CRISPR/Cas9-based Knockout Strategy Elucidates Components Essential for Type 1 Interferon Signaling in Human HeLa Cells

Victoria Urin[†], **Maya Shemesh**[†] **and Gideon Schreiber**

Department of Biomolecular Sciences, *Weizmann Institute of Science, Rehovot, Israel*

Correspondence to Gideon Schreiber: *gideon.schreiber@weizmann.ac.il*
https://doi.org/10.1016/j.jmb.2019.06.007
Edited by Dr M Yaniv

Abstract

Type I interferons (IFNs) have a central role in innate and adaptive immunities, proliferation, and cancer surveillance. How IFN binding to its specific receptor, the IFN α and β receptor (IFNAR), can drive such variety of processes is an open question. Here, to systematically and thoroughly investigate the molecular mechanism of IFN signaling, we used a CRISPR/Cas9-based approach in a human cell line (HeLa) to generate knockouts (KOs) of the genes participating in the type 1 IFN signaling cascade. We show that both IFNAR chains (IFNAR1 and IFNAR2) are absolutely required for any IFN-induced signaling. Deletion of either signal transducer and activator of transcription 1 (STAT1) or STAT2 had only a partial effect on IFN-induced antiviral activity or gene induction. However, the deletion of both genes completely abrogated any IFN-induced activity. So did a double STAT2–IFN regulatory factor 1 (IRF1) KO and, to a large extent, a STAT1 KO together with IRF9 knockdown. KO of any of the STATs had no effect on the phosphorylation of other STATs, indicating that they bound IFNAR independently. STAT3 and STAT6 phosphorylations were fully induced by type 1 IFN in the STAT1–STAT2 KO, but did not promote gene induction. Moreover, STAT3 KO did not affect type 1 IFN-induced gene or protein expression. Type 1 IFN also did not activate p38, AKT, or ERK kinase. We conclude that type 1 IFN-induced activities in HeLa cells are mediated by STAT1/STAT2/IRF9, STAT1/STAT1, or STAT2/IRF9 complexes and do not require alternative pathways.

Introduction

Type 1 interferons (IFNs) belong to the helical cytokine superfamily and were discovered more than half a century ago [1]. They are secreted proteins that promote antiviral (AV), antiproliferation (AP), and immunomodulatory activities in vertebrates [2,3] acting in practically every nucleated cell. Due to their wide range of activities, type 1 IFNs are used as treatment for various diseases, including multiple sclerosis, hepatitis C, and cancer [4–6]. In humans, the family consists of 13 IFN-α subtypes, IFNβ, IFNω, IFNκ, and IFNε. All type 1 IFNs bind the two common cell surface receptor components, IFNAR1 and IFNAR2 [7–9]. Binding results in ternary complex formation, which activates the intracellular JAK (janus kinase)–STAT (signal transducers and activators of transcription) pathway. Upon complex formation, the tyrosine kinases TYK2 and JAK1, which are constitutively associated with the IFNAR1 and IFNAR2 subunits, respectively, activate each other by phosphorylation, which drives the phosphorylation of key tyrosine residues located in the IFNAR1 and IFNAR2 intracellular regions [10,11]. Subsequently, STAT proteins are recruited and tyrosine-phosphorylated. The phosphorylated STAT1 and STAT2 form a trimeric complex together with IRF9, which translocates to the nucleus. This IFN-stimulated gene factor 3 (ISGF3) transcription activation complex drives the expression of numerous IFN-stimulated genes [10]. While this is the main type 1 IFN-induced signaling pathway, it is not the only one. The GAS pathway, activated by a homodimeric phosphorylated STAT1, and several accessory STAT independent signaling cascades, such as the p38 mitogen-activated protein kinase (MAPK) pathway, the phosphatidyl-inositol 3-kinase signaling pathway (PI3K/AKT), the extracellular signal-regulated kinase (ERK) MAPK kinase, and others were reported to be induced by type 1 IFN

[3,9,12–14]. Many of those pathways are cell type specific. Type 1 IFNs activate hundreds of genes and multiple cellular activities, some of them require only pM concentrations of IFN for a short time, while others are induced only by higher IFN concentrations and longer induction. The first is referred as "robust" activities (e.g., AV). The latter is referred as "tunable"; for example the IFN-induced antiproliferative activity [15].

Type 1 IFN receptors

As was mentioned earlier, all type 1 IFNs induce their diverse activities through the same receptor subunits. Both IFNAR1 and IFNAR2 are transmembrane proteins that belong to the class 2 helical cytokine receptors [7,16]. IFNAR2 is the high-affinity receptor, while IFNAR1 binds IFN with low affinity [17–19]. The intracellular domain of IFNAR1 and IFNAR2 binds the two kinases Tyk2 and Jak1, respectively. Aside from that, the receptors serve as docking site for most STATs [20]. Although the exact mechanism of this binding is not fully known, there were pieces of evidence of recruitment of STAT1 to the receptors via STAT2 [21,22]. STAT2 was reported to bind IFNAR2 also constitutively [23–25]. Although it is believed that both receptors are required for type 1 IFN-induced activity, there are reports in mice of an IFNAR2-independent activity through IFNAR1 alone. In this case, a different set of genes was found to be activated from that observed in the presence of both receptors [26].

The JAK/STAT signaling pathway

Type 1 IFN promotes a change in expression of over 1000 genes, leading to a wide variety of cellular responses [27]. The JAK/STAT pathway provides a fast membrane to nucleus mechanism for rapidly inducing gene expression [28]. It has been established that the proteins participating in the JAK/STAT pathway play crucial roles in the activation of the immune system [28,29]. STATs have key roles in macrophage and neutrophil functions [30,31], Th1 cells differentiation [32], and drive epigenetic modifications [33–35]. It was suggested that also the unphosphorylated form of STAT1 is important in signal activation upon type 1 IFN induction [36]. Uddin *et al*. [37] established that upon IFN binding to its receptors, phosphorylated JAK1 and TYK2 tyrosine kinases regulate also the IRS signaling pathway. It was also shown that type 1 IFN receptors engage in the AKT/mTOR signaling [38]. The main functions of the AKT (AKT1 and AKT2) proteins are cell survival, growth, and proliferation. It was suggested that AKT proteins are required for the IFN-dependent AV activity and take part in control of mRNA translation [38]. Moreover, besides the pro-apoptotic activity of type 1 IFNs, activated through the JAK–STAT pathway, an anti-apoptotic activity is induced by the PI3k/AKT pathway [39].

Much work has been invested to understand the complexity of type 1 IFN signaling. However, it was done in many different cell types and organisms, which may differ in their response from one another. The relatively low sequence conservation of type 1 IFNs and their receptors between different vertebrates further emphasizes that differences in mechanisms of type 1 IFN signaling in different vertebrates can be expected [40].

Here, we constructed human cells (HeLa) with KOs of the main components of the type 1 IFN signaling cascade using the CRISPR\Cas9 technology. This allowed us to investigate the different components of the system and their contribution to IFN signaling in the same genetic background in a controlled manner.

Results

Previously generated knockdowns (KDs) of IFNAR1, IFNAR2, STAT1, and STAT2 did not completely eliminate the activity of these genes [15,41]. Therefore, to explore type 1 IFN signaling, we generated knockout HeLa cells using the CRISPR \Cas9 technology for the above genes. In STAT1, a frameshift was introduced in the first exon, in STAT2 and IFNAR1 in the third exon, and in IFNAR2 in the fifth exon. To confirm the IFNAR1 and IFNAR2 KOs, we measured their surface expression by FACS using specific antibodies (Fig. 1a). No expression of IFNAR1 or IFNAR2 was observed in the IFNAR1 and IFNAR2 KO cells, respectively (Fig. 1a, panels 1 and 4). As expected, the levels of IFNAR2 in IFNAR1 KO and of IFNAR1 in IFNAR2 KO cells were similar to wild-type (WT) (Fig. 1a, panels 2 and 3). Reconstitution of the receptors by transient transfection restored their surface levels to WT.

STAT activation upon KO

We first evaluated STAT signaling in these four KO cell lines by assessing the phosphorylated (p) and total (t) protein levels of STAT1 and STAT2 using Western blot (WB). KO, reconstituted, and WT HeLa cells were treated with 2 nM IFNβ for 30 min and were compared to their non-treated (NT) controls. STAT1 and STAT2 KO resulted in the complete absence of the KO protein (total and phosphorylated) (Fig. 1b), while there was no effect on the protein levels of the other STATs (total or phosphorylated), which were similar to those in WT HeLa cells. Conversely, IFNAR1 or IFNAR2 KO cells did not promote any detectable levels of phosphorylated STATs (while levels of total STATs were unchanged; Fig. 1b). This does not only confirm that these cells are in fact KO cells for those genes, but also support

