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2 **Homeostatic and Interferon-induced gene expression represent different states of**

3 **promoter-associated transcription factor ISGF3**

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Abstract

Cells maintain the balance between homeostasis and inflammation by adapting and integrating the activity of intracellular signalling cascades, including the JAK-STAT pathway. Our understanding how a tailored switch from homeostasis to a strong receptor-dependent response is coordinated remains limited. We used an integrated transcriptomic and proteomic approach to analyze transcription-factor binding, gene expression and in *vivo* proximity-dependent labelling of proteins in living cells under homeostatic and interferon (IFN)- induced conditions. We show that interferons (IFN) switch murine macrophages from resting-state to induced gene expression by alternating subunits of transcription factor ISGF3. Whereas preformed STAT2-IRF9 complexes control basal expression of IFN-induced genes (ISG), both type I IFN and, unexpectedly, IFNγ cause promoter binding of a complete ISGF3 complex containing STAT1, STAT2 and IRF9. In contrast to the dogmatic view of ISGF3 formation in the cytoplasm, our results suggest a model wherein the assembly of the ISGF3 complex occurs on DNA.

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Introduction

Host defense by the innate immune system requires the establishment of antimicrobial states allowing cells to cope with microorganisms before the onset of the adaptive immune response. Interferons (IFN) are of vital importance in the establishment of cell-autonomous antimicrobial immunity. Particularly the type I-IFN species IFNα and IFNβ (collectively called IFN-I) or type III IFN (IFNλ) are tightly associated with the antiviral state enabling cells to inhibit viral propagation. On the other hand, type II IFN (IFNγ), while similarly capable of inducing the antiviral state, functions predominantly as a macrophage-activating cytokine [1,2]. The accepted scenario of signal transduction by the activated IFN-I receptor complex requires the Janus kinases TYK2 and JAK1 to phosphorylate the signal transducers and activators of transcription STAT1 and 2 on tyrosine. SH2 domain-mediated

47   heterodimerization enables STAT1-STAT2 to enter and reside in the cell nucleus. A third

48   subunit, the interferon regulatory factor 9 (IRF9) joins the heterodimer to complete

49   transcription factor ISGF3 which translocates to the nucleus and binds to interferon-

50   stimulated response elements (ISREs) in ISG promoters. The IFNγ receptor on the other

51   hand employs JAK1 and JAK2 to phosphorylate STAT1. STAT1 homodimers, a.k.a. gamma

52   interferon-activated factor (GAF), translocate to the nucleus and stimulate ISG expression by

53   binding to gamma interferon-activated sites (GAS) [3-5]. In addition to the canonical ISGF3 and

54   GAF, experiments in knockout cells suggest that transcription factors containing IRF9 and

55   either STAT1 or STAT2, but not both, have the potential to control ISG expression [6-8].

56   Furthermore, transcriptional activity of an ISGF3 complex assembled from unphosphorylated

57   STATs (uSTATs) was proposed [9]. The extent to which such noncanonical complexes form

58   and control ISG expression under conditions of a wild type cell remain elusive and are an

59   important aspect of this study.

60

61   As the emergence of cell-autonomous immunity is an arms race between pathogen

62   replication and restrictive mechanisms of the host, speed is a crucial attribute of the cellular

63   response to IFN. This is particularly true for antimicrobial gene expression that must rapidly

64   switch between resting-state and active-state transcription. Mechanisms to meet this

65   demand include remodeling and modification of promoter chromatin prior to the IFN

66   response [10]. Moreover, a host of studies support the concept that cells permanently produce

67   a small amount of IFN-I that stimulates a low, tonic signal by the IFN-I receptor [11,12]. This in

68   turn generates a baseline transcriptional response of IFN-induced genes (ISG). In support of

69   this idea, disrupting IFN-I production or signalling in resting-state cells was shown to cause a

70   drop in basal ISG expression. The enhancement of ISG transcription by an IFN stimulus

71   from basal to induced levels has been compared with the revving-up of a running engine [13].

