regulator of the level of USP18 and indirectly of its activity. Further studies are required to define the mechanism by which ISG15 regulates USP18.

General Discussion

General discussion

Desensitization of human cells to IFN α was described over 20 years ago (Larner et al., 1986). Since then, several mechanisms have been proposed to explain this change in cellular responsiveness. As mentioned in the Introduction, one group implicated the protein tyrosine phosphatase Tc-PTP. Another group correlated refractoriness of the cell with an increase in STAT1 content (see Introduction). A third study analyzed the responsiveness of human DC to bacterial LPS, a known DC maturation factor and inducer of type I IFN (Severa et al., 2006). The authors showed that immature DC are equally sensitive to IFN α2 and IFN β. On the other hand, LPS- or IFN β-matured DC fully respond to IFN β but are impaired in their response to IFN α2. This was the first report of α2/β differential desensitization. Interestingly, the level of IFNAR1 (surface and total content) was shown to be reduced in IFN β-matured DC as compared to immature DC, but the precise mechanism was not defined.

In collaboration with G. Uzé, we showed that α2/β differential desensitization occurs in primary and transformed cells of different lineages. In contrast to what seen in IFN β-matured DC, differential desensitization in fibroblastic-type cells and non-adherent cells (T-cell blasts) appears independent of surface receptor downregulation. Nonetheless, we found that, in desensitized fibroblasts, IFN α2 exhibits a reduced apparent binding affinity and consequently a lower activity in STAT-mediated signaling. On the other hand, the activity of IFN β is preserved, owing to its elevated affinity for the receptor.

We showed that the extent of differential desensitization is controlled by the amount of endogenous USP18, a cysteine protease specialized in removing the ubiquitin-like ISG15 from ISGylated proteins. We demonstrated that the constitutive expression of USP18 in naïve cells blunts IFN α response at the level of assembly with the receptor complex.

Previous to our work, murine Usp18 had been shown to negatively regulate type I IFN signaling. It was proposed that Usp18 interacts with the juxtamembrane box1-box2 motifs of IFNAR2 and consequently causes a displacement of Jak1 (Malakhova et al., 2006). For this analysis, the authors used murine Usp18 and human IFNAR2. The Usp18 binding site on IFNAR2 was mapped using a GST-IFNAR2 fusion in which the box1-box2-like sequence was deleted (Malakhova et al., 2006). It should be noted that IFNAR2 devoid of

the box1-box2-like sequence is expected not to bind Jak1, and this independently of the presence of Usp18.
By co-immunoprecipitation studies, we confirmed the ability of USP18 to interact with IFNAR2. However, we did not observe a displacement of Jak1 but, on the contrary, we observed a small but reproducible increase in the level of endogenous Jak1 associated with IFNAR2 in desensitized cells (Fig. 5A in Additional results).

An alternative possibility is that Usp18 interacts indirectly with IFNAR2, e.g by interacting with Jak1. To test this hypothesis in vitro assays using purified recombinant USP18-HA and full-length or truncated IFNAR2-GST could be used. Moreover, co-immunoprecipitation experiments between IFNAR2 and USP18 could be assayed in USP18-expressing cells silenced for Jak1.

Whether Usp18 interacts with the IFNAR2/Jak1 complex or only with Jak1, in both cases this could impair the formation of the binary ligand/IFNAR2 complex.
However, this model does not fit with the following observations. The current model of Type I IFN/receptor assembly is that IFN binds first to IFNAR2 and this binary complex recruits IFNAR1 to form the ternary complex (Gavutis et al., 2006). USP18-expressing cells are desensitized to IFN α2 and marginally to IFN β. These cells are also desensitized to a mutant of IFN α2 (IFN α2α8 tail) which is engineered to have higher affinity to IFNAR2 (comparable to the affinity of IFN β for IFNAR2), but are not desensitized to a mutant of IFN α2 (IFN α2-HEQ) which is engineered to have higher affinity to IFNAR1 (comparable to the affinity of IFN β for IFNAR1). These results suggest that the formation of the binary complex is less affected by USP18 than the formation of the ternary complex.