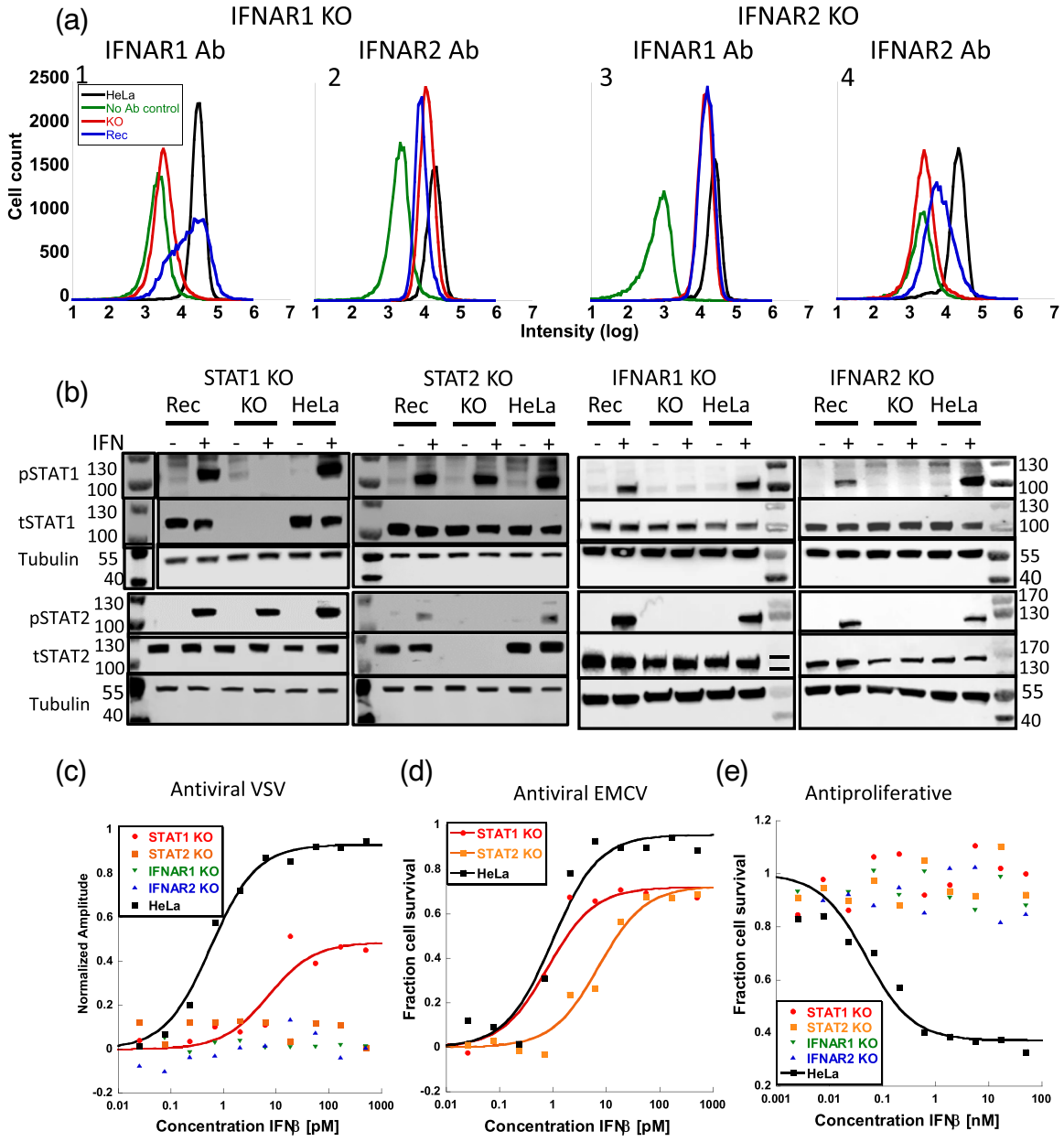

Fig. 1. KOs validation and activity assessment. (a) IFNAR1 KO cells (panels 1 and 2) and IFNAR2 KO cells (panels 3 and 4) were analyzed by FACS with and without reconstitution of IFNAR1 or IFNAR2, using anti-IFNAR1 (AA3) or IFNAR2 (117.7) antibodies. (b) Cells were treated with 2 nM IFNβ for 30 min and analyzed by WB using specific antibodies. The data presented are of phosphorylated (pSTAT1 and pSTAT2) and total (tSTAT1 and tSTAT2) protein levels in the WT, KO and KO reconstituted (Rec) with the WT gene, compared to the untreated cells. Quantitative analysis, normalized to tubulin is shown in Fig. S1. Markers that were cut and added from the same gel were placed in a separated box. Markers that were not seen because of repeated stripping were marked as black lines. (c) All cell lines were treated with IFNβ or left untreated (negative and positive control) for 4 h. VSV virus was added for 18 h, and cells were stained with crystal violet for cell viability. (d) As panel C but EMCV virus was added for 20 h before cells were stained with crystal violet for cell viability quantitation. (e) Antiproliferative activity was evaluated after 96 h of IFN treatment by crystal violet staining for cell survival. All the results were normalized to the non-treated KO cell line.

the notion that both receptors are needed for the initiation of IFN signaling. Reconstitution by transient transfection of the KO genes restored the activity to WT levels (Figs. 1b and S1). The results did not change when IFNα2 was used for induction instead of IFNβ (Fig. S2). Next, we measured STAT phosphorylation at different times of activation (30 min to 24 h; Fig. S3). As expected,

phosphorylation was the strongest at 30 min, followed by gradual decrease in phosphorylation (less for STAT2 than for STAT1 and STAT3). The same trend was observed for WT HeLa and the different KO cell lines.

AV and antiproliferative activity of IFNAR and STAT KOs

Type 1 IFN activities can be divided into robust and tunable ones. The AV activity of type 1 IFN is robust; that is, it is common to all cells and requires only low concentrations of IFN for a short time. On the other hand, the antiproliferative activity is tunable, and it is cell type specific and requires prolonged IFN activation at higher protein concentrations. AV activity was examined in the background of IFNAR and STAT KOs. Cells were treated with 10 tertiary dilutions of IFNβ starting from 500 pM. Four hours later, vasicular stomatitis virus (VSV) was added to the cells for 18 h. Cell survival was compared to non-treated KO cells as negative control and to cells treated only with VSV as the positive control. No AV activity was observed for the IFNAR1, IFNAR2, and STAT2 KO cells, while IFN still initiated a partial AV response in the STAT1 KO cells (Fig. 1c, Table S1). Monitoring AV activity against the encephalomyocarditis virus (EMCV) showed that both STAT1 and STAT2 KO cells promoted partial AV activity (Fig. 1d, Table S1). These results show that residual IFNβ-induced signaling is sufficient to promote some AV activity. Conversely to AV, none of the KOs (IFNAR or STAT) promoted any AP activity upon treating the cells with 10 tertiary dilutions of IFNβ from 50 nM to 0.4 pM. (Fig. 1e), in line with AP being a tunable activity.

Gene expression of IFNAR and STAT KOs

We next evaluated type 1 IFN-induced gene (ISG) expression in the different KO cells, probing a number of robust and tunable genes. For this, cells were treated with 2 nM IFNβ for different times and gene expression levels were determined. First, the level of expression of three robust (MX1 or MX2, OAS2, and IFIT1) and two tunable (CXCL10 and CXCL11) genes were quantified using qPCR. IFNAR KOs were assessed after 16 h (Fig. 2a) and the STAT KOs after 8 and 24 h (Fig. 2b and c). No induction in gene expression was detected in either IFNAR1 or IFNAR2 KO cells. Conversely, IFNβ induced low levels of gene expression in the STAT KOs. The reconstituted cells exhibited similar expression levels as the WT. Similar to IFNβ induction, IFNα2 also did not promote gene induction in the IFNAR KO cells, and partial induction in the STAT KO cells (Fig. S4). The difference here between IFNα2 and IFNβ treatment is the lower level of induction of the tunable CXCL10 and 11 genes by IFNα2. To obtain a more complete picture of gene expression, we used the fluidigm system, which analyzes the expression of up to 96 genes in 96 different samples (Fig. S5). Gene induction was determined after 3, 16, and 24 h for IFNAR KO cells and 8 and 24 h for STAT KO cells. As probes, we used common type 1 ISGs, both from the tunable and the robust set. To further expand gene induction on a genome wide level, we performed RNA-seq (Figs. 2d and S6). Both assays showed a total lack of induction of gene expression upon IFNβ treatment in both IFNAR1 and IFNAR2 KO cells and significant reduction in activation of gene expression in both STAT1 and STAT2 KO cells (Figs. 2d, S5, and S6). Interestingly, a different set of genes were induced by IFNβ in STAT1 and STAT2 KO cells (Fig. 2d and e). Submitting the list of genes uniquely upregulated in either STAT1 or STAT2 KO cells to the gene ontology software Panther [42] showed that genes uniquely upregulated in STAT1 KO cells (thus activated by STAT2) cluster as type 1 ISGs (FDR 8e-7), while genes uniquely upregulated in STAT2 KO cells (thus activated by STAT1) annotate as cellular response to IFNγ (including TRIM21, STAT1, CASP1, IRF1, GBP1, IRF9, and GBP5 genes, FDR 2e-6).

It has been previously shown that the basal gene expression (tonic) of some ISGs is reduced in IFNAR1 KO mice [43]. Here, we used the fluidigm to analyze whether IFNAR1, IFNAR2, STAT1, or STAT2 KO cells also show reduced tonic expression (Table S2). Indeed, reduced expression is observed for some of the IFNβ-induced genes in the KO cells. The difficulty to compare the levels of tonic expression of the different KOs may be attributed to IFN being only one of the factors controlling ISG gene expression. This could potentially explain why the genes with reduced tonic expression only partially overlay between the IFNAR1 and IFNAR2 KOs. This is in line with previous observations on the tonic expression of IFN receptor KOs in mice [43].

IFNAR1 and IFNAR2 do not have independent activity in HeLa cells

As detailed above, the RNAseq analysis showed complete lack of induction of gene expression in either IFNAR1 or IFNAR2 KO cells. This is true not only for type 1 ISGs as described above but also for the entire transcriptome (Fig. S6). As these results are different from those obtained from IFNAR2 KO mice [26] where IFNβ induced the transcription of a non ISG set of genes, we tested specifically a set of those genes reported to be induced by De Weerd *et al*. [26] using the fluidigm (Fig. 2f). None of the genes evaluated,whose expression was previously reported to be induced in an IFNAR2 KO mice were induced by IFNβ in human HeLa cells harboring the IFNAR2 KO.

