Focus: Type I interferons

The molecular basis for functional plasticity in type I interferon signaling

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Type I interferons (IFNs) are best known for their role in innate immunity, but they are also involved in other functions including immunomodulation, restricting proliferation, cancer surveillance, and the regulation of the adaptive immune response. All these responses are mediated through the interaction with a single cell surface receptor, albeit at different ligand and receptor concentrations, ligand subtypes, and time of activation. Here we review the functional plasticity of IFN signaling from a quantitative perspective, showing how variations in different ingredients of the system lead to differential IFN responses and how cells tune the system to maximize efficiency while minimizing detrimental effects. We present a basic model wherein the integrated action of different feedback mechanisms can provide sufficient temporal control to differentially drive cellular decisions.

IFNs as a paradigm of cytokine receptor plasticity

Cytokine surface receptors recognize signals from the outside and transmit them into the cell, driving a pleiotropy of cellular responses. Signal transmission across the plasma membrane is a result of the cytokine interacting with the extracellular domains of the receptor subunits, placing them in proximity to one another and/or driving conformational changes [1]. Receptor dimerization results in the activation of cytosolic Janus family tyrosine kinases (JAKs), which in turn initiate downstream signaling cascades that propagate the signal into the nucleus and regulate gene transcription, mainly via signal transducer and activator of transcription (STAT) proteins [2,3]. The translation of cytokine recognition by the receptor into cellular decisions is highly plastic and in many cases not only depends on the cellular context but also on the molecular determinants of the cytokine–receptor interaction [3]. Thus, numerous instances of differential cellular responses elicited by different cytokines through the same cell surface receptor have been reported [3,4].

Here we focus on providing a critical review of the plasticity of type I IFN signaling, which is encoded by the binding of different IFNs to a shared cell surface receptor comprising two subunits: IFNAR1 and IFNAR2 (Figure 1A) [5]. This, in turn, results in the activation and repression of over 2000 genes with a multitude of biological functions [6]. We are particularly intrigued by the nonlinear signaling, where a seemingly similar input (ligand–receptor interaction) promotes a multitude of biological outputs that are activated by different signaling cascades. Some of these outputs have key functions in innate and adaptive immune responses while others have been shown to be detrimental to health, apparently due to cytotoxicity and induced inflammation [7]. Thus, the tight control of this system is of foremost importance. New insights into this system provide a basic model where the actions of known activators, inhibitors, and feedback loops offer sufficient control of the system.

The molecular determinants of IFN receptor assembly

The human type I IFN system comprises 17 different ligands binding to two receptor subunits. The ligands include 13 IFNα subtypes, IFNβ, IFNω, IFNs, and IFNε. IFNs is a unique case as it is a signaling molecule during pregnancy and it is not discussed further here [8]. The various IFNαs share 80% sequence homology between them and IFNω and IFNβ have 50% and 30% homology, respectively. The divergence of multiple IFNα subtypes occurred separately in different species, supporting the concept that different vertebrate groups have independently expanded their IFN subtypes [9,10]. All type I IFNs bind the receptor subunits at the same location, producing structurally highly similar ternary ligand–receptor complexes [11–14]. The main difference between the various IFN subtypes lies in their binding affinity toward the receptor subunits (Figure 1B). In general, the binding affinity to IFNAR2 is high (of the order of nanomolar affinity) and to IFNAR1 it is low (micromolar affinity) [15–17]. Binding affinity, however, varies by about 1000-fold between the different ligands. The weakest binder to IFNAR2 is IFNα1 and the tightest is IFNβ (200 nM versus 0.2 nM affinity) [16,18]. All IFNαs bind IFNAR1 with similar low affinities of 1–5 μM, while IFNβ binds significantly more tightly, at 50 nM affinity [17,18]. Despite some allosteric crosstalk [19], the binding affinities of IFNs to each receptor subunit are hardly influenced by the interaction with the other receptor subunit [20]. IFNβ therefore has overall a substantially higher ‘integral’ affinity to the cell surface receptor compared with all other natural IFNs [18,21,22], which has been suspected to play a decisive role in the pronounced differential cellular activities of IFNβ. We have succeeded in reproducing the entire range of binding affinities of natural
IFNs by mutating IFNo2, showing that the variation in binding affinity accounts for much of the variation of different natural IFNs[12,23].