Is the deISGylase activity of USP18 required for differential desensitization?
We showed that USP18 catalytic activity is required for the regulation of the IFN response (Fig 1A in Additional results). Furthermore, our data showed that the ISGylation machinery is essential for USP18 to exert its function, at least in primed cells, and that the E3 enzyme EFP/TRIM25, rather than HERC5, is implicated in the ISGylation of a putative USP18 substrate(s) (Fig. 4A and B in Additional results). The substrate could be an ISGylated protein that is important for efficient IFN α-driven ternary complex formation.

However, the analyses of Usp18, ISG15 and Ube1L-deficient mice do not support an ISG15-targeted function of Usp18. Usp18-deficient mice die early from brain injury, they are hypersensitive to the IFN inducer poly I-C and MEF show sustained STAT1 phosphorylation in response to type I IFN (Malakhova et al., 2003). Furthermore, these mice are less susceptible to certain viral infections. This phenotype is perfectly compatible with a defect in the control of IFN α responses.

ISG15-deficient mice are healthy, albeit more sensitive to a subset of viruses and MEF do not show obvious defects in type I IFN signaling (Osiak et al., 2005).

Moreover, the Usp18 phenotype is not rescued in ISG15/Usp18 or Ube1L/Usp18 DKO mice, demonstrating that uncontrolled protein ISGylation is not the causality of the phenotype of Usp18-deficient mice (Knobeloch et al., 2005).

Therefore, the analyses of these KO mice raise serious doubts on the role of the deISGylase activity of USP18 in the negative control of the IFN response.

USP18, a de-ubiquitinase ?

Interestingly, several features of the ISG15 system are more closely related to the ubiquitin system than to other ubiquitin-like systems. Notably, ISG15 is the only ubiquitin-like molecule where the last six residues (LRLRGG) are identical to those present in ubiquitin. These similarities could be indicative of functional or regulatory overlap between these two pathways. For instance, the ISG15 E3 ligase EFP/TRIM25 can serve as an E3 ligase for K63-linked polyubiquitins (Gack et al., 2007).

To our knowledge, the Km for deISGylation and the Km for deubiquitination have never been determined for USP18. In a systematic search for deISGylases, Ploegh and co-workers identified, among many USPs, USP18 and USP5. Interestingly, this latter enzyme, also known as IsoT, is specific of the degradation of *unanchored* K63-linked polyubiquitins (Xia et al., 2009).

Thus, the possibility that USP18 acts on a ubiquitinated substrate cannot be dismissed.

Interestingly, it has recently been shown that, in an overexpression system, Tyk2 can be heavily ubiquitinated by K63-linked ubiquitin chains, but not by K48-linked ubiquitins (Piganis et al., 2011). The site(s) and the role of this modification is unknown. It was shown that overexpressed SOCS1, an early IFN-induced negative regulator of signaling, can reduce the level of these K63-linked ubiquitination of Tyk2, resulting in the destabilisation

of Tyk2 and exposure of IFNAR1 internalisation motif with subsequent IFNAR1 internalisation.

Modified Tyk2 could be a substrate of USP18. Deubiquitination of Tyk2 by USP18, similar to what was proposed for SOCS1, may promote the exposure of IFNAR1 internalisation motif and its subsequent internalisation and ubiquitination by βTrCP (see model in Fig. 10). This model could perhaps explain the poor surface replenishment of IFNAR1 in mature monocyte-derived DC (Severa et al., 2006).

USP18 and CYLD

Another possibility is that USP18 deISGylates a substrate which then undergoes rapid ubiquitination. Several examples have been documented where ISGylation of a protein protects it from ubiquitination (see Introduction, section 6.3). Interestingly, it has recently been reported that CYLD (cylindromatosis), a deubiquitinase that cleaves K63-linked ubiquitin chains, positively regulates Type I IFN signaling (Zhang et al., 2011). Upon IFN β stimulation, DC derived from CYLD-/- mice were shown to have a block in STAT1 phosphorylation, measured between 20 min and 3 hr of IFN stimulation, and showed a dramatic decrease in ISG induction as compared to WT DC. Upon IFN treatment or upon stressed conditions (such as viral infection), IFNAR1 is rapidly ubiquitinated via K63- and K48-linked ubiquitin chains and is internalised (Introduction, section 3.1). Thus, one possibility is that CYLD positively controls IFN receptor signaling by deubiquitinating the K63-linked polyubiquitin chains of IFNAR1. The level of IFNAR1 in CYLD -/- DC was not monitored.