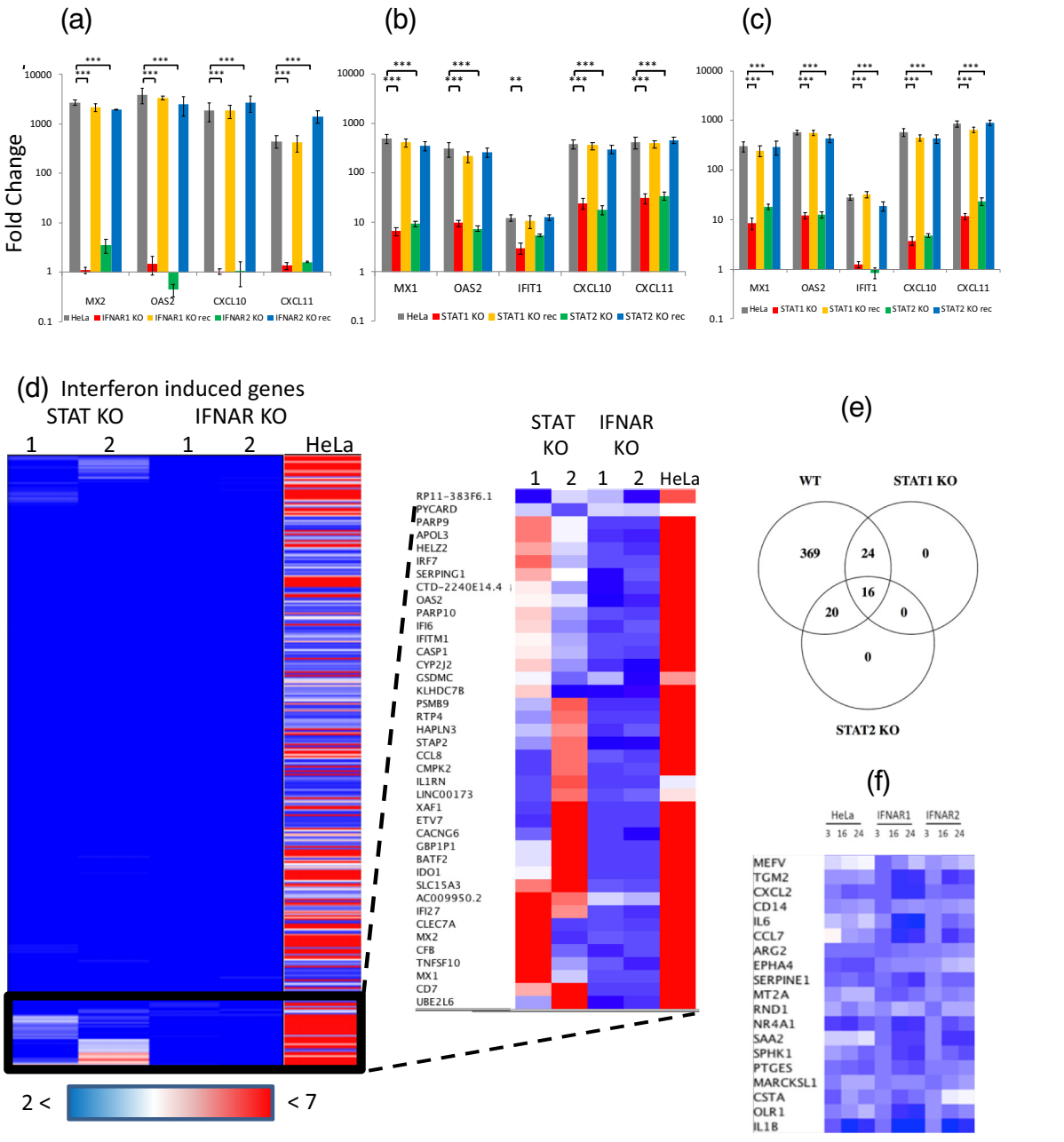

Fig. 2. Relative gene expression of KO cells in response to IFNβ treatment. (a) IFNAR KO cells were treated for 16 h with 2 nM IFNβ and analyzed by qPCR. The data presented are relative expression levels compared to those of untreated cells and normalized with HPRT1. Data presented are means calculated based on three independent experiments and their standard errors. (b and c) STAT KO cells were treated for 8 and 24 h, respectively, with 2 nM IFNβ and analyzed as described in panel A. Significance: *0.005 ≪ *P* ≪ 0.05, **0.0005 ≪ *P* ≪ 0.005, ***$P \ll 0.0005$. (d) Cells were treated for 16 h with 2 nM IFNβ and subjected to RNAseq. The data presented are of genes whose level of expression increased by ≫2-fold by IFN in WT HeLa cells. The color scale in the bottom is log(2). Magnified are genes that were induced in STAT1 or STAT2 KO in addition to WT HeLa cells. (e) Venn Diagram of genes whose level of expression increased by ≫2-fold by IFN treatment of WT HeLa cells as determined by RNAseq. The subpopulation of genes that was induced by ≫2-fold also in STAT1 KO cells and STAT2 KO cells (single-gene KO) is presented. (f) Fluidigm analysis of gene induction in IFNAR1 or IFNAR2 KO cells upon 2 nM IFNβ induction for different times. The selected genes were those reported in Ref. [26] as being induced in IFNAR2 KO mice. The numerical values of this figure are in Table S4.

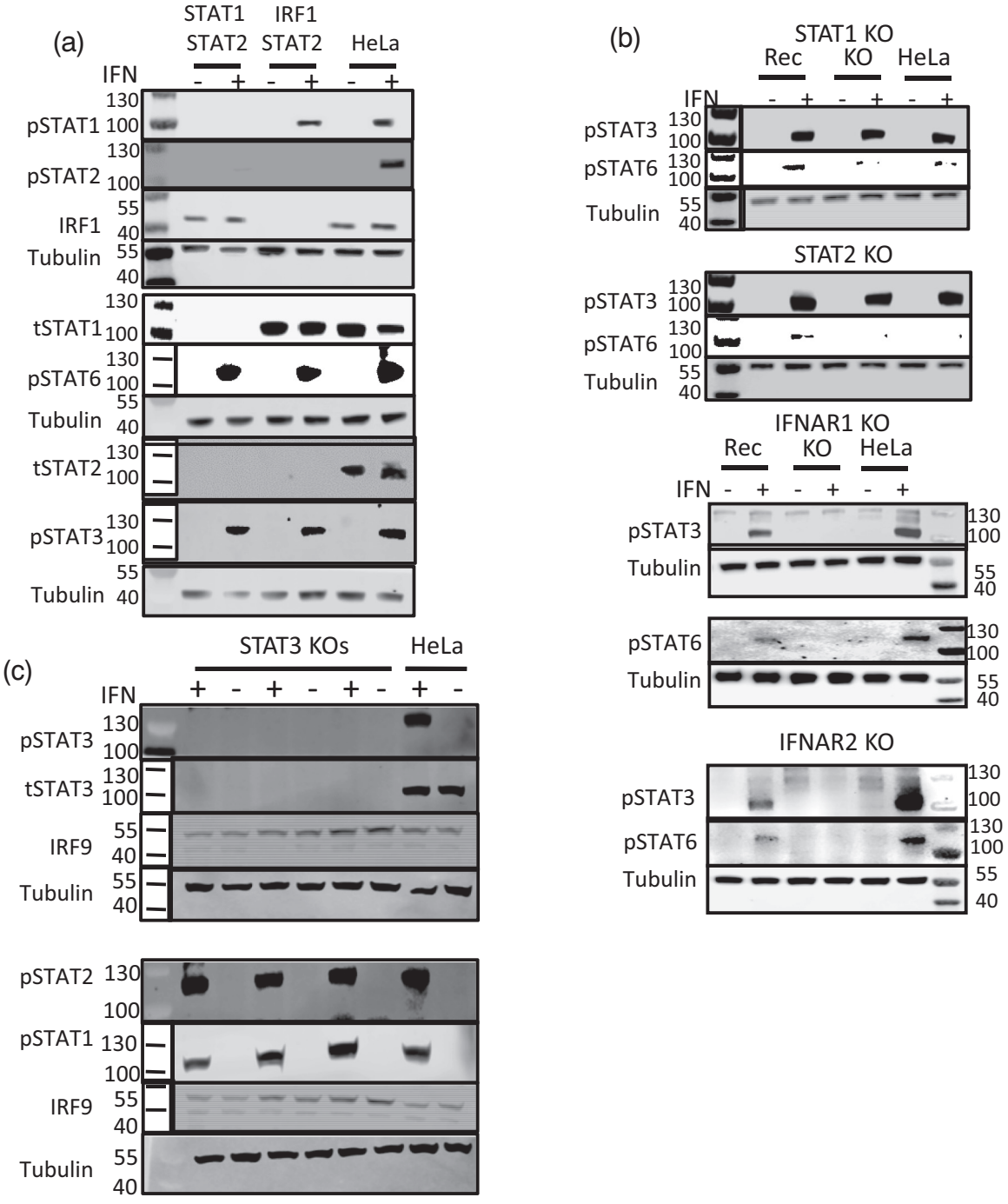

Fig. 3. Phosphorylated and total STAT protein levels in different KO cell lines. (a) STAT1/STAT2, IRF1/STAT2 double KO, and HeLa WT cells were treated with 2 nm IFNβ for 30 min. pSTAT and tSTAT protein levels were measured by WB. (b) STAT1, STAT2, IFNAR1, IFNAR2 KOs, and HeLa WT cells were treated as described in panel A, and STAT levels were determined. Rec is for cells reconstituted with the KO gene by transient transfection. (c) Three STAT3 KO clones obtained by CRISPR/Cas9 were treated with 2 nM IFNβ for 30 min. Quantitative analysis, normalized to tubulin, is shown in Fig. S7. Markers that were cut and added from the same gel were placed in a separated box. Markers that were not seen because of repeated stripping were marked with black lines.

