Phosphorylation of STAT2 on serine-734 negatively regulates the IFN-α-induced antiviral response

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ABSTRACT

Serine phosphorylation of STAT proteins is an important post-translational modification event that, in addition to tyrosine phosphorylation, is required for strong transcriptional activity. However, we recently showed that phosphorylation of STAT2 on S287 induced by type I interferons (IFN-α and IFN-β), evoked the opposite effect. S287-STAT2 phosphorylation inhibited the biological effects of IFN-α. We now report the identification and characterization of S734 on the C-terminal transactivation domain of STAT2 as a new phosphorylation site that can be induced by type I IFNs. IFN-α-induced S734-STAT2 phosphorylation displayed different kinetics to that of tyrosine phosphorylation. S734-STAT2 phosphorylation was dependent on STAT2 tyrosine phosphorylation and JAK1 kinase activity. Mutation of S734-STAT2 to alanine (S734A) enhanced IFN-α-driven antiviral responses compared to those driven by wild-type STAT2. Furthermore, DNA microarray analysis demonstrated that a small subset of type I IFN stimulated genes (ISGs) was induced more by IFNα in cells expressing S734A-STAT2 when compared to wild-type STAT2. Taken together, these studies identify phosphorylation of S734-STAT2 as a new regulatory mechanism that negatively controls the type I IFN-antiviral response by limiting the expression of a select subset of antiviral ISGs.

KEY WORDS: STAT2, Serine phosphorylation, JAK1, Interferon, Virus infection, Vesicular stomatitis

INTRODUCTION

Signal transducer and activator of transcription (STAT) 2 is a pivotal component of the type I interferon (IFN) signaling pathway (Leung et al., 1995; Steen and Gamero, 2013). Type I IFNs, which include IFN-α, -β, -ε, -κ and -ω, activate the tyrosine phosphorylation of both STAT2 and STAT1 through engagement of the heterodimeric type I IFN receptor composed of IFNAR1 and IFNAR2 (Darnell, 1997; Pestka et al., 2004). IFN binding to its receptor triggers the activation of protein tyrosine Janus kinases (JAK1) and TYK2 through reciprocal transphosphorylation in response to receptor dimerization (Barbieri et al., 1994; Muller et al., 1993; Velazquez et al., 1995; Yan et al., 1996). JAK1 and TYK2 phosphorylate STAT1 and STAT2 on a conserved tyrosine residue, resulting in dimerization of the two STATs, that together with the DNA-binding protein IRF9 form the heterotrimeric IFN-stimulated gene factor 3 (ISGF3) complex (Fu et al., 1990, 1992; Impota et al., 1994). ISGF3 translocates to the nucleus and binds to the promoters of multiple IFN-stimulated genes (ISGs) to activate their transcription (Fu et al., 1990; Li et al., 1997a). The resulting gene products drive the cellular responses to IFNs that include antiviral, antiproliferative and, in certain instances, pro-apoptotic responses (Chawla-Sarkar et al., 2001; de Veer et al., 2001).

The specific role of STAT2 in the ISGF3 complex is to provide its transactivation domain (TAD) for full transcriptional activity (Li et al., 1996). The STAT2 TAD is situated at the C-terminal end and spans ∼100 amino acid residues (amino acids 747–851) (Kraus et al., 2003) and binds p300 and CBP (hereafter p300/CBP, also known as EP300 and CREBBP, respectively) and GCN5 (also known as KAT2A), both of which are involved in facilitating transcription through their histone acetylation activities (Bhattacharya et al., 1996; Paulson et al., 2002). Earlier studies have suggested that the most crucial region for transactivation resides between residues 800 and 832 because deletion of this region abolished the transcriptional activity of ISGF3 (Qureshi et al., 1996). Later studies have suggested T800 and Y833 as additional phosphorylation sites (Farrar et al., 2000; Hornbeck et al., 2012; Shiomizu et al., 2013). Although mutation of Y833 to a phenylalanine residue impaired dimerization of STAT2–STAT4 induced by type I IFNs, evidence is lacking to support the biological significance of T800 and Y833 in their phosphorylated state (Hornbeck et al., 2012; Shiomizu et al. 2013).