Interestingly, CYLD is a known negative regulator of inflammation. It interacts with the cytosolic sensor RIG-I via the RIG-I N-terminal caspase recruitment domain (CARD). This interaction prevents EFP/TRIM25-mediated K63-linked ubiquitination of RIG-I and therefore inhibits the activation of RIG-I (Zhang et al., 2008). It has also been reported that CYLD is able to physically interact with the E3 ligase Itch, and that the Itch-CYLD complex sequentially cleaves K63-linked ubiquitin chains and catalyzes K48-linked ubiquitination of the kinase Tak1 to terminate inflammatory signaling initiated by tumor necrosis factor (TNF) (Ahmed et al., 2011).

USP18 and CYLD seem to have opposing effects. One appealing model would be that CYLD de-ubiquitinates IFNAR1, promoting its function at the cell surface, i.e binding and ternary complex formation

In IFN-primed cells, there is a considerable increase of ISG15, ISGylation enzymes and USP18. In contrast to what is observed in RIG-I regulation, CYLD and EFP could be friends instead of foes and ultimately promote the ISGylation of IFNAR1. ISGylated IFNAR1 might be protected from ubiquitination. In the presence of USP18, modified IFNAR1 will be deISGylated and handed over to ubiquitin E3 ligases (see model in Fig. 10).

In our study, the levels of IFNAR1 and IFNAR2 at the cell surface of IFN-primed cells were unchanged with respect to levels on naïve cells. Furthermore, no change in the level of the receptor subunits could be observed in naïve cells expressing constitutive USP18. It should be said that cell surface and total steady-state levels of IFNAR1, as signals in flow cytometry and western blot, are rather low. If a subtle change occurs upon expression of USP18, this may be difficult to appreciate.

However, other reports have shown that expression of USP18 does not affect the level of IFNAR1 or IFNAR2. The group of Zhang monitored the cell surface level of IFNAR1 and IFNAR2 in the leukemic KT-1 cells silenced for USP18 (Malakhova et al., 2006). No detectable differences in the steady-state or IFN-induced surface levels of either subunits of the IFN receptor were detected in USP18-silenced cells as compared with control cells.

Furthermore, in a high-throughput screen to identify deubiquitinases that regulate the level of EGF-R in squamous cell carcinoma, Duex et al identified USP18 as a positive regulator of the expression of EGF-R (Duex and Sorkin, 2009). Overexpression of USP18 elevated EGF-R levels in a manner requiring the catalytic cysteine of USP18. Importantly, these authors also reported as « data not shown » that USP18 does not alter the expression of IFNAR1 and IFNAR2 at the cell surface.

Our results together with these published data suggest that USP18 does not affect the level of IFNAR1 and IFNAR2 at the cell surface. However, a deeper analysis of a possible functional link between USP18 and IFNAR1 level is needed. Monocyte-derived dendritic cells isolated from WT or Usp18-/- mice could represent a good cell system to study the effect of USP18 on IFNAR1 level.

A non-exclusive possibility to account for the effect of USP18 on the ligand binding activity of the receptor - not implicating a reduction in receptor level - is that USP18, once it associates to IFNAR2, alters the spatio-temporal dynamics of IFNAR2 and/or IFNAR1 at the plasma membrane. Thus, in collaboration with J. Piehler (Osnabruck University), single molecule tracking of IFNARs at the plasma membrane has been set up in order to measure potential differences in trajectories, diffusion and/or confinement of the receptors in control cells versus USP18-expressing cells.