72   The 'revving-up' or 'autocrine loop' model predicts a tight coupling between homeostatic and

73   receptor mediated interferon signalling and that transcriptional ISG activation in resting and

74   activated states differs in its intensity, but abides by the same mechanism.

4

75   Our study challenges this notion. By combining ChIP-seq and transcriptome analysis we find

76   that basal expression of many ISGs is controlled by a preformed STAT2-IRF9 complex,

77   whose formation does not require signalling by the IFN-I receptor. IFN treatment induces a

78   rapid switch from the STAT2-IRF9 to the canonical ISGF3 complex, revving up ISG

79   transcription. Quantitative proteomic analysis and in vitro interaction studies suggest a

80   model wherein the majority of IRF9 resides in the nucleus under homeostatic conditions and

81   the assembly of the ISGF3 complex occurs on DNA. In conclusion, combining high-

82   throughput data enabled us to reveal mechanisms by which different states of promoter-

83   associated transcription factor ISGF3 control the switch from homeostatic to interferon

84   induced gene expression.

85

86   **Results**

87   **Different transcription factors bind to ISG promoters under resting and induced**

88   **conditions.**

89   We used macrophages to study mechanisms contributing to constitutive ISG expression.

90   Basal expression of ISGs controlled via ISRE promoter sequences (Irf7, Usp18 and Oas1a)

91   in bone marrow-derived macrophages (BMDM) was strongly reduced by ablation of any of

92   the three ISGF3 subunits (Fig. 1a). Surprisingly, genes regulated predominantly by STAT1

93   dimer binding to GAS sequences such as Irf1 and Irf8 were largely unaffected by the gene

94   deficiencies including STAT1 (Fig. 1b). Since IRF9 is the DNA binding subunit of all ISRE-

95   associated transcription factors, our results point towards an important role of this protein

96   and its associates for basal ISG expression. Consistent with this, RNA-Seq and gene set

97   enrichment analysis (GSEA; Fig. 1c) underscore the impact of IRF9 loss on global ISG

98   transcription in resting cells. To determine whether IRF9 dependence reflected the formation

99   of an ISGF3 complex we performed ChIP-Seq in wt cells. The integrated experimental

100   approach is shown in supplementary Fig.1. This first-ever examination of all three ISGF3

101   subunits simultaneously was made possible by the generation of a novel anti-IRF9

102   monoclonal antibody, 6FI-H5, which yields excellent signal-to-noise ratios in ChIP

103   (Supplementary Fig. 2a). IRF9-dependence of STAT1/2 binding was confirmed by ChIP-Seq

104   in Irf9-/- BMDM.

105   The combination of RNA-Seq (plotting wt versus Irf9-/- cells) and ChIP-Seq data (revealing

106   promoter occupancy in wt cells) in a scatter plot showed promoters of a majority of IRF9-

107   dependent genes associated with STAT2 and IRF9, but not with STAT1 (Fig.. 2a, d. A

108   quantitative representation of promoters binding STAT2-IRF9, ISGF3 or STAT1 dimers is

109   given in the pie chart inserts and genes are listen in supplementary table 1). A much smaller

110   fraction was associated with all subunits of an ISGF3 complex. A brief treatment with either

111   IFN-I or IFNγ caused a vast majority of promoters, including many of those associated with

112   STAT2-IRF9 in resting state, to bind ISGF3 (Fig. 2b,c and 2e,f). Promoters associated with

113   STAT1 dimers were prominently represented among IFNγ–induced genes, but not among

114   IFN-I-induced ISG. According to the prevailing JAK-STAT paradigm transcriptional IFN-I

115   responses are ISGF3/ISRE based while those to IFNγ use STAT1 dimers/GAS. Surprisingly

116   we observed a prominent contribution of ISGF3 to IFNγ-induced ISGs as well as the de novo

117   formation of STAT2-IRF9 complexes at both IFN-I and IFNγ-induced ISGs. Among the

118   overlapping peak sets a small number of IRF9 complexes can be found which, upon visual

119   inspection, appeared to be co-bound by STAT2 and are therefore more likely the result of

120   type II peak prediction error (Supplementary Fig. 2b). Taken together the data are consistent

121   with the notion that the switch from resting- to activated-state transcription is caused by a

122   transition from STAT2-IRF9 to ISGF3 at a majority of ISG promoters.