Aside from tyrosine phosphorylation, serine phosphorylation modulates the transcriptional activity of the STATs. STATs are phosphorylated on serine residues located predominantly in the N-terminal region of the TAD, usually 20–30 amino acid residues C-terminal of the conserved tyrosine (Decker and Kovarik, 2000). STAT1, STAT3, STAT4, STAT5a and STAT5b all share a conserved motif consisting of two prolines flanking either a single serine residue or a serine residue preceded by a methionine residue, usually referred to as the ‘p(M)SP’ motif, that is targeted for phosphorylation. STAT2 and STAT6 are the exception as they lack the PMSP motif. STAT6, however, is phosphorylated at a serine residue in the TAD, but its function is to inhibit gene activation (Shirakawa et al., 2011). Most recently, we made the discovery that S287-STAT2, which is mapped to the coiled-coil domain, is phosphorylated in response to IFN-α stimulation (Steen et al., 2013). In contrast to other STATs, but similarly to STAT6, phosphorylation of S734-STAT2 did not augment, but rather inhibited, gene transcription.

In this study, we report that S734 is a new STAT2 phosphorylation site targeted by type I IFN stimulation. This molecular event relies on JAK kinase activity and STAT2 tyrosine phosphorylation. Phosphorylation of S734-STAT2 plays a specific
role in type I IFN signaling by contributing to the inactivation of the antiviral, but not the anti-proliferative, response to IFN-α and IFN-β. Our findings indicate that phosphorylation of STAT2 at specific serine residues dictates the specificity and kinetics of an IFN-α and IFN-β response.

RESULTS

Identification of a new IFN-α-induced S734-STAT2 phosphorylation by mass spectrometry

To gain further insight into the regulation of type I IFN signaling by post-translational modifications of STAT2, we reconstituted STAT2-null U6A cells with human STAT2. We treated these cells with or without IFN-α and immunoprecipitated STAT2 from these cells, which was then subjected to mass spectrometry. From this analysis, we identified phosphorylation of S734 as a new IFN-α-induced post-translational modification of STAT2 (Fig. 1). S734 maps to the TAD of STAT2 (Fig. 1B) and appears to be evolutionarily restricted to primates (Fig. 1C). To confirm our mass spectrometry data and show that STAT2 was indeed phosphorylated on S734 after stimulation with type I IFNs, we generated a polyclonal rabbit antibody against an immunopeptide containing phosphorylated (p)S734 and its surrounding amino acids. We established the specificity of the pS734-STAT2 antibody in two ways. First, we showed the absence of a pS734-STAT2 signal in immunoblots of protein lysates from parental U6A cells and U6A cells stably expressing mutant STAT2 in which S734 was changed to alanine (S734A) (Fig. 2A; Fig. S1A). Of note, a second band was often detected migrating immediately below the pS734-STAT2-specific band. Given that this band was also detected in STAT2-null U6A cells and in S734A-STAT2-expressing U6A cells, we interpret this lower protein band to be non-specific. Second, to confirm that the observed protein band recognized by our pS734-STAT2 antibody was indeed a phosphorylated amino acid, immunoprecipitated wild-type (WT-)STAT2 was treated with or without lambda protein phosphatase (LPP). LPP treatment eliminated the protein band corresponding to pS734-STAT2 (Fig. 2B). With these two sequential approaches, we convincingly demonstrate the specificity of the anti-pS734-STAT2 antibody.

S734-STAT2 phosphorylation by IFN-α is observed in multiple cell lines

We consistently detected constitutive phosphorylation of S734-STAT2 in resting U6A cells reconstituted with WT-STAT2. The level of phosphorylated S734-STAT2 was increased with IFN-α.
after 1 h of treatment that then peaked at 4 h (Fig. 2A). We next evaluated the basal phosphorylation and the kinetics of IFN-α-induced phosphorylation of S734-STAT2 in cell lines that express endogenous STAT2. No basal levels of phosphorylated S734-STAT2 were observed in immortalized human mammary epithelial hTERT-HME1 cells. In response to IFN-α treatment, induction of
serine phosphorylation was first detected at 8 h of cytokine exposure that peaked around 12 h and decreased thereafter (Fig. 2C). As expected, given that STAT2 is an ISG, total STAT2 levels also increased over time in hTERT-HME1 cells. The observed decrease in pS734-STAT2 detected at 24 h did not correlate with elevated STAT2 levels, indicating at least a partial independence between STAT2 levels and S734-STAT2 phosphorylation in hTERT-HME1 cells. Similarly, IFN-α treatment also induced S734-STAT2 phosphorylation in two human colon carcinoma cell lines (Fig. 2D,E). Additionally, we show that human STAT2 was phosphorylated on S734 when reconstituted in Stat2−/− mouse embryonic fibroblasts (MEFs) following treatment with murine IFN-β (Fig. 2F; Fig. S1B). It is important to remark that in all the cell lines we studied, with the exception of T29 cells, IFN-α-stimulated phosphorylation of S734-STAT2 peaked later when compared against Y690-STAT2 phosphorylation. Furthermore, we found no differences in the kinetics of tyrosine phosphorylation of STAT2 and STAT1 between U6A cells expressing wild-type and mutant S734A-STAT2 at up to 8 h of IFN-α treatment (Fig. 2A). However, when examined at 24 h, we found STAT2 tyrosine phosphorylation to be prolonged in S734A-STAT2 U6A cells and barely detectable in WT-STAT2 U6A cells (Fig. 7D), indicating that S734-STAT2 plays a role in regulating STAT2 activation.