A STAT1-STAT2 double-knockout eliminates type 1 IFN-induced activity

Both STAT1 and STAT2 KO cells responded partially to type 1 IFN activation. To explore the molecular pathways leading to induction of gene expression with either KOs, we generated a double STAT1–STAT2 KO. No total or phosphorylated STAT1 or STAT2 proteins were detected in this strain before or after addition of IFNβ (Figs. 3a and S7). No gene induction following IFNβ treatment of the double KO cells was observed on a selected set of genes (Fig. 4a). To validate the qPCR results on a genomic level, we performed RNAseq (using the MARS sequencing method) for STAT1 and STAT2 KO and the double KO of STAT1–STAT2 (Fig. 4d and e). The MARS seq data confirmed that no significant transcription activation is observed upon IFNβ induction in the double-KO cell lines. In line with nullified gene induction, IFNβ did not stimulate an AV state against either VSV or EMCV, independent on its concentration (Fig. 4f). Overall, these data confirm that type 1 IFN activation is dependent on having either STAT1 or STAT2 (for partial activation) or both (for full activation).

Alternative type 1 IFN-induced signaling pathways

Several alternative signaling pathways were suggested to be activated by type 1 IFN. One such pathway is the gamma-activated sequence (GAS) pathway. GAS elements bind pSTAT1 homo-dimers activated by IFNγ [29] and also by type 1 IFN [44]. IRF1 expression is induced both by type 1 and type 2 IFNs, with a GAS element located 100 base pairs upstream of the transcription start site. IRF1 is also one of the genes whose expression is induced by IFNβ in STAT2 KO cells (Fig. 4a, most probably through the STAT1/STAT1 dimer). IRF1 regulates expression of target genes by binding to ISRE [29,44–46]. To evaluate whether IFNβ induces genes on the background of STAT2 KO through IRF1, we generated a double KO of STAT2 and IRF1 (see Figs. 3a, and S7 for WB of the KO). Indeed, HeLa cells harboring this double KO did not induce the expression of selected genes that were induced in STAT2 KO cells (Fig. 4b). The lack of IFNβ-induced gene expression was further confirmed genome wide using MARSseq (Fig. 4d and e). In addition, IFNβ failed to induce an AV response against either VSV or EMCV in the double KO cells (Fig. 4f). Thus, IRF1 is required for maintaining the partial IFN activity observed in the STAT2 KO.

It was suggested that the pSTAT2 protein can induce gene activation by binding IRF9 also in the absence of pSTAT1. Here, we opted to use siRNA generated against IRF9, as we have previously verified the efficiency of this KD (see also Fig. S8, which shows a 90% reduction in IRF9 protein level). IRF9 KD in STAT1 KO cells reduced gene induction to near basal levels for genes that were induced in the STAT1 KO cells (Fig. 4c). However, some gene activation was still observed in the MARSseq experiment (Fig. 4d and e). This may be due to alternative pathways of activation or due to residual expression of IRF9 when using siRNA. It is also interesting to note that IRF9 levels were ~15% higher in STAT1 KO in comparison to HeLa WT cells. The results presented above suggest that type 1 IFN induces gene activation in the HeLa epithelial cell line mainly through pSTAT1/pSTAT2, but that partial signaling goes also through the pSTAT1/pSTAT1 and pSTAT2/IRF9 complexes. No other signaling pathways were evident.

STATs, other than STAT1 and STAT2, were shown to be activated by type 1 IFNs. Probing pSTAT3 and pSTAT6 protein levels in IFNAR and STAT KOs showed that in the absence of either of the IFNARs, there was no phosphorylation of STAT3 or STAT6 in comparison to reconstituted cells or HeLa WT. Conversely, phosphorylation of STAT3 or STAT6 was not dependent on STAT1 or STAT2, with similar to WT levels of phosphorylation being observed upon type 1 IFN treatment (Figs. 3b, S2 and S3). Next, pSTAT3 and pSTAT6 levels were examined in the STAT1–STAT2 and STAT2–IRF1 double-KO cells. Again, there was no effect on STAT3 or 6 phosphorylation (Figs. 3a and S7). These findings led us to generate a STAT3 KO cell line. WBs to detect pSTAT3 or tSTAT3 protein levels in STAT3 KO cells showed complete KO in three independent clones (Figs. 3c and S7). STAT3 KO did not affect the phosphorylation of STAT1 or STAT2 upon IFNβ induction (Figs. 3c and S7). Next, we characterized IFNβ-induced activities in STAT3 KO cells. As can be seen in Fig. 5, STAT3 KO had no noticeable effect on either AV, AP, or gene induction upon IFNβ treatment. To validate our gene induction findings on genomic level, we performed MARSseq on STAT3 KO cells (Fig. 4d and e). No significant deviation from gene induction of the control HeLa WT cells was observed for the STAT3 KO cells. STAT3 was previously implicated as an adapter to couple PI3K to IFNAR1 (through p85) [47,48], leading to the activation of AKT [38], which is required for IFN-inducible engagement of the mTOR/p70 S6 kinase pathway, leading to up-regulation of key type 1 IFN-inducible proteins. These were shown to be important in activating the AV and AP type 1 IFN responses. To directly test this in HeLa, we monitored phosphorylation of AKT, p38 and ERK upon addition of IFNβ and did not find any change (Fig. S9A and B), in line with previous publications on multiple other human cell lines [49]. Next, we monitored the expression of two IFN-induced proteins (STAT1 and STAT2). Expression levels of both proteins were highly induced after 24 h

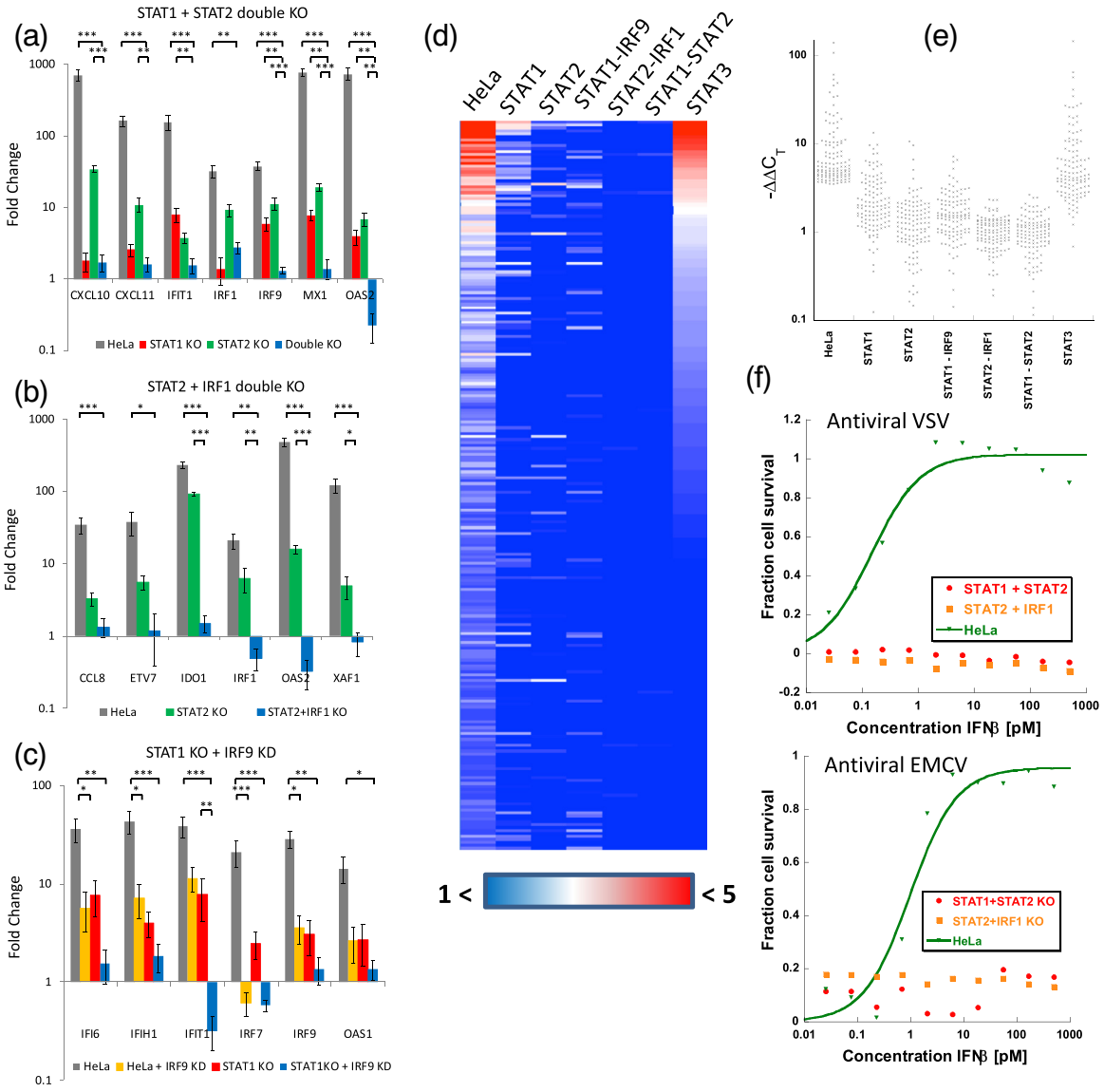

Fig. 4. Activity assessment of double-KO cells upon IFNβ treatment. (a) STAT1 + STAT2 double-KO cells were treated for 8 h with 2 nM IFNβ and analyzed by qPCR. Data presented are means calculated based on three independent experiments and their standard errors. (b) STAT2 + IRF1 double-KO cells were treated for 8 h with 2 nM IFNβ and analyzed as described in panel A. (c) STAT1 KO + IRF9 KD cells were treated for 8 h with 2 nM IFNβ and analyzed as described in panel A. Significance: *0.005 ≪ P ≪ 0.05, **0.0005 ≪ P ≪ 0.005, ***P ≪ 0.0005. (d) HeLa WT and different KO cells were treated for 24 h with 2 nM IFNβ and subjected to MARSseq for genome with expression analysis. The data presented are the difference between the treated cells to its non-treated control. (e) ISGs by ≫2.5-fold in WT HeLa cells were selected, and their level of expression in the different KO cells is shown. Each dot represents a gene. (f) All cell lines were treated with IFNβ or left untreated (negative and positive control) for 4 h. VSV or EMCV virus were added for 18 or 20 h, respectively, and cells were stained with crystal violet for cell viability evaluation.