In cell-based assays, IFN activity is most often probed by its ability to induce JAK/STAT phosphorylation and gene induction, as well as antiviral and antiproliferative cellular responses. The antiviral state is observed in all cell lines on activation for a few hours by picomolar IFN concentrations and is maintained even after subsequent removal of the IFN [24]. We therefore designated the antiviral response a ‘robust’ activity of IFNs (Figure 1B). By contrast, the antiproliferative activity of IFNs is cell-type specific, requires about 1000-fold higher interferon concentrations, is activated at physiological IFN concentrations only by IFNβ, requires continuous receptor activation over prolonged periods (days), and is susceptible to the concentration of cell surface receptors [25,26]. Such IFN activities, which strongly depend on the cellular context, were designated ‘tunable’. Studies of various natural IFNs and IFN mutants have shown a clear linear correlation between binding affinity and antiproliferative activity, as long as the affinity toward IFNAR1 is not reduced below approximately 100 μM [18,25,27]. Antiviral activity, by contrast, is promoted even with extremely weak-binding IFN mutants that do not promote antiproliferative activity. In contrast to antiproliferative activity, the antiviral potency of IFNs is maximized already at medium receptor binding affinity (possessed by most IFNαes), with the higher binding affinity of IFNβ hardly increasing the antiviral potency above that of IFNo2 or even IFNo1 (Figure 1B) [25,26].

The IFN receptor subunits comprise an extracellular domain containing four and two fibronectin type III domains for IFNAR1 and IFNAR2, respectively, a single transmembrane helix, and a cytoplasmic domain of 100 and 251 amino acids for IFNAR1 and IFNAR2, respectively, that is mostly unstructured (Figure 1A). The structures of the isolated extracellular portions of the receptors and IFNs, as well as the receptors bound to IFNo, IFNo2, and IFNβ, have been solved to high resolution [11,12,28]. The recognition of IFNs by IFNAR1 and IFNAR2 has been recently reviewed [29]. To summarize, the high-resolution structures revealed that different type I IFNs bind the receptors at the same location, resulting in a very similar ternary structure independent of the ligand subtype. The variation in amino acid sequence between the different type I IFNs represents a multitude of parallel solutions that support binding at various affinities [12]. Comparing the unbound with the bound structure reveals rearrangements of the membrane-proximal domains of the receptors in relation to the membrane-distal domains on ligand binding. This reordering is particularly obvious for IFNAR1 [12], where a
first-order rate constant of approximately 1 s\(^{-1}\) was determined [30]. The role of the observed structural rearrangement on the biological activity of the receptors is currently under investigation. Alanine scanning of the IFN–IFNAR2 interface has identified a classical arrangement of binding energies found in protein–protein interfaces, with the hot-spot residues in the center surrounded by less important residues and the peripheral residues not contributing significantly to binding [31–33]. Interestingly, the most varied sequence in IFNα is the C-terminal unstructured tail, where little homology is observed between the subtypes. Replacing the tail of IFNα2 with that of IFNα8 increased its binding affinity and biological activity by approximately 15-fold [34].

Alanine scanning of the low affinity IFN–IFNAR1 interface did not reveal any hotspots, with several mutations resulting in a decrease of three- to fivefold in binding affinity [35]. However, replacing Arg120 on IFNα2 with glutamic acid almost completely eliminates binding to IFNAR1 [36]. Surprisingly, mutation of any one of the three clustered amino acids His57, Glu58, and Gln61 to alanine increases binding by about threefold, with the triple HEQ mutant increasing binding by 20-fold [23]. Further optimization using phage display generated the triple mutation H57Y, E58N, and Q61S (YSN), which binds IFNAR1 60-fold tighter than IFNα2 and three fold tighter than IFNβ [37]. The conservation of His, Glu, and Gln at positions 57, 58, and 61 in all IFNs subtypes suggests that weak binding to IFNAR1 is an evolutionarily conserved trait. Strikingly, the largely overlapping activity profiles of YNS and IFNβ corroborate that differential IFN activity is directly related to receptor binding affinities (Figure 1B) [23,37].

Assembly and dynamics of the signaling complex in the plasma membrane

The striking correlation between the receptor binding properties and activity profiles of IFNs suggests a key role of receptor assembly and dynamics in signaling specificity. The mechanism of cytokine receptor assembly has been debated during the past decade. The original observation of human growth hormone receptor dimerization caused by simultaneous interaction of the ligand with the receptor subunits has long been viewed as the dominant mechanism [38]. This mechanism is in line with the asymmetric binding affinities of the ligand to the receptor subunits, which is also prominent in the case of the IFN receptor. The latter suggests a binding mechanism, where ligand binding to the high-affinity subunit is followed by recruitment of the low-affinity subunit at the plasma membrane (see Figure I in Box 1). However, following the observation of a