**IFN-α can induce phosphorylation of STAT2 at S734 only in the presence of JAK kinases and if its Y690 is phosphorylated**

To define the signaling requirements for S734-STAT2 phosphorylation, we first examined the role of STAT2 tyrosine phosphorylation as a potential pre-requisite event, as previously demonstrated with STAT1 serine phosphorylation (Sadzak et al., 2008). We chose two well-characterized STAT2 mutants for this analysis: Y690F-STAT2 and R409A/K415A-STAT2 (RKAA). The Y690F-STAT2 mutant cannot be phosphorylated by type I IFNs and is thus unable to translocate to the nucleus (Li et al., 1997b). The RKAA-STAT2 mutant is retained in the cytosol due to a non-functioning nuclear localization signal, but preserves its capacity to become phosphorylated on tyrosine (Melen et al., 2001). Accordingly, we reconstituted STAT2-null U6A cells with WT-, RKAA-, or Y690F-STAT2. The STAT2-reconstituted U6A cells were treated with or without IFN-α and assayed for pS734-STAT2 by immunoblot analysis. We detected a basal level of pS734-STAT2 with all three versions of STAT2 (Fig. 3A). In response to IFN-α treatment, RKAA-STAT2 showed an increase in S734 phosphorylation (Fig. 3A). In contrast, Y690F-STAT2 did not undergo S734-STAT2 phosphorylation, supporting the notion that tyrosine phosphorylation of STAT2 is necessary for the inducible phosphorylation of S734-STAT2 by type I IFNs. To test the potential contribution of JAK kinases in the phosphorylation of S734-STAT2, U6A cells expressing WT-STAT2 were pre-treated with pan-JAK inhibitor followed by treatment with IFN-α. Western blot analysis demonstrated that JAK activity was necessary for IFN-α-dependent induction of pS734-STAT2 (Fig. 3B). To further validate these results, we evaluated pS734-STAT2 in JAK1-deficient U4A cells. Lack of JAK1 not only impaired pS734-STAT2 by IFN-α, but also basal STAT2 serine phosphorylation (Fig. 3C). Collectively, these results identify tyrosine phosphorylation on Y690 as a prerequisite for enhancing basal S734 phosphorylation of STAT2 in response to IFN-α treatment. Furthermore, IFN-α-induced S734-STAT2 phosphorylation appears to take place in the cytosol as evidenced by the behavior of the RKAA-STAT2 mutant.