USP18-mediated establishment of α/β differential activities : physiological relevance

IFN α2 and IFN β exhibit comparable potencies in early STAT activation, ISGF3-driven transcription and antiviral activity against a large panel of viruses. However, their non-redundant function is best illustrated by their different potency in growth suppression or apoptosis. We showed that these α2/β differential activities are governed in part -if not entirely- by the expression of USP18 (Francois-Newton, Biochem J. *in revision*).

It is remarkable that, at least in humans, the 13 IFN α gene sequences have been selected for non optimal affinity to the receptor chains and that precisely this weakness allows α/β differential bioactivities and differential desensitization. Thus, in a viral infection, IFN α is likely to be expressed at high concentration from the multiple genes. It limits the spread of the virus by exerting its potent antiviral action in a timely regulated mode on cells that will then be desensitized. On the other hand, the single IFN β - induced alone or co-induced with IFN α upon viral infection - is optimized to bind the receptor chains with high affinity and thus can function at low dose and it retains activity on cells desensitized for IFN α. This exclusive property of IFN β may be critical for cellular homeostasis. After a viral infection, Type I IFN signaling must be downregulated by USP18 to preserve the cells from the apoptotic effect of IFN. At the same time, the low constitutive production of IFN β will modulate the homeostatic balance. In the early eighties, it was proposed that IFN is induced by ongoing low-grade exposure of the mucosa to external pathogens, by tissue remodeling or damage (Bocci, 1980). The importance of constitutive IFN β in maintaining immune balance was then revealed by studies examining the aberrant phenotype of mice lacking IFNAR1. IFNAR1-deficient mice have decreased numbers of splenic NK cells and B220-positive B lymphocytes and increased $CD11c^{+}$ myeloid cells (Swann et al., 2007). In the absence of constitutive IFN β signaling, murine hematopoietic cells exhibit enhanced

proliferative responses to low doses of CSF-1 (colony stimulating factor-1) and increased expression of the activation markers CD11c and CD11b (Gough et al., 2012 ; Honda et al., 2003).

The role of USP18 in cellular homeostasis may not be negligeable. A recent study in a murine infection model showed that Usp18 can be critical to the establishment of antiviral immune responses (Honke et al., 2011). By restraining IFN responses in macrophages resident in the splenic marginal zone, basal Usp18 allows local permissive VSV infection that is necessary to secure sufficient antigen production and activation of the adaptive immune response.

Moreover, Usp18 appears to regulate the development of DC, since Usp18 -/- mice show a 50% reduction in the frequency of conventional CD11b+ DC in the spleen. Furthermore, in the presence of GM-CSF, bone marrow-derived DC are less efficiently generated from Usp18-/- bone marrow than from control bone marrow. This appears to be due to upregulation of SOCS1 and SOCS3 proteins occurring in Usp18-/- cells (Cong et al., 2012).

On the other hand, in clinical settings, USP18 may counteract the efficacy of therapeutic IFN α as, for example, in chronically HCV infected patients, where a high USP18 level in pre-treatment livers has been associated with poor response to treatment (Chen et al., 2005; Sarasin-Filipowicz et al., 2008).
Being a protease, the development of small molecule inhibitors of USP18 catalytic activity could be envisaged. These compounds may be used to boost the antiviral response of cells to IFN and its administration to chronically infected HCV patients may increase the efficacy of the IFN treatment. On the other hand, this may lead to hypersensitivity to Type I IFN that could promote auto-immune manifestations.

Type I and type III IFNs are produced by similar stimuli and exhibit common bioactivities and synergize in antiviral activity towards several viruses, including HCV. Our data demonstrate that priming of cells with IFN λ renders them desensitized to IFN α but not to IFN β or IFN λ, via the induction of USP18. Importantly, this was observed also in human hepatocytes that indeed express the IFN λ-specific receptor subunit IFNLR1 (Doyle et al., 2006). Ongoing clinical trials for treatment of HCV chronically infected patients with IFN λ are giving promising results (Muir et al., 2010). The sustained sensitivity to IFN β and

IFN λ, despite preactivation of signaling, provides support for further clinical exploration of treatment of IFN α-nonresponders with IFN β and IFN λ.