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**Box 1. Spatiotemporal dynamics of IFN receptor assembly and activation**

Ligand-induced receptor assembly and the formation of dynamic ternary complexes is controlled by several rate constants, as depicted in Figure 1. IFN binding to the cell surface is dominated by the approximately tenfold higher association rate constant of the interaction with IFNAR2 (\(k^{+}_{0}\)) compared with IFNAR1 [62,95]. Subsequently, IFNAR1 is recruited by 2D interactions in the plasma membrane to form the ternary signaling complex. In model membranes, this reaction has been demonstrated to be far from diffusion control despite an approximately 100-fold reduced mobility of the interacting partners [62,95]. The dynamic equilibrium between binary and ternary complexes at the plasma membrane is determined by the 2D IFNAR1 equilibrium dissociation constant (\(K^{+}_{D}\)) versus the receptor concentration. Thus, the 100-fold higher IFNAR1 binding affinity of IFNβ compared with IFNα allows efficient IFNAR1 recruitment at much lower receptor concentrations. Importantly, the total IFN binding affinity to the cell surface receptor depends on the equilibrium between the binary and ternary complexes, because the dissociation probability (and thus the equilibrium dissociation constant of cell surface binding) of IFN simultaneously interacting with IFNAR1 and IFNAR2 is substantially decreased (more than 100-fold for IFNα2 [17]) compared with the dissociation from IFNAR2 alone (\(K^{+}_{D}\)). The molecular lifespan of individual signaling complexes is determined by the 2D dissociation rate constant, \(k^{\circ}\). Until dissociation occurs, receptor phosphorylation occurs followed by signaling activity. However, in the absence of further stabilizing mechanisms (e.g., endocytosis; cf. Figure 2 in main text), complexes can dissociate and are subject to dephosphorylation. It is possible that dissociated receptor subunits remain signaling competent, which could allow serial activation of multiple receptors by a single ligand [64]. However, plasma membrane mosaicity may introduce further determinants of receptor dynamics. Diffusional encounters by the receptor subunits are more complicated due to the diffusion barriers caused by the cortical actin skeleton [membrane skeleton (MSK)]; inside coralls of approximately 100 nm in diameter, fast local diffusion is possible, which is reduced by a factor >10 over micrometer distances due to the reduced probability of crossing the actin-mediated fences [96]. For receptor dimerization, both receptor subunits need to be localized within the same corral. Importantly, transient receptor confinement may also ensure reassociation of dissociated receptor dimers and thus maintain active ternary signaling complexes over longer time periods. Such ‘signaling bursts’ have been theoretically predicted.

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**Figure 1.** Putative ligand-induced dimerization and dynamics of the interferon (IFN) receptor. After IFN binding to the high-affinity subunit IFNAR2, IFNAR1 is recruited on the membrane to form the ternary signaling complex. Activated by mutual interactions, Jak1 and Tyk2 phosphorylate each other as well as tyrosine residues in the cytoplasmic receptor domains. Dissociation of the complexes, signaling is abrogated by dephosphorylation. More details are provided in Box 1. [TRENDS in Immunology]
ligand-independent dimer within a crystal structure of the erythropoietin (Epo) receptor ectodomain [39], increasing evidence for the preassembly of several cytokine receptors has emerged [40–43]. For the homodimeric cytokine receptors (growth hormone, Epo), pre-dimerization via the transmembrane domains has been proposed [40–42]. These findings were followed by other heterodimeric cytokine receptors [44–49] including IFNAR [50], for which pre-dimerization was suggested. Interestingly, an important role of the associated JAKs in receptor pre-dimerization has also been proposed [50]. Taken together, these findings suggest that receptor dimerization may be mediated not only by the extracellular domains but also by other parts of the transmembrane receptor. These concepts are in line with recent studies on the epidermal growth factor receptor (EGFR), for which a subtle interplay of interactions, including the transmembrane domains and the kinase domains, was demonstrated [51]. Importantly, these rather weak interactions may only marginally contribute to receptor dimerization but play a key role in JAK activation [52] and in regulating receptor activity [51]. However, these weak interactions may also be responsible for inducing receptor dimerization on overexpression, which is required for most dimerization assays. Autoactivation of receptors caused by receptor overexpression has been reported in some cases (e.g., for the EGFR), which may be caused by shifting the equilibrium toward receptor dimers. Moreover, there is ample evidence that, at physiological receptor expression levels, receptor dimerization is induced by the ligand. This includes the role of receptor expression levels in the observed cell surface binding affinity (Box 1) as well as the bell-shaped dose–response curves observed for homodimeric receptors [53–55].