Moreover, immunoprecipitation of STAT1 from U6A WT-STAT2 cells revealed that, in the absence of IFN-α treatment, STAT2 associated with STAT1 was serine phosphorylated at a basal level (Fig. 3D). Treatment with IFN-α increased pS734-STAT2, but did not alter pS734-STAT2 binding to STAT1, indicating that pS734-STAT2 is neither enriched in nor excluded from IFN-α-induced STAT2–STAT1 dimers. Moreover, analysis of cytoplasmic and nuclear extracts from IFN-α-treated 2iG7H cells (the parent of U6A cells) showed a time-dependent accumulation of nuclear pS734-STAT2. After 1 h of IFN-α stimulation, the amount of cytoplasmic pS734-STAT2 was decreased, which coincides with an initial accumulation of nuclear pS734-STAT2. Between 4 h and 8 h of IFN-α treatment, pS734-STAT2 peaks, and a higher level is seen in the nucleus with some detected in the cytoplasm, although at reduced levels (Fig. 4A). We also detected pS734-STAT2 accumulation in the nucleus of IFN-α-treated hTERT cells (Fig. 4B). As expected, phosphorylation of S734-STAT2 in hTERT cells required a much longer incubation time with IFN-α, as we previously determined in Fig. 2C. Our data, therefore, show that JAK1-dependent tyrosine phosphorylation of STAT2 is a prerequisite for enhancing STAT2 serine phosphorylation with optimal S734-STAT2 phosphorylation peaking when STAT2 is tyrosine phosphorylated. This finding correlated with a steady increase in the level of phosphorylated S734-STAT2 in the nucleus. Further, U6A cells expressing the RKAA-STAT2 mutant showed intact IFN-α-induced pS734-STAT2 (Fig. 3A). Thus, these results reveal that phosphorylation of S734-STAT2 by type I IFNs must be occurring in the cytoplasm before STAT2 translocates to the nucleus.

**S734-STAT2 phosphorylation does not affect the IFN-α-induced antiproliferative activity, but diminishes the IFN-α-induced antiviral state**

To elucidate the biological relevance of phosphorylated S734-STAT2 in type I IFN signaling, we compared the anti-proliferative responses to IFN-α in U6A cells expressing WT-STAT2 or S734A-STAT2. First, no significant differences in growth rates were observed between U6A cells expressing WT-STAT2 and those expressing S734A-STAT2 (Fig. 5A). Second, the anti-proliferative effects of IFN-α were also unaffected by S734A-STAT2. Similar results were obtained with the phosphomimetic mutant STAT2-S734D (Fig. 5B).

Next, we asked whether phosphorylation on S734 affected IFN-α-mediated antiviral responses. To this end, we left untreated or pre-treated U6A cells expressing WT-STAT2 or S734A-STAT2 with 1000 U/ml of IFN-α, before infection with a prototypic IFN-sensitive virus vesicular stomatitis virus (VSV) that also encodes for GFP expression (VSV-GFP) (Fig. 6A). Quantifying progeny virus yield from these cells demonstrated that, although there was no detectable difference at 24 h between cells expressing WT-STAT2, and phospho-null or phosphomimetic STAT2 mutants (data not shown), virion production at 36 h from S734A-STAT2-containing cells was significantly reduced compared to cells expressing WT-STAT2 (∼3×10⁵ pfu ml⁻¹ versus ∼4×10⁵ pfu ml⁻¹) or phosphomimetic S734D-STAT2 mutant (Fig. 6A). Note that expression of S734A-STAT2 or WT-STAT2 in the absence of IFN-α treatment rendered U6A cells similarly susceptible to VSV infection. It is also important to mention that S734A-STAT2 led to increased STAT2 tyrosine phosphorylation compared to WT-STAT2 in U6A cells pre-treated with IFN-α at 3 h but not at 36 h post-VSV infection (Fig. S2). No differences were found in the level of tyrosine-phosphorylated STAT1 under these conditions. Expression of S734D-STAT2 had no significant effect on VSV yield at either 24 or 36 h post infection, compared to WT-STAT2-
expressing cells. We speculate that the effect of the phosphomimetic mutant is masked by phosphorylation of S287-STAT2 because, as reported by us previously (Steen et al. 2013), it negatively regulates type I IFN and because pS287-STAT2 led to a much stronger phenotype than pS734-STAT2. To confirm our findings using a different cell line, we infected Stat2−/− MEFs expressing either human WT-STAT2 or S734A-STAT2 that had been pre-treated with or without IFN-β with VSV-GFP. We quantified expression of VSV-GFP 36 h later by flow cytometry to determine the degree of viral infection. We found that compared to IFN-β-pre-treated Stat2−/− MEFs expressing human WT-STAT2, IFN-β-pre-treated Stat2−/− MEFs expressing human S734A-STAT2 showed enhanced viral protection at 36 h post infection (Fig. 6B). Taken together, these results indicate that phosphorylation of STAT2 on S734 serves to negatively regulate the type I IFN antiviral response.
STAT2 phosphorylation enhances the expression of a subset of IFN-stimulated antiviral genes