123

124   **Complex formation and intracellular proximity of ISGF3 subunits.**

125   Co-immunoprecipitation studies in human epithelial cell lysates concluded that STAT2 and

126   IRF9 form complexes devoid of STAT1. Corroborating this result, recent work solved the

127   structure of the binding interface and determined that STAT2 binds IRF9 with 500-fold higher

128   affinity than STAT1 [14]. Besides, STAT2-IRF9 as well as STAT1-STAT2 complexes could be

129   co-immunoprecipitated from resting-cell extracts [15-18]. Our results in mouse BMDM are in

130   agreement with these observations. Reciprocal immunoprecipitations demonstrated a clear

131   association between STAT2 and IRF9 that increased after IFN-I treatment (Fig. 3a).

132   Furthermore, we observed a weak association between STAT2 and STAT1 that, in line with

133   earlier observations, did not increase after IFN-I treatment [15,17]. STAT1 and IRF9 could not

134   be coprecipitated despite earlier studies assigning transcriptional activity to STAT1-IRF9

135   complexes [19,20]. Thus, while STAT2-IRF9 and a significantly smaller amount of STAT1-

136   STAT2 complexes can be demonstrated in resting cells, we observed neither ISGF3 nor

137   other complexes containing both STAT1 and IRF9. To corroborate these findings and to rule

138   out that the data reflected stability under IP conditions rather than complex formation in cells,

139   we used the BioID proximity labelling technology as depicted in Supplementary Fig. 1.

140   Modified biotin ligase BirA* fusion proteins [21] biotinylate proteins within a distance of

141   approximately 10nm [22]. Raw 264.7 macrophages, which show the same response to IFN as

142   BMDM (Supplementary Fig. 3a), were engineered to express a doxycycline (Dox)-inducible,

143   myc-tagged IRF9-BirA* fusion gene and used to study complex formation in vivo. The fusion

144   protein restored IFN signalling in Irf9-/- mouse embryonic fibroblasts (Supplementary Fig.

145   3b). To avoid overexpression artefacts, we matched IRF9-BirA* with endogenous IRF9

146   levels (Supplementary Fig. 3c) and used cells expressing an N-terminally myc tagged BirA*

147   gene as controls. We examined STAT-IRF9 proximity prior to IFN treatment by parallel

148   reaction monitoring (PRM), an approach allowing to specifically acquire information about

149   peptides of interest and their quantities [23]. PRM revealed that IRF9-BirA* biotinylated both

150   itself and STAT2 in two biological replicates (Fig. 4a, Supplementary table 2). In contrast,

151   STAT1 was not enriched by Streptavidin-mediated affinity purification compared to the

152   control cells. A 90 min pulse with either IFN-I or with IFNγ did not cause detectable STAT1-

153   IRF9 proximity (Fig. 4b) although it caused ISGF3 formation on DNA (Fig. 2). STAT2 on the

154   other hand was among the top interactors under these conditions (Fig. 4b; Supplementary

155   Fig. 4a). Further analysis of the constitutive IRF9 interactome revealed an association

156   between IRF9 and a number of chromatin modifiers (Fig. 4b-d; Supplementary Fig. 4a).

157   To rule out that the lack of interaction between IRF9 and STAT1 resulted from the

158 unavailability of lysines for biotinylation, we generated Raw 264.7 macrophages expressing

159 a Dox-inducible, myc-tagged STAT2-BirA* fusion gene. STAT2-BirA* expressed at

160 endogenous levels was functional and restored the IFN response of Stat2-/- MEFs

161 (Supplementary Fig. 3d, e). PRM confirmed IRF9 proximity to STAT2-BirA* and additionally

162 revealed proximity between STAT2 and STAT1 (Fig. 4e). The latter finding was further

163 verified in western blots of STAT2-BirA*cell lysates with HRP-coupled streptavidin

164 (Supplementary Fig. 3e). Treatment with IFN further increased the interaction between

165 STAT2 and IRF9 (Supplementary Fig. 4b). Importantly, the spectrum of STAT2 interactors

166 partially overlapped with that produced in the IRF9-BioID approach (Fig. 4b, f,

167 supplementary table 2).