of treatment with IFNβ and were similar in the STAT3 KO cells and WT HeLa cells (Fig. S9C). Finally, we evaluated the effects of the KOs on the two negative feedback regulators of type 1 IFN signaling, SOCS1 and USP18. Monitoring their level of induction shows that the STAT1, STAT2, and STAT1–STAT2 double KOs strongly reduced their induction, while STAT3 KO had no effect on their IFNβ induced induction (Fig. S10). These results clearly show that the basic functions of type 1 IFN (gene induction, protein expression, AV, and AP) are controlled only by STAT1 and STAT2 in HeLa cells, and that alternative pathways are not required for them to be activated. These findings made us to investigate whether activation of STAT3 by another ligand, IL6, would generate an effect on gene induction in HeLa cells. In Fig. S11A, we show that IL6 indeed is activating phosphorylation of STAT3. However, as

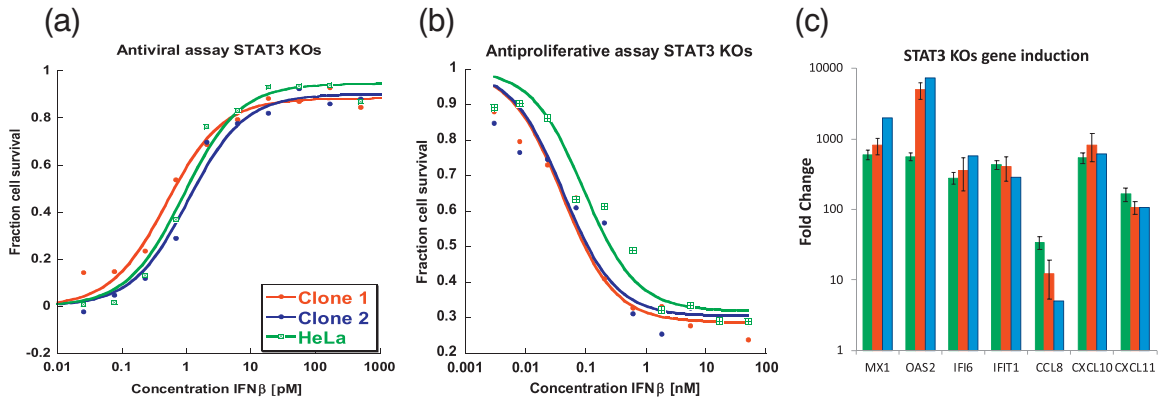

Fig. 5. Characterization of STAT3 KO clones. (A) AV activity: cells were treated with IFNβ for 4 h before infection with the VSV virus for 18 h. Cells were stained with crystal violet for cell viability. (B) AP assay: cells were treated with IFNβ for 96 h before staining with crystal violet for cell viability. (C) Cells were treated with 2 nM IFNβ for 24 h, and gene expression was evaluated using qPCR. Data presented are means calculated based on three independent experiments and their standard errors. Significance: *$0.005 \ll P \ll 0.05$, **$0.0005 \ll P \ll 0.005$, ***$P \ll 0.0005$.

shown in Fig. S11B and C, also IL6 did not result in gene activation (similar to that observed for IFN), whether we probed ISGs or IL6-induced genes. Thus, gene expression in HeLa cells may not be induced by pSTAT3.

Discussion

Type 1 IFNs play a crucial role in the innate and adaptive immunity. They were between the first biological drugs used as treatment in a variety of illnesses, such as multiple sclerosis, hepatitis C, and cancer [4,5]. Their mechanism of action against these three diseases is fundamentally different: In multiple sclerosis, IFNβ has an anti-inflammation effect on the myelin layer. In hepatitis C, IFNα has an AV effect and, in cancer, type 1 IFN apparently has an immunomodulatory effect. However, how binding of the same protein to its cell surface receptor is initiating these multitudes of responses is not well understood, hampering the efforts to maximize the efficiency of using type 1 IFNs as drugs [9,50,51]. For those reasons, it is important to fully understand the different pathways that are induced by type 1 IFNs, both the common pathways and the tissue-dependent ones. While type I IFNs activate all cells, the responses are cell type specific. Moreover, type 1 IFN and its receptors diverged in evolution much more than other cytokines, suggesting that type 1 IFN action may differ as well [38]. This makes it difficult to combine evidence from different studies to obtain a coherent description of type 1 IFN-induced signaling. To obtain such description, we knocked out the major components of the type 1 IFN signaling pathway in one cell type—human epithelial HeLa cells, to make comparable studies. CRISPR/Cas9 opened the door for such endeavor, which was previously not possible. It should be noted that HeLa cells are a non-physiological system, as are all cell lines, and further studies are needed using primary type 1 IFN-responsive cells. However, the problem here is that KOs cannot be generated in human primary cells, and the mouse system may differ in signaling from the human one. A summary of the signaling pathways activated in the different KO cells is provided in Fig. 6. While all the work was done in HeLa cells, the conclusions should be valid in other human cell lines, as the basic signaling mechanism of type 1 IFN is conserved.

Type 1 IFN receptors

Up until now, there were no human IFNAR1 or IFNAR2 KO cell lines that were generated in a controlled manner and were validated for the KO both in the genome and the protein levels. In IFNAR1 and IFNAR2 KO cells, IFNα2 or IFNβ did not activate STAT1, STAT2, STAT3, or STAT6 phosphorylation (Figs. 1, 3, 6,S2 and S3). AP and AV assays also showed no activity for IFNβ in these KOs. Gene expression analysis by three methods—qPCR, fluidigm, and genome-wide RNAseq did not show any gene induction, either of type 1 IFN-stimulated genes or any other gene in the absence of either one of the receptors. These results support the notion that both receptors are absolutely required in human HeLa cells to initiate any type 1 IFN signaling. To specifically rule out induction of genes that were previously found to be induced in IFNAR2 KO mouse cells [26], we validated the induction of these genes by qPCR and fluidigm, confirming the lack of gene induction upon IFNβ treatment. The differences observed between our study and previous studies in mice could relate to specie or cell type specificity

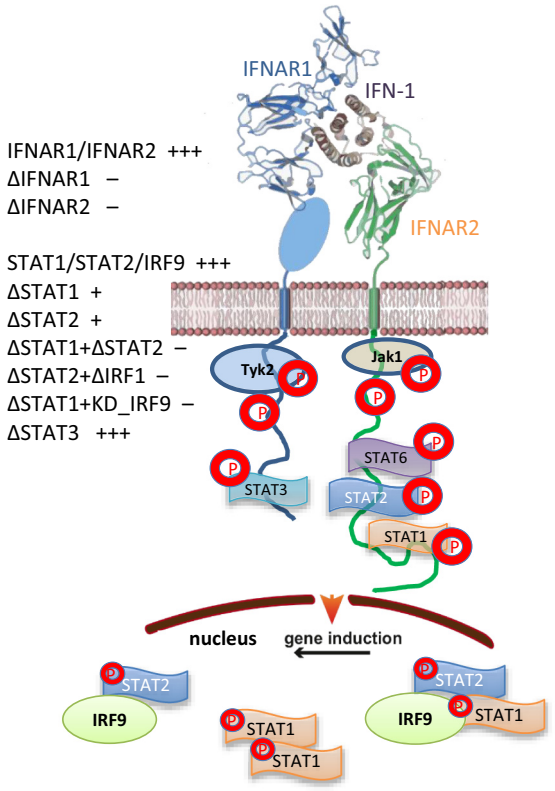

Fig. 6. Illustration of type 1 IFN-induced activities in HeLa cells, as deduced from the various gene KOs. The list on the top left side shows the activity as determined for the different KOs. Complete gene induction is obtained by pSTAT1/pSTAT2/IRF9 heterotrimer. Partial gene induction is obtained either by pSTAT2/IRF9 heterodimer or by pSTAT1/pSTAT1 homodimer. Lack of both STAT1 and STAT2 or STAT2 and IRF9 results in complete abolition of type 1 ISG induction and biological activity, while STAT3 KO had no apparent biological effect.

in the type 1 IFN system, or methodological differences in the generation of the KOs.