As is the case for many other cytokine receptors, the physiological expression level of IFNAR1 and IFNAR2 is very low – typically a few hundred copies per cell [15]. Traditional ensemble fluorescence microscopy cannot be applied at such cell surface receptor concentrations, requiring the use of single-molecule imaging techniques. Single-molecule tracking of individual IFNAR1s and IFNAR2s at physiological cell surface expression levels has revealed a random distribution and independent diffusion in the plasma membrane [56,57]. On addition of IFN, a minor decrease in receptor mobility was observed, which probably can be attributed to ligand-induced dimerization of IFNAR1 and IFNAR2 [58]. Similar spatiotemporal dynamics have been observed for fluorescent IFNα2 bound to endogenous IFNAR [59]. By contrast, super-resolution imaging revealed strong co-clustering of overexpressed IFNAR1 and IFNAR2 [60], corroborating the potential experimental bias caused by high densities of receptors. While such effects are clearly not detectable at physiological receptor expression levels, transient receptor confinement in actin-dependent membrane microdomains was identified [57], which was also related to STAT recruitment in transient signaling zones [57]. The spatiotemporal organization of receptor subunits and the signaling complex identified by single-molecule tracking suggests an even more complex interplay of interactions and receptor confinement involved in the dynamics of IFN signaling complexes.

Based on available evidence, we propose that, at physiological receptor levels, the molecular interactions of IFN with its receptor subunits dominate receptor dimerization. This assumption is in line with the key role of IFN binding affinity toward IFNAR1, which has been shown to be responsible for the differential activity between IFNα2 and IFNβ [23]. Assuming a two-step assembly mechanism as depicted in Figure I in Box 1, the interaction between IFN and IFNAR1 determines the 2D equilibrium between binary (IFNAR2/IFN) and ternary (IFNAR1/IFNAR2/IFN) complexes in the plasma membrane. In the case that the 2D equilibrium dissociation constant ($K_d^2$) significantly exceeds the cell surface concentrations of IFNAR1 and IFNAR2, efficient complex formation is abrogated. Importantly, this equilibrium can only partially be compensated by IFN concentrations, as only IFN bound to IFNAR2 contributes to ternary complex formation. In vitro studies of receptor dimerization in artificial membranes suggested that the binding affinities of IFNs toward IFNAR1 limit ternary complex formation at physiological receptor expression levels, while no significant contribution of the transmembrane domain to receptor dimerization was detectable [61–63]. Thus, substantially more efficient ternary complex formation by IFNβ compared with IFNα2 can be assumed at physiological receptor expression levels, which is in line with the observation that the differential activity of IFNβ compared with IFNs is abrogated on increasing receptor cell surface expression [64]. Notably, increased binding affinity toward IFNAR1 is accompanied by an increased lifespan for individual signaling complexes (cf. Box 1). The exact role of complex lifespan in signaling activity has remained unclear. Indications that IFNα2 may even surpass the potency of IFNβ at high receptor expression levels have been interpreted by a model of ‘serial activation’; that is, the ability of a single ligand to sequentially activate multiple receptors (see Figure I in Box 1) [64]. Moreover, differential binding affinities have also been linked to differential receptor endocytosis [23] – probably another key event in the regulation of (differential) IFN activity (see below). Although the exact correlation between molecular interaction properties and the spatiotemporal dynamics of receptor assembly and signal propagation remain to be elucidated, its key role in understanding functional receptor plasticity is clear.