168 Together, the co-IP experiments and the BioID approach demonstrate the presence of

169 STAT2-IRF9 and of STAT1-STAT2 heterodimers in resting cells and confirm our finding that

170 neither resting conditions nor IFN-treatment produce detectable amounts of complexes

171 containing both STAT1 and IRF9. This suggests that ISGF3 complexes may not form in

172 absence of DNA. To examine this possibility, we carried out DNA-mediated precipitation

173 studies (Fig. 3b). Oligonucleotides representing ISG15 or Oas1a ISREs precipitated STAT2-

174 IRF9 from resting-cell extracts, but the entire ISGF3 complex after stimulation with IFN-I (the

175 weak STAT1 band in resting cell extracts (lane 1) was also observed in absence of DNA

176 (lanes 5 and 6) and results from nonspecific association to the carrier material). This

177 behaviour resembles complex binding in ChIP-Seq (Fig. 2). We conclude that ISRE binding

178 either stabilizes ISGF3 or is required for its formation.

179

180 **Signalling requirements for STAT2-IRF9 formation**

181 The revving-up model of innate immunity predicts that the molecular machinery for

182 constitutive ISG expression requires a low chronic signal from the IFN receptor. It further

183 implies that a small quantity of ISGF3 subunits should be nuclear in untreated cells. To test

184 these assumptions, we first analysed the cellular localization of ISGF3 subunits. We have

185 recently shown that IFN-induced nuclear localization of STAT2 is reduced in Irf9-/- BMDMs

186 [24]. It follows that a portion of IRF9 should be localized to the cell nucleus and that nuclear

187 shuttling of at least a sub-fraction of STAT2 requires IRF9. Indeed, the majority of IRF9 in

188 resting cells was found in the nucleus by immunofluorescence and IFN-I treatment did not

189 further enhance nuclear accumulation (Fig. 5a). Raw 264.7 cell fractionation and western

190 blots revealed that nuclei from resting cells contained IRF9 and a small fraction of STAT2,

191 but not STAT1 (Fig. 5b). The small quantity of STAT1 bound to chromatin according to

192 ChIP-Seq appears to be below the detection limit. To assess whether IFN receptor signalling

193 was important for STAT2 nuclear accumulation, we treated cells with Staurosporine, a

194 potent kinase inhibitor, prior to IFN stimulation. The inhibitor did not reduce nuclear STAT2

195 amounts in resting cells. By contrast, IFN-induced nuclear translocation of STAT2 was

196 abrogated. The small STAT2 quantity found in the nucleus of IFN-I and inhibitor treated cells

197 equaled that of unstimulated cells (Fig. 5b). Next, the cells were treated with a specific JAK

198 inhibitor, P6, which completely abrogated STAT1 and STAT2 tyrosine phosphorylation in

199 IFN-I and IFNγ treated BMDMs (Fig. 5c). As anticipated, the addition of P6 prior to a short

200 IFN treatment led to a complete loss of STAT1 phosphorylation at Y701 and inhibition of

201 STAT1 translocation to the nucleus (Fig. 5d). In contrast, a small fraction of STAT2 was

202 detected in the nucleus of both Raw 264.7 macrophages (Fig. 5e) and BMDM (Fig. 5f),

203 despite a complete loss of Y689 phosphorylation. Taken together these observations

204 support the conclusion that the formation and nuclear localization of STAT2-IRF9 complexes

205 occur independently of a continuous signal from the IFN-I receptor.