We next conducted whole-genome DNA microarray analysis to identify potential differences in the transcriptional responses to IFN-α between WT-STAT2 and S734A-STAT2 that might explain how phosphorylation of STAT2 on S734 regulated the type I IFN antiviral state. We used total RNA extracted from U6A cells expressing WT-STAT2 or S734A-STAT2 that had been stimulated with or without IFN-α for 4 h. This genomic approach identified 10 ISGs that were induced more by IFN-α in U6A cells expressing S734A-STAT2 than they were in cells expressing WT STAT2 (Fig. 7A; full microarray data is available in the Gene Expression Omnibus under accession number GSE57017). We then selected representative genes from this list to validate by quantitative real-time PCR (qPCR). At 6 h, we consistently detected an enhanced induction of OAS1 and IFIT2 by IFN-α in U6A cells containing S734A-STAT2 than they were in cells expressing WT STAT2 (Fig. 7B). Similarly, we found that Stat2<sup>−/−</sup> MEFs reconstituted with human S734A-STAT2 and stimulated with murine IFN-β showed enhanced transcription of Oas1b and Isg15 when compared to IFN-β-treated MEFs expressing human WT-STAT2 (Fig. 7C). Although differences in Oas1b mRNA levels were not evident until 24 h after IFN-β stimulation, this could be attributed to the activation of human STAT2 in mouse cells. To confirm these results at the protein level, U6A cells reconstituted with either WT-STAT2 or S734A-STAT2 were stimulated with 1000 U/ml of IFN-α for 24 and 60 h and analyzed by western blot analysis for ISG15 and OAS1 expression (Fig. 7D). We found ISG15 protein levels to be similar in WT-STAT2 and S734A-STAT2 U6A cells at 24 h. At 60 h, however, ISG15 protein levels were reduced in WT-STAT2 cells, but remained elevated in S734A-STAT2 cells. In contrast, expression of OAS1 was found to be slightly elevated only at 24 h in S734A-STAT2 cells when compared to WT-STAT2 cells. This observation also correlated with increased STAT2 tyrosine phosphorylation in cells expressing S734A-STAT2 at 24 h, indicating that S734 regulates STAT2 activity. Thus, these results support the idea that phosphorylation of S734-STAT2 contributes to the transcriptional regulation of a small subset of ISGs with antiviral activity.

DISCUSSION

Regulation of type I IFN signaling through STAT2 has remained understudied chiefly because the diversity of STAT TADs and the sequence divergence of the STAT2 TAD among different species have made it challenging to predict amino acids in STAT2 that might be important for proper transcriptional activity (Park et al., 1999; Paulson et al., 1999). For example, serine phosphorylation within the P(M)SP motifs found in several STAT transactivation domains has been shown to be required for STAT-dependent gene
regulation. In contrast, STAT2 lacks this conserved motif, and this opens the door to investigate the biological role of serine-phosphorylated STAT2.

Our study provides new evidence that serine phosphorylation of STAT2 plays a pivotal role in selectively regulating the biological actions of type I IFNs. We have identified a serine phosphorylation

Fig. 6. S734A-STAT2 enhances IFN-α-induced protection against VSV infection. (A) The U6A cell panel was left untreated or pre-treated with IFN-α (1000 U/ml) for 16 h and then infected with GFP-tagged vesicular stomatitis virus (VSV) for 36 h. Viral titers after 36 h post-viral infection (h.p.i.) were determined by plaque assay. Viral titers are shown on a log scale. (B) Western blot analysis of STAT2 expression in Stat2−/− MEFs reconstituted with either human WT- or S734A-STAT2. (C) Stat2−/− MEFs expressing human WT- or S734A-STAT2 were pre-treated or not with murine IFN-β (1000 U/ml) for 16 h and VSV infection was quantified by flow cytometry analysis of GFP-positive cells. Data are shown as the mean±s.e.m. from three or four independent experiments. **P<0.01 (Student’s t-test). NS, not statistically significant.