**Effectector activation at the IFNAR signaling complex**
The cytoplasmic domains of the IFNAR subunits are associated with various effector proteins responsible for propagating the signal to the nucleus to regulate gene activation mainly via the JAKSTAT pathway [65]. Key effector proteins are the JAKs Jak1 and Tyk2, which are associated with IFNAR2 and IFNAR1, respectively. Dimerization of IFNAR1 and IFNAR2 probably promotes interactions between Jak1 and Tyk2, thereby allowing further activation of kinase activity via cross-phosphorylation. Furthermore, several tyrosine residues of IFNAR1 and IFNAR2 are phosphorylated by the activated JAKs, which serve as docking sites for other effector proteins. The hallmark of IFN signaling is the formation of a pSTAT1/pSTAT2 heterodimer, which in complex with IFN regulatory factor 9 (IRF9) forms the transcription
factor IFN-stimulated gene factor 3 (ISGF3), which promotes transcription of key ISGs (Figure 1A). While STAT proteins are supposedly recruited to pTyr via their SH2 domains, STAT2 was found to bind IFNAR2 via a constitutive, phosphorylation-independent binding site [66]. STAT1 in turn was shown to be recruited via STAT2 [66]. However, a tyrosine phosphorylation site of IFNAR2 is required for STAT phosphorylation [67]. Recently, live-cell micropatterning was established to probe the interaction of STAT proteins with the IFNAR in living cells. This method is based on spatially reorganizing bait proteins in the plasma membrane by means of micropatterned capturing groups on a solid support [68]. Thus, effector interactions with IFNAR can be monitored in situ and quantified with respect to complex stabilities. These studies confirmed constitutive STAT2 binding to IFNAR2 as well as docking of STAT1 via STAT2 [68]. The role of the constitutive STAT2 binding site in STAT1/STAT2 phosphorylation remains under debate, as mutation studies suggested that constitutive STAT2 binding may not be mandatory [69]. Interestingly, increased STAT recruitment to the receptor was observed on formation of the ternary signaling complex [68], which could be caused by cooperative STAT binding to IFNAR2 via constitutive and phosphorylation-dependent sites. Beside pSTAT1/pSTAT2 heterodimers, homodimeric pSTAT1 is also generated on IFNAR activation, which is responsible for the regulation of IFN-γ-activated sequence (GAS) elements, although STAT2 was reported to be essential for STAT1 phosphorylation [70]. However, uncompromised STAT1 phosphorylation on RNAi-mediated silencing of STAT2 has been observed [71], suggesting more complicated and potentially redundant mechanisms of STAT1 recruitment. While the fundamental mechanism of ISGF3 formation appears to be distinct from classic STAT activation, the control of homo- versus heterodimer formation may play a critical role in the functional plasticity of IFN signaling.

Characteristics of robust and tunable IFN activities

IFN activities can be divided into robust and tunable, as discussed above [26]. The most obvious robust activity is the antiviral activity of IFNs, which is common to all cells and serves as the first line of defense against viral and other pathogen attacks [8]. This response is a basic constituent of innate immunity and its absence is one of the reasons for the high lethality associated with Ebola virus infection [72]. Interestingly, robust responses are induced with similar potencies by all IFNs despite their highly varying receptor binding affinities. By contrast, tunable responses are induced at physiological IFN concentrations mainly by the high affinity binding IFN. Thus, a primary cause of receptor plasticity is the different affinity–activity relationships of robust and tunable cellular responses (Figure 2).

Examples of the tunable activities of IFNs are their antiproliferative (apoptosis and cell cycle arrest) activities and at least some of their immunomodulatory functions. The robust and tunable activities have a strong signature in IFN-induced gene induction [27]. Robust genes that are rapidly induced by low IFN concentrations for a short time are annotated with Gene Ontology biological process terms from the Database for Annotation, Visualization, and Integrated Discovery (DAVID) relating to response to viruses, biotic stimulus, and MHC class I. Tunable genes are annotated with cytokine and chemokine activities, taxis, and inflammatory and antiproliferative activities [25]. Promoter analysis of robust genes has shown a strong signature of the canonical IFN response elements (ISREs), while tunable gene promoters seem to be driven mainly by other response elements [25].

In contrast to robust IFN activity, the tunable activity of IFNs is highly cell-type specific. For example, IFNs induce strong tunable activity, as monitored by antiproliferative activity and specific gene activation, in OVCAR3 (human ovarian carcinoma) cells but not in T47D (breast cancer) cells [25]. As discussed above, the activation of robust activities requires IFN binding to a few cell surface receptors, while tunable activities require IFN binding to most receptors [26]. Reducing the number of surface receptors (for example by using siRNA or by natural processes such as those observed in certain cancer cells [26,73]) hinders cells from activating tunable activities. Conversely, increasing the number of cell surface receptors enables the cell to respond in a tunable manner [64]. Importantly, IFNAR1 binding affinity could be confirmed as a key determinant of tunable IFN activities. We have generated an IFNα2 mutant with strongly reduced IFNAR1 binding but increased IFNAR2 binding (IFN-1αnt [36]) that can activate robust responses only, independent of IFN-1αnt concentration (Figure 1B) [25]. This observation highlights the key relevance of IFNAR1 recruitment into the ternary complex as a determinant of the initiation of tunable responses.