206

Tonic IFN receptor signalling maintains expression of ISGF3 subunits

208 The continued presence of STAT2-IRF9 in the nucleus suggests that basal ISG expression

209 should be largely unaffected by JAK inhibition. To test this hypothesis, untreated or P6-

210 treated WT BMDM were compared to the ISGF3-subunit knock-outs (knockout data are as

211 in Fig. 1 and included here for ease of comparison). In agreement with our assumption,

212 basal expression of genes with pre-bound STAT2-IRF9 was largely maintained in the

213   presence of inhibitor (Irf7, Usp18, Oas1a; Fig. 6a). All three knockouts strongly affected

214   STAT2-IRF9-dependent genes. Genes induced via GAS sequences (Irf1, Irf8) were

215   unaffected by either the inhibitor or the knockouts. Thus, shutting down signalling while

216   maintaining ISGF3 subunit levels, as is the case in our inhibitor experiments, sustains basal

217   ISG expression independent of tonic IFNAR signalling.

218   The persistence of nuclear STAT2-IRF9 upon JAK inhibition raises the question of why

219   genes whose basal expression is sustained by this complex are sensitive to a permanent

220   disruption of signalling in cells lacking the IFN-I receptor or STAT1, or that express a

221   STAT1Y701F mutant [24]. A straightforward answer to this question is provided by the results

222   in Fig. 6b showing that ISGF3 subunits are bound to the promoters of each of their genes in

223   resting cells and suggesting they all contribute to each other's basal expression. Consistent

224   with this, Stat1 and Irf9 promoters were associated with ISGF3 and the Stat2 promoter

225   bound STAT2-IRF9. Thus, gene deletion of all three ISGF3 subunits is expected to lower

226   IRF9 levels and therefore any ISRE-dependent basal expression. Consistently, knockout of

227   each subunit caused a severe reduction of the two other subunits (Fig. 6c). Thus, cells

228   lacking the ability to form an ISGF3 complex express low amounts of all its subunits and are

229   therefore unable to sustain STAT2-IRF9-dependent basal gene expression. The data

230   emphasize the importance of studying STAT complex formation in wt conditions. An

231   integrated model depicting the proposed interplay between tonic signalling-dependent and

232   independent events for basal ISG expression and the changes occurring upon IFN treatment

233   are depicted in Fig. 7.

234   **Discussion**

235   Recent findings suggest that unphosphorylated STAT complexes control ISG expression [8].

236   Our study shows an important contribution of STAT2-IRF9 complexes, formed independently

237   of IFN-I receptor signalling, to constitutive ISG expression in resting cells. This added

238   mechanistic insight calls for a reinterpretation of the 'revving-up' model and the implications

239   of tonic signalling by the IFN-I receptor [12,13]. Both models provide a mechanistic framework

240   to explain how cells are prepared for the race between pathogen multiplication and innate

241   resistance. They posit that IFN-dependent responses such as the antiviral state or

242   macrophage activation are not established de novo during immune responses, but represent

243   the enhancement of a pre-existing condition compatible with resting-state physiology. We

244   confirm that tonic signalling is indeed of critical importance for the maintenance of ISGF3

245   subunits and show that the signal-independent formation of STAT2-IRF9 complexes,

246   previously considered as non-canonical, is an integral component of ISG regulation. The

247   change from STAT2-IRF9 to ISGF3 functions is a transcriptional mode switch between

248   resting and active states for many ISGs. Importantly, our observations rely on wt cells. The

249   data with knockout cells show that deletion of one STAT subunit not only extinguishes the

250   function of that protein, but also changes the stoichiometry of the remaining ISGF3

251   components. Component stoichiometry is an important determinant for the proper biological

252   output of JAK-STAT signalling. For example, IL-6 signalling in the absence of STAT3

253   exhibits a transcriptional profile similar to that of IFNγ by preferentially using STAT1 [25].

254   Basal ISG expression requires the constant secretion of miniscule IFN-I amounts to prime

255   cells for a rapid response to microbial infections. This is illustrated by IFNβ promoter-

256   luciferase reporter mice that show weak luminescence in the entire body [26]. Earlier studies

257   revealed IFN-I mRNA and protein in tissues under pathogen-free conditions [27,28]. Besides

258   priming cells for enhanced cytokine responsiveness, this homeostatic mechanism reveals its

259   importance in the maintenance and mobilization of the hematopoietic stem cell niche [29,30].