Fig. 7. S734-STAT2 regulates the expression of a subset of ISGs. (A) Microarray analysis identified a small subset of ISGs for which mRNA levels were found to be enhanced in S734A-STAT2 U6A cells when compared against WT-STAT2 U6A cells after 4 h of IFN-α (1000 U/ml) treatment. Genes marked in A with an asterisk were subsequently validated by qPCR using (B) U6A or (C) Stat2−/− MEFs reconstituted with either human WT- or S734-STAT2 treated with or without IFN-α or IFN-β (1000 U/ml) for 6, 18 or 24 h. The mRNA levels were normalized to actin expression and calculated by using ΔΔCt method. Data are presented as the mean±s.e.m. fold change from untreated cells from three or four independent experiments. *P<0.05, **P<0.01 (Student’s t-test). (D) WT-STAT2 and S734A-STAT2 U6A cells were stimulated or not with IFN-α (1000 U/ml) for 24 or 60 h and levels of ISG15 and OAS1 as well as pY-STAT2, pY-STAT1, STAT1, STAT2 and GAPDH as indicated, were assessed by western blot analysis.
site (S734) on STAT2 that resides in the TAD, but that is away from the crucial region previously mapped for transactivation. This new site appears to specifically regulate the antiviral activity of type I IFNs, without disturbing the anti-proliferative activity. We consistently show that S734-STAT2 is phosphorylated in response to IFN-α stimulation in several cell lines, with kinetics that are distinctly slower than those observed for STAT2 tyrosine phosphorylation. We found JAK1 activity and STAT2 tyrosine phosphorylation to be necessary for pS734-STAT2 induction. Furthermore, we present evidence that IFN-α-induced pS734-STAT2 translocates to the nucleus. Although the serine kinase responsible for phosphorylating S734 is yet unknown, with the use of prediction tools, we have identified two candidate serine kinases that we are currently evaluating for their role in pS734-STAT2 phosphorylation.

The function of this newly discovered post-translational modification appears to differ from the S287 site we recently identified on STAT2. In response to IFN-α stimulation, disabling phosphorylation of S287-STAT2 has two consequences. First, it augments the growth inhibitory effects of type I IFN and, second, it protects cells from viral infection resulting in prolonged cell survival due to reduced virus replication (Steen et al., 2013), two features not entirely shared with S734-STAT2. Although additional studies are underway to determine the combinatorial effect of STAT2 dually phosphorylated on serine residues 287 and 734 in type I IFN signaling, we propose that S734-STAT2 phosphorylation has a specific contributory role in negatively regulating the antiviral effects of type I IFN.

How might phosphorylation of S734-STAT2 negatively regulate type I IFN signaling? Early experiments using U6A cells reconstituted with C-terminally truncated versions of STAT2 showed that deletions up to amino acid 831 did not affect type I IFN signaling and ISG induction (Qureshi et al., 1996). However, removing residues to amino acid 812 led to a partial reduction in STAT2 function, and truncating STAT2 to residue 800 completely disrupted gene induction. The interaction between the STAT2-TAD and p300/DBP has been recently mapped by NMR and confined to amino acids 788–816 of STAT2, with some stabilizing, but non-critical interactions between residues 817 and 833 (Wojciak et al., 2009). Owing to the deleterious effects of C-terminal truncations after removing the first 50 residues, the importance of the N-terminal part of the TAD (residues ~700–800) has not been carefully studied. Compared to other STATs, STAT2 has a very large TAD that includes the region C-terminal of Y690. Adding to that, STAT2 is different from all other STATs in that it does not stably interact with DNA (Bluysen and Levy, 1997). In type I IFN signaling, this task is performed by STAT1 and IRF9, which bind DNA directly while STAT2 contributes its transactivation function in the transcriptional response. Because this particular post-translational modification seems to only affect the level of induction of a select subset of ISGs, we hypothesize that the proteins interacting with STAT2 at the loci of these ISGs are what is providing selectivity that is not seen with other ISGs.

Based on these observations together with our recent report on S287 phosphorylation of STAT2, we propose a preliminary model (Fig. 8) in which dynamic S734 phosphorylation regulates the antiviral activities of type I IFNs. Overall, this discovery highlights striking differences in the role of serine phosphorylation of STAT2 compared to other STATs, including STAT1, wherein phosphorylation on serine enhances, rather than antagonizes, the transcriptional response to type I IFNs.