Spatiotemporal regulation of IFN signaling

How do ‘robust’ and ‘tunable’ translate at a signaling level? Surprisingly, the potencies of IFNα2 and IFNβ with respect to the activation of STAT phosphorylation are very similar despite their different binding affinities. This suggests that additional signaling pathways activated by IFNAR, such as mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) signaling, are likely to be involved in promoting differential IFN activities. However, specific roles for these pathways in differential IFN activity have remained vague. By contrast, interplay between different feedback mechanisms regulating the assembly and activity of signaling complexes could explain specific features of receptor plasticity [74]. There are at least three layers of negative regulation of IFN signaling: endocytosis and endocytic trafficking [75]; suppressor of cytokine signaling 1 (SOCS1), which is a potent inhibitor of JAKs and binds to phosphorylated Tyk2 [76]; and ubiquitin-specific protease 18 (USP18) binding to the cytosolic domain of IFNAR2 [77]. While endocytosis is induced immediately after receptor activation, SOCS1 and USP18 are ISGs, which are substantially upregulated after around 4 h. Inadequate activity of any one of these three systems, as well as constant high expression of IFNs, is associated with various diseases [74,78–82].

Among these feedback regulators, endocytosis and USP18 have been proposed to critically contribute to differential
IFN activity (Figure 2). Endocytosis of IFN signaling complexes was demonstrated to be initiated via ubiquitination of IFNAR1, which exposes an endocytic motif masked by Tyk2 in the inactive state [83,84]. Interestingly, receptor endocytosis does not immediately abrogate signaling and even appears to enhance STAT phosphorylation activity [85,86]. While immediate regulation of receptor activity by endocytosis has not been directly demonstrated (Box 2), endocytosis also potentially contributes to receptor assembly: while the receptor subunits are highly diluted in the plasma membrane, uptake into early endosomes locally concentrates the receptor and the IFN into extremely small compartments. Receptor assembly can be considered essentially irreversible in endosomes, while reversible binding applies at the plasma membrane. Under these conditions, endocytosis of intact signaling complexes could account for the similar potencies of IFNα2 and IFNβ in the early phase of receptor activation, despite the approximately 1000-fold difference in their receptor binding affinities. As this higher binding affinity is caused by increased complex stability, endocytosis rather than ligand dissociation limits the lifetime of the ligand–receptor complex at the plasma membrane (Figure 2). Thus, steady-state binding, endocytosis, and degradation/recycling rather than the law of mass action determine the total concentration of active signaling complexes. Moreover, nuclear translocation of pSTAT could be facilitated by endosomal uptake (cf. Box 2), which could also account for the decreased pSTAT levels observed on inhibition of endocytosis. However, the final fate of endocytosed IFNAR is lysosomal degradation [87], which possibly contributes to downregulation of IFN signaling activity.

Another key regulator of IFN signaling is USP18 which is upregulated on IFN stimulation and responsible for efficient receptor desensitization after the first wave of gene induction [88]. Interestingly, the ISG15-specific protease activity of USP18 is not required for IFNAR desensitization. Rather, binding to IFNAR2 was shown to be responsible for its activity as a negative feedback regulator [88]. Interestingly, USP18 desensitizes receptor activation by IFNα2 much more efficiently compared with IFNβ [77,89], identifying USP18 as a key regulator of differential activity. Intriguingly, desensitization by USP18 was demonstrated to be tightly linked to IFNAR1 binding affinity, as the IFNα2 HEQ variant (see above) also showed reduced desensitization compared with wild type IFNα2 [89]. Ligand-dependent IFNAR desensitization by USP18 was
Box 2. Role of receptor endocytosis in regulating cytokine signaling

Cytokine receptors undergo endocytosis via clathrin-dependent and clathrin-independent pathways and highly specific features of endocytic trafficking have been observed for different receptors [86,97,98]. While these characteristic features suggest specific biological functions, the exact regulatory mechanisms exerted via receptor endocytosis remain debated. Originally, endocytosis was considered to control downregulation of cytokine signaling by depleting receptors and/or associated cytokines via lysosomal degradation. Enhanced signaling activity was achieved by altering endosomal sorting via engineered cytokines promoting recycling rather than degradation [99]. A key role of Epo receptor shuffling to control the dynamic range of signaling was recently demonstrated by experimental studies combined with quantitative simulations [100]. However, beside the control of total concentration by endosomal trafficking, several observations suggest that cytokine receptor complexes remain signaling competent in endosomes. Thus, efficient STAT3 association with endosomal vesicles was observed and regulatory control of cytosolic transport and nuclear translocation as well as post-translational modification via endosomal trafficking was proposed [101,102]. Strikingly, reduced STAT1 phosphorylation was observed for IFNAR but not IFNGR signaling on inhibition of endocytosis [86]. Based on these findings, control of signaling specificity by providing a specific signaling environment in endosomes was proposed [85]. In this context, membrane microdomains have been speculated to play an important role by controlling the spatiotemporal organization of signaling complexes at the plasma membrane.