A strong predictive factor of spontaneous HCV clearance and successful treatment of chronically HCV infected patients with pegIFN α and ribavirin is the IFN λ3 (IL-28B) genotype. Paradoxically, the good response IFN λ variant, i.e. predicting higher success rate of IFN α-based therapy was found to be associated with higher viral load. The prediction of higher success rate of IFN α-based therapy is also associated with weak expression of hepatic ISG (Ge et al., 2009; Suppiah et al., 2009; Thomas et al., 2009). These consistent observations have spurred intensive studies to try to relate IL28B genotype with the level of hepatic ISGs. To date, contrasting data have been reported that do not provide a clear picture. Likewise, we are still missing analyses of which of the variants, if any, alters the expression level and/or the potency of IFN λ.

Perspectives

In conclusion and with the critical contribution of many colleagues in the laboratory, I have shown that the cysteine isopeptidase USP18 dampens specifically the response of human cells of different lineages to multiple IFN α subtypes while leaving nearly intact the response to IFN β and IFN λ1. A number of approaches were used to try to uncover the specific mode of action of USP18. However, important issues remain unanswered and these may be grouped into the following two main questions.

How does USP18 regulate type I IFN signaling?

One key question is whether the catalytic activity of USP18 is needed for its negative regulatory role. Experiments presented in the section Additional results were performed using human fibroblastic "naïve" cells stably expressing wt or catalytically inactive USP18. However, in this context the need for catalytic activity may be "masked" or abolished due to the absence or the low level of ISGylated substrate(s).

The group of Knobeloch has recently engineered C61A Usp18,knock-in mice. Using primary murine embryonic fibroblasts (MEF) from these mice, kindly provided to us, we will be able to truly monitor the catalytic role of Usp18 on dampening the IFN response in physiological-like conditions of cells stimulated with IFN, ie where Usp18 C61A is co-induced with ISG15 and the level of ISGylated proteins is increased. Stat activation will be

analyzed in WT and C61A knock-in MEFs after prolonged IFN-stimulation and after priming.

Moreover, if the catalytic activity proves to be required for negative function of Usp18, it will be important to ascertain whether this represents a deISGylase or a deubiquitinase activity.

We know that USP18 blocks IFN signaling at an early step of the cascade, ie at the level of activation of the Jak enzymes. Thus, potential substrates of USP18 include IFNAR2, IFNAR1, Tyk2 and Jak1.

We were able to detect a small proportion of ISGylated IFNAR2 in USP18-silenced/primed cells. Thus, ISGylated IFNAR2 could be a *bona fide* substrate of USP18. Nonetheless, we cannot rule out the possibility that ISGylated IFNAR2 could be dispensable for USP18 action or that it could act as recruitment site for USP18. It would therefore be interesting to identify which lysine residues in IFNAR2 are ISGylated by mass spectrometry and reconstitute the IFNAR2-deficient U5A cells with lysine mutated forms of IFNAR2. The response of these cells to IFN α2 and IFN β will then be monitored.

Another potential substrate of USP18 is Tyk2. Tyk2 was shown in Piganis *et al* to be basally modified by K63-linked polyubiquitin chains. Could such ubiquitinated Tyk2 be a substrate of USP18? In this event, a deubiquitinase action of USP18 could be invoked. It would therefore be interesting to study the ubiquitination state of basal and activated Tyk2 in the presence or in the absence of USP18.

How does free ISG15 sustain USP18 level?

Another unexpected observation that I have made is that free (unconjugated) ISG15 plays a role in sustaining the level of USP18. Based on results obtained using the protein synthesis inhibitor cycloheximide, one possibility is that ISG15 protects USP18 from degradation. To test this hypothesis the extent of ubiquitination of USP18 will be monitored in 293T cells transiently co-transfected with USP18 and ubiquitin in the presence or absence of ISG15.

Additional experiments using proteasome or lysosome inhibitors will be needed to monitor whether USP18 is less stable and more prone to degradation in ISG15-silenced cells.

Furthermore, the effect of ISG15 on Usp18 needs to be studied in the murine system. For this, the level of Usp18 that accumulates after IFN stimulation will be compared in wt MEF and in ISG15-/- MEF (a generous gift of Dr Knobeloch).

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