JAK–STAT activation

As mentioned above, type 1 IFN has been suggested to activate multiple pathways, but direct evidence on the molecular mechanism of activation is controversial, partially due to the multiple different cellular backgrounds of the different studies. We first generated HeLa KO cells of STAT1 and STAT2. In both cell lines, type I IFNs induced normal phosphorylation of other STATs and partial gene induction and AV activity (depending on the challenging virus). Still, AP activity was not induced, in line with AV being a robust outcome of type 1 IFN while AP being tunable. Interestingly, many of the IFNβ-induced genes in the STAT1 KO differ from those induced in the STAT2 KO cells (although all genes induced in either KO were induced also in WT HeLa cells). Gene ontology analysis of genes uniquely upregulated by either STAT1 or STAT2 KO cells [42] showed that unique genes to STAT1 KO cluster as type 1 ISGs, while unique genes to STAT2 KO were identified as IFNγ induced genes. Indeed, the GAS pathway, which is induced mainly by IFNγ, is known to be activated also by type 1 IFN [44]. The GAS pathway is activated by a dimerization of two phosphorylated STAT1 molecules, which promote the transcription of the IRF1 transcription factor, which further promotes the transcription of additional ISRE genes [29]. To validate the STAT1–IRF1 pathway, we generated a double KO of STAT2 and IRF1. This KO nullified gene transcription initiation by IFNβ and eliminated completely the partial AV activity observed for the STAT2 KO alone. This clearly shows that indeed the GAS pathway is the only one activated in the absence of STAT2. Still, it is interesting to note that only a small fraction of IFNγ-activated genes are also activated in STAT2 KO cells, despite strong STAT1 phosphorylation, suggesting that other factors play a role here as well.

In the STAT1 KO cell line, we suspected that a STAT2 (monomer or homodimer) forms a complex with IRF9 as complementary pathway for type 1 IFN activation. This pathway was previously described as inducing transcriptional response and partial AV activity in the absence of the STAT1 protein [52]. Here, residual activity was examined upon KD of IRF9 using a specific siRNA, in the background of STAT1 KO cells. The results show significant reduction in type 1 ISG expression relative to the STAT1 KO alone. Our results suggest that STAT1 and STAT2 are obligatory involved in all type 1 IFN related activities in HeLa cells. To prove this assumption, we generated a STAT1–STAT2 double KO. Indeed, the double KO of STAT1–STAT2 exhibited no ISG expression or AV activity (Figs. 4 and 6). These data also clearly show that type 1 IFN activation through NFκB, PI3K–AKT, AKT/mTOR, and ULK1/autophagy pathways either act in synergy with the STAT1/2 pathway or do not take part in type 1 IFN-induced signaling in HeLa cells. Indeed, in HeLa cells we (Fig. S9) and others [49] did not observe activation of AKT, ERK, or p38 upon induction by type 1 IFN. Moreover, we did not see any effect of the STAT3 KO on IFNβ-induced protein translation levels (which connects IFNAR1 activation to AKT through PI3K [38]).

Interestingly, while the STAT1–STAT2 KO exhibited no type 1 IFN-related activities, it preserved the phosphorylation of STAT3 and STAT6 to similar levels as seen in HeLa cells. This is perplexing, as phosphorylated STAT3 protein was found to drive a variety of cellular activities such as cell migration and proliferation, survival, and apoptosis [53]. Moreover, it was described as a negative regulator of type 1 IFN-related AV response [54]. One has to point out that those findings were described in different cell lines than HeLa. To verify the importance of STAT3

for type 1 IFN signaling, we prepared a STAT3 KO cell line and assessed how it altered IFNβ-induced activities. STAT3 KO exhibited no influence on any of the evaluated type 1 IFN-related activities. Our results are clearly different from those in mice. For example, numerous genes were induced by IFNγ in the absence of STAT1, due to alternative STAT3 signaling, which is dominant in the absence of STAT1 through a change in receptor occupancy by the dominant STAT, causing a switch from one activated STAT to another [55–57]. In our case, STAT3 was phosphorylated by IFNβ to the same extend in the STAT1–STAT2 double-KO cells as in WT, but without resulting in gene induction. All those findings support the fact that STAT1 and STAT2 are the main mediators of type 1 IFN signaling in HeLa cells, despite the activation of other STATs in the absence of STAT1 and STAT2.

In summary, in this manuscript, we established the type I-induced signaling pathway in human HeLa cells to high resolution. For this pathway to be activated, both IFNAR1 and IFNAR2 are required, as well as STAT1 and STAT2. In addition to the main activity mediated by STAT1–STAT2–IRF9, we also found residual activity through STAT1–IRF1 and STAT2–IRF9. We did not observe any role for activation of STAT3 or STAT6. Also, if NFκB, PI3K–AKT, AKT/mTOR, or ULK1/autophagy pathways are involved, they have to be downstream of STAT1 or STAT2. As all the experiments were done in HeLa cells, this does not preclude the possibility that in other backgrounds (different cell types or other species), other pathways will be of major importance in IFN signaling. Indeed, one of the challenges of this system, which is activated in all vertebrates and cell types, is to identify the common signals, from the cell type-specific signals to obtain a more complete understanding on the importance of IFN in health and disease.

Materials and Methods

Cell lines

HeLa is a human cervical cancer cell line. In HeLa cells, type 1 IFN activates both its robust and tunable activities. In addition, they are adherent cells and easily transfected. The genome of HeLa is stable, was mapped, and karyotyped with the number of alleles of each of our KO genes being known [58]. Both IFNARs have three alleles (chromosome 21), and STAT1 (chromosome 2) and STAT2 (chromosome 12) have two and three alleles respectively. This is important consideration for generating KOs. It should be noted that HeLa cells harbor defined HPV genome integration sites and HPV-encoded proteins, which may affect IFN signaling. HeLa cells were grown in Dulbecco's modified Eagle's medium (Gibco 41965-039) with 10% fetal bovine serum (Gibco 12657-029), 1% pyruvate (biological industries 03-042-1B), and 1% penicillin–streptomycin (biological industries 03-031-1B).

CRISPR/Cas9

All targets were chosen using the benchling design tool (https://benchling.com/crispr). Primers were annealed using T4 PNK (NEB- M0201 L) and ligated using T4 ligase (NEB–M0202L) into Px459 nuclease or Px462 nickase cas-9 vectors (Addgene). Cells were transfected with the vectors using the JetPrime reagent (PolyPlus 114-07) or Lipofectamine™ 2000 Transfection Reagent (Thermofisher 11668027). Clones were selected using puromycin resistance. Multiple STAT1, STAT2, STAT1 + STAT2 double KO, STAT2 + IRF1 double KO, and STAT3 KO clones were generated. All clones generated were sequenced and verified as KO in all gene's alleles. Gene induction and STAT activation assays were performed and compared for at least two clones, with no clone specific results being observed. The here presented data are of one selected clone from each KO.

AV and AP assays

1.2×10^4 HeLa WT or KO cells were grown ON on flat-bottomed 96-well plates for the AV assay and 4×10^3 for the AP assay. Cells were treated with 10 tertiary dilutions of IFNβ starting from 500 pM for the AV and 50 nM for the AP assay. AV activity against VSV and EMCV was assessed by determining the extent of viruses' cytophatic effect inhibition [59,60]. Four hours after the addition of IFN, VSV or EMCV was added for 18 or 20 h, respectively. AP was monitored 96 h after the addition of IFNβ. In both assays, the cell viability was determined by crystal violet staining as described previously [60]. The EC_{50} values and cell sensitivity to the indicated IFNs were determined from the IFN dose–response curve, as previously described [60].

WB analysis

STAT phosphorylation was measured by WB as described earlier [15] using the following antibodies: rabbit polyclonal anti-pSTAT1 (Tyr701) (Cell signaling 9167s), rabbit polyclonal anti-pSTAT2 (Tyr690) (cell signaling 4441s), rabbit polyclonal anti-STAT1 (p84/p91) (Santa Cruz Biotechnology Inc. sc-346), mouse monoclonal anti-STAT2 (A-7) (Santa Cruz Biotechnology Inc. sc-1668), rabbit polyclonal anti-pSTAT3 (Tyr 705) (Santa Cruz Biotechnology Inc. sc-7993-r), and mouse monoclonal anti-pSTAT6 (pY641.18) (Santa Cruz Biotechnology Inc. sc-136,019). Mouse monoclonal anti-α-tubulin (Sigma) (T9026-2 ml) was used as normalizing

protein: Py-ERK (pT202/Py204.22A) (Santa Cruz Biotechnology Inc. sc-136,521 monoclonal mouse Ab), Py-p38 (D8) (Santa Cruz Biotechnology Inc. sc-7973 monoclonal mouse Ab), and Py-Akt (S473) (Cell Signaling 4060S rabbit polyclonal Ab). Two size markers are shown in all WBs. In some cases, multiple stripping and re-blotting of the same membrane resulted in the size markers to be invisible. In these cases, we manually draw their positions according to their location before stripping.

Quantitative PCR analysis

The extent of expression of IFN stimulated genes was measured using qPCR as described previously [15]. The sequences of the primers used are detailed in Table S3. High-throughput qPCR was performed with BioMark 96 × 96 Dynamic Array (Fluidigm Corporation) according to the manufacturer's protocol. cDNAs (50 ng/μl) were pre-amplified with all the primers and analyzed with the BioMark real-time PCR instrument. Initial data analysis was performed with the fluidigm real-time PCR analysis software. Clustering was performed using the Netwalker software.