**MATERIALS AND METHODS**

**Cell culture**

STAT2-deficient human U6A fibrosarcoma cells (obtained from Dr Ana Costa-Pereira, Imperial College London, London, UK), STAT2-null mouse embryonic fibroblasts (provided by Dr Chris Schindler, Columbia University, New York, NY), human colorectal carcinoma cells F6-8 and T29 (obtained from Dr Bert Vogelstein, Johns Hopkins University, Baltimore, MD), and 293FT cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Cellgro Mediatech, Inc., Manassas, VA) supplemented with 10% fetal calf serum (Gemini Bio-Products), 100 units/ml penicillin (Cellgro), 100 μg ml−1 streptomycin (Cellgro), and 1× GlutaMAX (InVitrogen, Waltham, MA). Human fibrosarcoma 2FTGH cells and epithelial mammary cells hTERT-HME1 (kindly provided by Dr George Stark, Cleveland Clinic Foundation, Cleveland, OH) and 293FT cells purchased from Life Technologies (Grand Island, NY) were grown in complete DMEM (Cellgro Mediatech). hTERT-HME1 cells required DMEM further supplemented with mammary epithelium growth medium.
containing bovine pituitary extract, hydrocortisone, insulin, epithelial growth factor, and gentamicin and amphotericin-B (Clonetics, Basel, Switzerland). All cells were grown at 37°C in a humidified atmosphere containing 5% CO₂, authenticated and checked for contamination.

Reagents and antibodies
Recombinant human IFN-α-2a (specific activity 2×10⁷ units ml⁻¹) was purchased from PeproTech, Inc. (Rock Hill, NJ). Recombinant murine IFN-β was a kind gift from Biogen, Inc. (Cambridge, MA). JAK inhibitor was obtained from EMD Millipore (Bedford, MA). Rabbit anti-STAT2 (C-20, cat. no. sc-476, 1:1000), rabbit anti-STAT1 (E-23, cat. no. sc-346, 1:1000) and rabbit anti-Lamin B1 (C-5, cat. no. sc-365962, 1:1000) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-phospho-Y690-STAT2 (cat. no. 07-224, 1:1000) was obtained from Millipore, mouse anti-pY701-STAT1 (cat. no. 612233, 1:1000) was purchased from Proteintech Group, Inc. (Chicago, IL). Affinity purified rabbit polyclonal anti-actin (cat. no. 60008-1, 1:5000) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-phospho-Tyr701-STAT1 (cat. no. sc-476, 1:1000), rabbit anti-STAT1 (E-23, cat. no. sc-346, 1:1000) was a kind gift from Biogen, Inc. (Cambridge, MA). JAK inhibitor was a kind gift from Biogen, Inc. (Cambridge, MA). The complete data files are available at NCBI, GEO accession number GSE57017.

Immunoprecipitation assays were performed by first pre-clearing protein lysates with the Alpha-Innotech HD2 imaging system (ProteinSimple, San Jose, CA). Further statistical analyses were performed using BRB-ArrayTools (Simon et al., 2007). Gene classification into ontology categories (GO) was performed using Blast2GO® version 2.6.4 (Biobam Bioinformatics, Valencia, Spain). The complete data files are available at NCBI, GEO accession number GSE57017.

Statistical analysis
Prism software (GraphPad, San Diego, CA) was used for statistical analysis. Student’s t-test was applied to discern significant statistical differences between samples. P<0.05 was considered significant.
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Competing interests
The authors declare no competing or financial interests.

Author contributions
A.M.G. prepared the manuscript and designed experiments; H.C.S. and K.P.K. conducted and designed experiments, and helped write the manuscript; S.N. and M.Y.H. conducted some of the experiments, and S.B. helped with manuscript preparation and experimental design.

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Data availability
The microarray data reported in this study are available from the Gene Expression Omnibus database under accession number GSE57017 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE57017).

Supplementary information
Supplementary information available online at http://jcs.biologists.org/lookup/doi/10.1242/jcs.185421

References


**Fig S1.** STAT2 protein levels in cells reconstituted with either wild type (WT)- or S734A-STAT2. (A) U6A and (B) Stat2KO MEFs transduced with RFP lentivirus encoding WT or mutant STAT2 were analyzed by flow cytometry analysis using the FL2 channel. Cells were found to be 100% positive for STAT2 based on RFP detection.
**FigS2. Tyrosine phosphorylation of STAT1 and STAT2 in VSV infected cells.** U6A cells reconstituted with wild type (WT)-STAT2 or S374A-STAT2 were pre-treated with IFN-α (1000U/ml) for 16 h, washed with PBS, and then infected with GFP-tagged VSV for either (A) 3h or (B) 36 h. Cells were then harvested and protein extracts prepared. Tyrosine phosphorylated STAT1 and STAT2, total STAT1, STAT2 and actin were assessed by Western blot analysis.