Beyond these still rather hypothetical concepts of specific endosomal signaling pathways, uptake of intact cytokine receptor complexes into endosomes may also contribute to increase the number of active signaling complexes and to enhance signaling by increasing the local concentration. Steady-state conditions of continuous receptor uptake into signaling-competent endosomes followed by degradation and/or recycling (see Figure 2 in main text) may be responsible for the highly robust, affinity-independent early signaling response observed for many cytokines. By integrating these possible functions into the spatiotemporal regulation of signaling activity, a more detailed, quantitative understanding of these processes is required to unravel the cellular basis of functional receptor plasticity.

correlated with reduced cell surface binding affinity of IFNα2 but not IFNα2 HEQ, suggesting that USP18 regulates IFN signaling at the level of receptor assembly. In combination with receptor endocytosis, such regulation of receptor binding affinity could provide the mechanistic bases for the functional plasticity of IFN receptors.

The stochastic nature of IFN activities

The ability of cells to accurately control gene expression levels in response to extracellular signals is limited by the inherently stochastic nature of signal transduction and transcriptional regulation. Cell-to-cell variability in expression (noise) changes as a function of transcription factor activity, which leads to variability at the level of the individual cell [90,91]. Key steps of virus-induced signal transduction, IFNβ expression, and induction of IFN-stimulated genes are stochastic events in individual cells. The heterogeneity of IFN and IFN-induced gene production is of cellular origin, arising from intrinsic noise, and thus is temporal and unpredictable [92]. However, the antiviral response is activated in all cells despite the stochastic response of single cells to viruses. This is achieved through a combination of two mechanisms. One is the very low threshold for the initiation of the antiviral response (which is a robust function of IFN) and the second is autocrine response amplification, where secreted IFN buffers the stochastic nature of the single-cell response to viruses.

A different outcome is observed for the IFN tunable responses. Stochastic gene expression results in only 60–75% of WISH (human amniotic epithelial) cells treated with very high concentrations of IFN undergoing apoptosis. A direct link was observed between average receptor number and percentage of cells undergoing apoptosis [26,64]. Single-cell analysis has shown that WISH cells require a minimum number of surface receptors to be able to activate tunable functions (such as apoptosis) and that stochastic cell-to-cell variation in the number of receptors dictates whether a specific cell will have sufficient receptor numbers to be able to induce tunable responses [26]. Basically, tunable responses require the system to transmit signals to its maximum capacity (a high concentration of tightly binding IFN occupying most cell surface receptors to generate a maximum signal). A reduction in any one of these parameters in the single cell will affect its tunable signaling. This allows cell-to-cell variability as well as cell type-specific signaling of tunable functions. Thus, for example, OVCA13 cells promote a strong tunable signal while this is almost absent in T47D cells [25,26]. By contrast, the stochastic nature of receptor numbers is much less important for robust functions, as only a few receptors and weak signaling are required for close-to-optimal activation [25,26]. These observations highlight the key relevance of stochasticity in governing some but not all cell functions and that the requirement for different levels of receptor engagement for short- versus long-term cellular responses is a primary cause of functional receptor plasticity.

Interestingly, the percentage of cells undergoing apoptosis increased to >95% and the time of activation shortened from days to hours when cFLIP (an inhibitor protein of caspase-8) was downregulated using gene knock down, yet without affecting the IFN EC50 [93]. The magnitude of apoptosis is a direct consequence of the balance between cFLIP and caspase-8, and as such is also stochastic. This shows that not only the initiation of tunable IFN functions dictates their outcome, but also their execution.