Reconstitution of KO proteins

Transfection of the knocked-out proteins was performed either by JetPrime reagent (PolyPlus 114-07) for STAT KOs using addgene no. 12301 eGFP STAT1 WT for STAT1 and pSEMS vector for STAT2 transfection or by electroporation (NEPA21 Super Electroporator, NEPAGENE) for the IFNAR KOs (by using pDisplay vector). Twenty-four to 48 h later, cells were treated with IFNβ to evaluate AV activity, gene induction, and STAT protein levels. Since AP activity requires 96-h IFN induction, it is impossible to evaluate it by using transient transfection. For IRF9 KD, HeLa and KO cells were transfected for 48 h with human siRNAs ON-TARGETplus IRF9 or ON-TARGETplus Non-Targeting siRNA no. 2 (control siRNA) (Dharmacon). Transfection was performed with INTERFERin (Polyplus-transfection) according to the manufacturer's recommendations.

Cell surface receptor analysis using FACS

IFNARs surface levels were monitored as described in Ref. [15] using monoclonal antibodies AA3 and 117.7, which are specific for IFNAR1 and IFNAR2, respectively. Adherent cells were first detached with phosphate-buffered saline with 5 mM EDTA and re-suspended in phosphate-buffered saline containing 3% fetal bovine serum (Gibco 12657-029). Gating of live cells was done by using Propidium Iodide (Sigma-Aldrich p-4864). Samples were analyzed with the BD Accuri™ C6 personal flow cytometer.

RNAseq

Cells were treated with 2 nM IFNβ for 16 h. RNA samples were extracted using 5-Prime PerfectPure RNA purification kit (Gentra 2302340). Total RNA (500 ng) was fragmented followed by reverse transcription and second strand cDNA synthesis. The double-strand cDNA was subjected to end repair, A base addition, adapter ligation, and PCR amplification to create libraries. Libraries were evaluated by Qubit and TapeStation. Sequencing libraries were constructed with barcodes to allow multiplexing of 12 samples in 1 lane. Around 20 million single-end 60-bp reads were sequenced per sample on Illumina HiSeq 2500 V4 instrument. The RNAseq data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus [61] and are accessible through GEO Series accession number GSE118551. UTAP [62] is the transcriptome analysis pipeline used here to analyze the RNAseq and MARseq data.

MARseq

HeLa, STAT single- and-double KO cell lines were treated with 2 nM IFNβ for 24 h. RNA samples were extracted using the Qiagen RNeasy Mini Kit (Cat No./ID: 74104). cDNA preparation and RNA sequencing were performed as described by Jaitin *et al*. [63].

Error analysis

All the experiments were performed using multiple biological replicates. For qPCR data, the average of at least three independent biological replicates is shown, with their affiliated standard errors. Significance was calculated using ANOVA with tukey post-test as implemented in the Kaleidagraph software package version 4.5, with significance of *$0.005 \ll P \ll 0.05$, ** $0.0005 \ll P \ll 0.005$, and *** $P \ll 0.0005$. For RNA-seq, 96% of $\Delta\Delta$CT data of biological replicate experiments were within 2-fold of one another. Therefore, we chose as significant level induction of $\gg$2-fold.

Acknowledgments

We want to thank Dr. Daniel Harari for performing the MARSseq and the INCPM for performing the RNAseq experiments detailed in this manuscript. This research was funded by the United States–Israel Binational Science Foundation Grant 2011093 and the I-CORE Program of the Planning and Budgeting Committee of the Israel Science Foundation Grant 1775/12.

Conflict of Interest Statement: The authors declare that they have no conflicts of interest with the contents of this article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmb.2019.06.007.

Received 28 February 2019;
Received in revised form 26 May 2019;
Available online 15 June 2019

Keywords:
type I interferon;
signal transduction;
protein phosphorylation;
gene regulation;
antiviral activity

†co-first authors, contributed equally to the paper

Abbreviations used:
IFN, interferon; AV, antiviral; AP, antiproliferation; JAK, janus kinase; STAT, signal transducers and activators of transcription; KD, knockdown; WB, Western blot; WT, wild type; VSV, vasicular stomatitis virus; EMCV, encephalomyocarditis virus; ISGs, IFN-induced genes; GAS, gamma-activated sequence

References

[1] A. Isaacs, J. Lindenmann, Virus interference. I. The interferon, Proc. R. Soc. Lond. B Biol. Sci. 147 (1957) 258.
[2] O. Haller, G. Kochs, F. Weber, The interferon response circuit: induction and suppression by pathogenic viruses, Virology 344 (2006) 119.
[3] L.C. Platanias, Mechanisms of type-I- and type-II-interferon-mediated signalling, Nat Rev Immunol 5 (2005) 375.
[4] W.G. Cooksley, T. Piratvisuth, S.D. Lee, V. Mahachai, Y.C. Chao, T. Tanwandee, A. Chutaputti, W.Y. Chang, F.E. Zahm, N. Pluck, Peginterferon alpha-2a (40 kDa): an advance in the treatment of hepatitis B e antigen-positive chronic hepatitis B, J. Viral Hepat. 10 (2003) 298.
[5] D.S. Goodin, The use of interferon Beta and glatiramer acetate in multiple sclerosis, Semin. Neurol. 33 (2013) 13.
[6] Y.V. Katlinskaya, K.V. Katlinski, A. Lasri, N. Li, D.P. Beiting, A.C. Durham, T. Yang, E. Pikarsky, C.J. Lengner, F.B. Johnson, Y. Ben-Neriah, S.Y. Fuchs, Type I interferons control proliferation and function of the intestinal epithelium, Mol. Cell. Biol. 36 (2016) 1124.
[7] G. Uze, G. Schreiber, J. Piehler, S. Pellegrini, The receptor of the type I interferon family, Curr. Top. Microbiol. Immunol. 316 (2007) 71.
[8] B. Cohen, D. Novick, S. Barak, M. Rubinstein, Ligand-induced association of the type I interferon receptor components, Mol. Cell. Biol. 15 (1995) 4208.
[9] M.M. Brierley, E.N. Fish, Review: IFN-alpha/beta receptor interactions to biologic outcomes: understanding the circuitry, J. Interf. Cytokine Res. 22 (2002) 835.
[10] G.R. Stark, I.M. Kerr, B.R. Williams, R.H. Silverman, R.D. Schreiber, How cells respond to interferons, Annu. Rev. Biochem. 67 (1998) 227.
[11] N.A. de Weerd, S.A. Samarajiwa, P.J. Hertzog, Type I interferon receptors: biochemistry and biological functions, J. Biol. Chem. 282 (2007), 20053.
[12] M.M. Brierley, E.N. Fish, Stats: multifaceted regulators of transcription, J. Interf. Cytokine Res. 25 (2005) 733.
[13] N.A. de Weerd, T. Nguyen, The interferons and their receptors—distribution and regulation, Immunol. Cell Biol. 90 (2012) 483.
[14] J.E. Darnell, Interferon research: impact on understanding transcriptional control, Curr. Top. Microbiol. Immunol. 316 (2007) 155.
[15] D. Levin, D. Harari, G. Schreiber, Stochastic receptor expression determines cell fate upon interferon treatment, Mol. Cell. Biol. 31 (2011) 3252.
[16] S.V. Kotenko, J.A. Langer, Full house: 12 receptors for 27 cytokines, Int. Immunopharmacol. 4 (2004) 593.
[17] P. Lamken, M. Gavutis, I. Peters, J. Van der Heyden, G. Uze, J. Piehler, Functional cartography of the ectodomain of the type I interferon receptor subunit ifnar1, J. Mol. Biol. 350 (2005) 476.
[18] G. Schreiber, J. Piehler, The molecular basis for functional plasticity in type I interferon signaling, Trends Immunol. 36 (2015) 139.
[19] C. Thomas, I. Moraga, D. Levin, P.O. Krutzik, Y. Podoplelova, A. Trejo, C. Lee, G. Yarden, S.E. Vleck, J.S. Glenn, G.P. Nolan, J. Piehler, G. Schreiber, K.C. Garcia, Structural linkage between ligand discrimination and receptor activation by type I interferons, Cell 146 (2011) 621.
[20] G. Schreiber, The molecular basis for differential type I interferon signaling, J. Biol. Chem. 292 (2017) 7285.
[21] S. Leung, S.A. Qureshi, I.M. Kerr, J.E.J. Darnell, G.R. Stark, Role of STAT2 in the alpha interferon signaling pathway, Mol. Cell. Biol. 15 (1995) 1312.
[22] K. Blaszczyk, H. Nowicka, K. Kostyrko, A. Antonczyk, J. Wesoly, H.A.R. Bluyssen, The unique role of STAT2 in constitutive and IFN-induced transcription and antiviral responses, Cytokine Growth Factor Rev. 29 (2016) 71.
[23] X. Li, S. Leung, I.M. Kerr, G.R. Stark, Functional subdomains of STAT2 required for preassociation with the alpha interferon receptor and for signaling, Mol. Cell. Biol. 17 (1997) 2048.
[24] O.W. Nadeau, P. Domanski, A. Usacheva, S. Uddin, L.C. Platanias, P. Pitha, R. Raz, D. Levy, B. Majchrzak, E. Fish, O. R. Colamonici, The proximal tyrosines of the cytoplasmic domain of the beta chain of the type I interferon receptor are essential for signal transducer and activator of transcription (Stat) 2 activation. Evidence that two Stat2 sites are required to reach a threshold of interferon alpha-induced Stat2 tyrosine phosphorylation that allows normal formation of interferon-stimulated gene factor 3, J. Biol. Chem. 274 (1999) 4045.
[25] H.C. Steen, A.M. Gamero, STAT2 phosphorylation and signaling, JAKSTAT 2 (2013), e25790.
[26] N.A. de Weerd, J.P. Vivian, T.K. Nguyen, N.E. Mangan, J.A. Gould, S.J. Braniff, L. Zaker-Tabrizi, K.Y. Fung, S.C. Forster, T. Beddoe, H.H. Reid, J. Rossjohn, P.J. Hertzog, Structural basis of a unique interferon-beta signaling axis mediated via the receptor IFNAR1, Nat. Immunol. 14 (2013) 901.