260   More recent studies suggest that multipotent stem cells achieve ISG expression and an

261   antiviral state independently of IFNAR signalling [31]. The results strengthen the idea that

262   mobilization of ISG promoters via STATs can be uncoupled from IFN-I receptor signalling.

263   The uSTAT model provided a first proof of principle for this possibility. Accordingly, the

264   prolongation of ISG expression occurs via formation of a uISGF3 complex following an early,

265   IFNAR-dependent response that increases the levels of ISGF3 subunits [9]. In hepatic cells a

266   uISGF3 was proposed to function under homeostatic conditions and without a previous

267   IFNAR-dependent signal [32]. However, a mechanism explaining IFNAR-independent ISG

268   expression under conditions of a wildtype cell remained elusive. Here we show that the

269   switch of many ISGs from basal to rapid IFN-induced expression requires exchanging

270   STAT2-IRF9 for ISGF3 complexes. Our model receives strong support from a study

271   comparing basal gene expression in Ifnar1-/- and Tyk2-/- cells, both lacking tonic signalling

272   [33]. This revealed a large overlap with genes whose expression was impaired by the loss of

273   IRF9 in our study. Importantly, tonic-sensitive loci showed higher STAT2 binding at baseline

274   [33,34]. Strong support for a role of IRF9-STAT2 in basal gene expression is also provided by

275   the finding that an IRF9 fusion protein with the STAT2 transactivating domain is sufficient to

276   increase basal ISG expression and to induce an antiviral response in absence of added IFN

277   [35].

278   Earlier studies showing pre-association of STAT2 and IRF9 proposed cytoplasmic

279   localization of the complex, due to a dominant STAT2 nuclear export sequence [36]. Our data

280   revise this idea by showing that a proportion of STAT2-IRF9 resides in the nucleus of resting

281   cells. Importantly, our antibody- and DNA-mediated co-precipitation studies argue in favor of

282   an ISGF3 formation on DNA. In support of this, earlier biochemical studies have failed to

283   demonstrate a DNA-independent, complete ISGF3 complex [37]. More generally, the switch

284   from STAT2-IRF9 to ISGF3 poses the question how these complexes manage constitutive

285   and IFN-induced ISG expression. One reason for the transcriptional increase associated

286   with ISGF3 binding is most likely the improved stability on DNA. While IRF9 makes contacts

287   to the core ISRE, STAT1 increases ISGF3 affinity by additionally interacting with 5' flanking

288   sequences [38]. A higher off-rate of STAT2-IRF9 would also explain how this transcription

289   factor can be rapidly exchanged for ISGF3 after IFN treatment. An additional implication

290   might be transcription factor cooperativity. For example, a STAT2-IRF9 complex reportedly

291   allows for co-regulation of the human IL6 gene by IFN and NFκB-activating agents because

292   STAT2 binds IRF9 as well as NFκB p65 [39]. Consistently, data by Mariani et al. suggest that

293   STAT2-IRF9 mediates cooperativity required for the enhanced induction of genes in

294   response to IFNβ and the NFκB-activating cytokine TNF [40]. Thus, it is possible that STAT2-

295   IRF9 and ISGF3 represent alternative platforms for the integration of transcription factor

296   signalling at promoter level.

297   In vivo biotinylation shows IRF9 to be in close proximity to a large number of proteins

298   involved in chromatin organization and transcription initiation. This points to a putative role of

299   IRF9 in altering the chromatin landscape and accessibility prior to interferon signalling.

300   Some of these interactors, such as BRD4 have previously been linked to interferon

301   signalling [41,42]. Another novel IRF9 interactor, TRIM28, forms complexes with STAT1 and

302   STAT3 [43]. BioID with STAT2-BirA* identified several of the interactors identified in the IRF9

303   proximity screen. Human RVB1 and RVB2, which are highly conserved ATP binding proteins

304   contained in chromatin-remodeling complexes such as Tip60/NuA4, interact with the

305   transactivation domain of STAT2 in the nuclei of IFN-stimulated cells and are required for

306   robust activation of IFNα-stimulated genes [44]. Although we could not find RVB1 or RVB2

307   among biotinylated proteins, Ep400, which is also a subunit of the Tip60/NuA4 complex, was

308   shown in close proximity to both IRF9 and STAT2.