Translating differential activity into a digital code

The assembly of published experimental quantitative data obtained with WISH cells provides a view of the complexity of the temporal sequence of events of IFN-induced responses and the key role of receptor engagement levels. In Figure 3, we plot the timeline of the various activities and their magnitudes resulting from two regimens of activation: 1 pM IFNα2 (A) and 1 nM IFNγ (B). Low IFNα2 activates only robust activities, while high IFNγ activates both robust and tunable activities. Due to feedback mechanisms, IFN induces wave-like responses, with receptor complexation and STAT phosphorylation occurring within minutes. Interestingly, STAT phosphorylation peaks after around 30 min and then declines, independent of the magnitude of treatment (pSTAT1 declines faster than pSTAT2). The decline in pSTAT, caused by negative feedback regulation including receptor endocytosis and
degradation (Box 2) as well as SOCS activity, constitutes the first layer of negative feedback on the system. Gene activation, which follows the assembly of ISGF3, is observed from 2 h of continuous IFN induction onward, independent of the type of IFN, a time point where pSTAT is already declining. Interestingly, the very small wave of pSTAT induced by 1 pM IFNα2 is sufficient to drive sufficient transcription of robust (but not tunable) genes, thus yielding full antiviral protection and showing the sensitivity of the system. Tunable gene expression starts later, is maximized at 16–24 h of continuous IFN stimulation, and is not induced by 1 pM IFNα2 but only by 1 nM IFNβ. Interestingly, phosphorylated STAT1 and STAT2 can no longer be detected at this time point and thus uncoupling between STAT phosphorylation and tunable gene transcription is observed. However, the formation of ISGF3 is essential for tunable gene induction, suggesting a second layer of gene regulatory control possibly related to the transcriptional activity of unphosphorylated STATs [94]. USP18 expression is upregulated similarly to the other robust genes. As a result, from approximately 8 h onward an additional negative feedback is induced that reduces complex assembly at the plasma membrane and, particularly, abrogates signaling with the 1 pM IFNα treatment. In the absence of USP18, one sees significantly stronger transcription induced by IFNαs. However, the stronger-binding IFNβ has the ability to partially overcome the USP18-induced inhibition. In line with the massive induction of robust genes even with 1 pM IFNα2, it is already sufficient to induce an antiviral response after 4 h of induction. Conversely, it is not sufficient to induce an antiproliferative response, for which longer times and a stronger signal are required.

**Concluding remarks**

The molecular and cellular mechanisms controlling specificity in pleiotropic signaling by many cytokines have remained enigmatic and controversially debated in this field. For IFNs as a paradigm for cytokine receptor plasticity, compelling determinants are emerging, which we have summarized in this review. Based on comprehensive, quantitative structure–function analyses, affinity–activity correlations, and analysis of gene expression patterns, together with enormous progress in mechanistic understanding of receptor assembly, signal activations, and regulatory mechanisms, strikingly simple principles have been identified. Our concept proposes that, rather than qualitative differences in signaling pathways, the temporal evolution of signaling activity plays a decisive role in cellular decision making. In combination with very different signaling thresholds for different cellular responses, differential activity can be explained by the different abilities of IFNs to maintain receptor activation. For this concept, feedback mechanisms that modulate receptor activity over time in a ligand affinity-dependent manner are key. This quantitative understanding of differential IFN activity has important implications for the future development of IFNs as drugs, both to increase their benefits and reduce their harmful side effects. Thus, IFNs with highly specific activities have been engineered [25] and the efficacy of cell marker-specific targeting IFNs could be tremendously enhanced [59]. Moreover, the exposure of patients treated with IFN can be systematically optimized. For particular therapies, we can now ask whether a more constant drug concentration, achieved by frequent injections or by extending the in situ IFN half-life, is beneficial and which type of IFN-induced activity is sought. These considerations may shift the way we develop cytokine drugs in the future and choose

### Box 3. Outstanding questions

IFNs have a prominent role in many aspects of innate and acquired immunity. However, despite almost 60 years of research in the field, we are still surprised by the complex integration of molecular information into cellular decision making. While some basic mechanistic concepts of IFN receptor plasticity are emerging, several key questions remain unsolved.

- What is the molecular mechanism of STAT activation at the cytosolic IFNAR signaling complex?
- How is STAT activation spatiotemporally regulated in the context of different feedback mechanisms (receptor assembly, endocytosis) and further post-translational modifications?
- How do IFN signaling and gene transcription proceed in the absence of STAT phosphorylation?
- What mechanisms are responsible for differentially controlling robust and tunable gene expression?
- Which are the specific cellular determinants regulating cell-specific decision making?

Answering these specific cellular questions will require a much more detailed picture of the IFNAR signaling interactome and transcriptome. Moreover, novel probes and reporters to quantitatively monitor protein interactions and conformational dynamics at the IFNAR signaling complex as well as signal propagation and gene transcription in situ will be essential.
their mode of administration. However, the exact molecular and cellular mechanisms controlling the temporal evolution of IFN signaling remains largely unclear (Box 3). In particular, a much better understanding how IFN signaling proceeds after potent abrogation of STAT phosphorylation by negative feedback regulators and how tunable genes are specifically induced will be essential. This will require not only a detailed spatiotemporal inventory of the IFNAR interactome and transcriptome, but also novel tools to monitor downstream signaling events with subcellular resolution on multiple temporal scales.

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