[27] J. Piehler, C. Thomas, K. Christopher Garcia, G. Schreiber, Structural and dynamic determinants of type I interferon receptor assembly and their functional interpretation, Immunol. Rev. 250 (2012) 317.
[28] J.J. O'Shea, R. Plenge, JAK and STAT signaling molecules in immunoregulation and immune-mediated disease-Immunity 36 (2012) 542.
[29] G.R. Stark, J.E.J. Darnell, The JAK-STAT pathway at twenty, Immunity 36 (2012) 503.
[30] H. Nguyen-Jackson, A.D. Panopoulos, H. Zhang, H.S. Li, S. S. Watowich, STAT3 controls the neutrophil migratory response to CXCR2 ligands by direct activation of G-CSF-induced CXCR2 expression and via modulation of CXCR2 signal transduction, Blood 115 (2010) 3354.
[31] H. Zhang, H. Nguyen-Jackson, A.D. Panopoulos, H.S. Li, P. J. Murray, S.S. Watowich, STAT3 controls myeloid progenitor growth during emergency granulopoiesis, Blood 116 (2010) 2462.
[32] E.G. Schulz, L. Mariani, A. Radbruch, T. Höfer, Sequential polarization and imprinting of type 1 T helper lymphocytes by interferon-gamma and interleukin-12, Immunity 30 (2009) 673.
[33] L. Durant, W.T. Watford, H.L. Ramos, A. Laurence, G. Vahedi, L. Wei, H. Takahashi, H.W. Sun, Y. Kanno, F. Powrie, J.J. O'Shea, Diverse targets of the transcription factor STAT3 contribute to T cell pathogenicity and homeostasis, Immunity 32 (2010) 605.
[34] L.L. Elo, H. Järvenpää, S. Tuomela, S. Raghav, H. Ahlfors, K. Laurila, B. Gupta, R.J. Lund, J. Tahvanainen, R.D. Hawkins, M. Oresic, H. Lähdesmäki, O. Rasool, K.V. Rao, T. Aittokallio, R. Lahesmaa, Genome-wide profiling of interleukin-4 and STAT6 transcription factor regulation of human Th2 cell programming, Immunity 32 (2010) 852.
[35] L. Wei, G. Vahedi, H.W. Sun, W.T. Watford, H. Takatori, H.L. Ramos, H. Takahashi, J. Liang, G. Gutierrez-Cruz, C. Zang, W. Peng, J.J. O'Shea, Y. Kanno, Discrete roles of STAT4 and STAT6 transcription factors in tuning epigenetic modifications and transcription during T helper cell differentiation, Immunity 32 (2010) 840.
[36] M.R. Rani, R.M. Ransohoff, Alternative and accessory pathways in the regulation of IFN-beta-mediated gene expression, J. Interf. Cytokine Res. 25 (2005) 788.
[37] S. Uddin, E.N. Fish, D.A. Sher, C. Gardziola, M.F. White, L.C. Platanias, Activation of the phosphatidylinositol 3-kinase serine kinase by IFN-alpha, J. Immunol. 158 (1997) 2390.
[38] S. Kaur, A. Sassano, B. Dolniak, S. Joshi, B. Majchrzak-Kita, D.P. Baker, N. Hay, E.N. Fish, L.C. Platanias, Role of the Akt pathway in mRNA translation of interferon-stimulated genes, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 4808.
[39] H. Lei, P.J. Furlong, J.H. Ra, D. Mullins, R. Cantor, D.L. Fraker, F.R. Spitz, AKT activation and response to interferon-beta in human cancer cells, Cancer Biol Ther 4 (2005) 709.
[40] D. Harari, R. Abramovich, A. Zozulya, P. Smith, S. Pouly, M. Koster, H. Hauser, G. Schreiber, Bridging the species divide: transgenic mice humanized for type-I interferon response, PLoS One 9 (2014), e84259.
[41] A. Apelbaum, G. Yarden, S. Warszawski, D. Harari, G. Schreiber, Type I interferons induce apoptosis by balancing cFLIP and caspase-8 independent of death ligands, Mol. Cell. Biol. 33 (2013) 800.
[42] H. Mi, X. Huang, A. Muruganujan, H. Tang, C. Mills, D. Kang, P.D. Thomas, PANTHER version 11: expanded annotation data from Gene Ontology and Reactome pathways, and data analysis tool enhancements, Nucleic Acids Res. 45 (2017) D183.
[43] S. Mostafavi, H. Yoshida, D. Moodley, H. LeBoité, K. Rothamel, T. Raj, C. Ye, N. Chevrier, S.-Y. Zhang, T. Feng, M. Lee, J.-L. Casanova, J. Clark, M. Hegen, J.-B. Telliez, N. Hacohen, P. De Jager, A. Regev, D. Mathis, C. Benoist, Parsing the interferon transcriptional network and its disease associations, Cell 164 (2016) 564.
[44] L.B. Ivashkiv, L.T. Donlin, Regulation of type I interferon responses, Nat Rev Immunol 14 (2014) 36.
[45] A. Kumatori, D. Yang, S. Suzuki, M. Nakamura, Cooperation of STAT-1 and IRF-1 in interferon-gamma-induced transcription of the gp91(phox) gene, J. Biol. Chem. 277 (2002) 9103.
[46] A. Rettino, N.M. Clarke, Genome-wide identification of IRF1 binding sites reveals extensive occupancy at cell death associated genes, J Carcinog Mutagen (2013), S6-009. https://doi.org/10.4172/2157-2518.S6-009 (Spec Iss Apoptosis).
[47] L.M. Pfeffer, J.E. Mullersman, S.R. Pfeffer, A. Murti, W. Shi, C.H. Yang, STAT3 as an adapter to couple phosphatidylinositol 3-kinase to the IFNAR1 chain of the type I interferon receptor, Science 276 (1997) 1418.
[48] C.H. Yang, A. Murti, S.R. Pfeffer, L. Basu, J.G. Kim, L.M. Pfeffer, IFNalpha/beta promotes cell survival by activating NF-kappa B, Proc. Natl. Acad. Sci. U. S. A. 97 (2000), 13631.
[49] M. Livingstone, K. Sikström, P.A. Robert, G. Uzé, O. Larsson, S. Pellegrini, Assessment of mTOR-dependent translational regulation of interferon stimulated genes, PLoS One 10 (2015), e0133482.
[50] J.A. Langer, Interferon at 50: new molecules, new potential, new (and old) questions, Sci. STKE 2007 (2007) pe53.
[51] A.T. Reder, X. Feng, How type I interferons work in multiple sclerosis and other diseases: some unexpected mechanisms, J. Interf. Cytokine Res. 34 (2014) 589.
[52] S.T. Perry, M.D. Buck, S.M. Lada, C. Schindler, S. Shresta, STAT2 mediates innate immunity to Dengue virus in the absence of STAT1 via the type I interferon receptor, PLoS Pathog. 7 (2011), e1001297.
[53] C.K. Lee, R. Raz, R. Gimeno, R. Gertner, B. Wistinghausen, K. Takeshita, R.A. DePinho, D.E. Levy, STAT3 is a negative regulator of granulopoiesis but is not required for G-CSF-dependent differentiation, Immunity 17 (2002) 63.
[54] W.B. Wang, D.E. Levy, C.K. Lee, STAT3 negatively regulates type I IFN-mediated antiviral response, J. Immunol. 187 (2011) 2578.
[55] Y. Qing, G.R. Stark, Alternative activation of STAT1 and STAT3 in response to interferon-gamma, J. Biol. Chem. 279 (2004), 41679.
[56] P.J. Murray, The JAK–STAT signaling pathway: input and output integration, J. Immunol. 178 (2007) 2623.
[57] A. Majoros, E. Platanitis, E. Kernbauer-Hölzl, F. Rosebrock, M. Müller, T. Decker, Canonical and non-canonical aspects of JAK–STAT signaling: lessons from interferons for cytokine responses, Front. Immunol. 8 (2017).
[58] A. Adey, J.N. Burton, J.O. Kitzman, J.B. Hiatt, A.P. Lewis, B.K. Martin, R. Qiu, C. Lee, J. Shendure, The haplotype-resolved genome and epigenome of the aneuploid HeLa cancer cell line, Nature 500 (2013) 207.
[59] J.W. Schoggins, S.J. Wilson, M. Panis, M.Y. Murphy, C.T. Jones, P. Bieniasz, C.M. Rice, A diverse range of gene products are effectors of the type I interferon antiviral response, Nature 472 (2011) 481.
[60] L.C. Roisman, D.A. Jaitin, D.P. Baker, G. Schreiber, Mutational analysis of the IFNAR1 binding site on IFNalpha2 reveals the architecture of a weak ligand–receptor binding-site, J. Mol. Biol. 353 (2005) 271.

[61] R. Edgar, M. Domrachev, A.E. Lash, Gene expression omnibus: NCBI gene expression and hybridization array data repository, Nucleic Acids Res. 30 (2002) 207.
[62] R. Kohen, J. Barlev, G. Hornung, G. Stelzer, E. Feldmesser, K. Kogan, M. Safran, D. Leshkowitz, UTAP: user-friendly transcriptome analysis pipeline, BMC Bioinformatics 20 (2019) 154.
[63] D.A. Jaitin, E. Kenigsberg, H. Keren-Shaul, N. Elefant, F. Paul, I. Zaretsky, A. Mildner, N. Cohen, S. Jung, A. Tanay, I. Amit, Massively parallel single-cell RNA-seq for marker-free decomposition of tissues into cell types, Science 343 (2014) 776.