309   We conclude that complexes containing different ISGF3 subunits, both phosphorylated and

310   unphosphorylated, serve distinct and relevant functions in controlling ISG expression. Our

311   study focused on the STAT2-IRF9 to ISGF3 transition, but work by others addressed the

312   importance of STAT1-STAT2 dimers. For example, their constitutive association may be

313   relevant for the rapid formation of the tyrosine-phosphorylated heterodimer after IFN

314   treatment [15] and it prevents dissociation after dephosphorylation [45]. Furthermore,

315   hemiphosphorylated pSTAT1-STAT2 dimers resulting from the preferential tyrosine

316   phosphorylation of STAT1 dampen the response to IFNγ by restricting STAT1 access to the

317   nucleus[18]. The multipurpose employment of ISGF3 subunits thus represents a striking

318   example of the cell's economy in the management of complex regulatory tasks.

319

320 **Acknowledgments**

330

331 **Author contributions**

332 E.P. designed and performed experiments, analyzed results and contributed to

333 computational analyses of BioID data. D.D. and C.C. performed experiments and analyzed

334 results A.S. performed experiments. M.H. and T.G. performed LC-MS and analyzed BioID

335 protein interactors. M.M. provided intellectual input and expertise. M.N. performed

336 computational analyses for RNA-seq and ChIP-seq. T.D. conceived the study, designed

337 experiments, provided intellectual input, helped interpret data, provided supervision and

338 secured funding. T.D. and E.P. wrote the manuscript.

339

340 **Competing interests.**

341 The authors declare no competing interests.

342

343 **Materials & Correspondence.**

344 Correspondence and requests for materials should be addressed to T.D.

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459

460 **METHODS**

461 **Mice, animal experiments**

462 Animal experiments were approved by the institutional ethics and animal welfare committee

463 of the University of Veterinary Medicine, Vienna, and the national authority (Austrian Federal

464 Ministry of Education, Science and Research) according to §§26ff of Animal Experiments

465 Act (Tierversuchsgesetz TVG 2012, BGBl. I Nr 114/2012) under the permission license

466 numbers BMWF 68.205/0032-WF/II/3b/2014 and BMWFW-68.205/0212-WF/V/3b/2016.

467 Animal husbandry and experimentation was performed under the Austrian national law and

468 the ethics committees of the University of Veterinary Medicine Vienna and according to the

469 guidelines of FELASA which match those of ARRIVE. C57BL/6N, Irf9-/-, Stat1-/-, and Stat2-

470 /- mice[1-3] were backcrossed for more than 10 generations on a C57BL/6N background were

471 housed in the same specific-pathogen-free (SPF) facility under identical conditions

472 according to recommendations of the Federation of European Laboratory Animal Science

473 Association and additionally monitored for being norovirus negative.

474

475 **Cell culture**

476 Bone marrow-derived macrophages (BMDMs) were differentiated from bone marrow

477 isolated from femurs and tibias of 8- to 12-week-old mice from both sexes. Femur and tibia

478 were flushed with Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich) and cells

479 were cultured in DMEM supplemented with 10% of fetal calf serum (FCS) (Sigma-Aldrich),

480 10% L929-cell conditioned medium as a source of colony-stimulating factor 1 (CSF-1), 100

481 units/ml penicillin, and 100 ng/ml streptomycin (Sigma-Aldrich). Cells were kept at 37°C and

482 5% CO_2 and differentiated for 10 days. For ChIP-seq and RNA-seq experiments, BMDMs

483 were differentiated in DMEM containing recombinant M-CSF (a kind gift from L. Ziegler-

484 Heitbrock, Helmholtz Center, Munich, Germany). BMDMs and Raw 264.7 cells were

485 stimulated with 10 ng/ml murine interferon gamma (IFNγ eBioscience; Cataog #14-8311-63)

486 or 250 IU/mL of IFNβ (PBL Assay Science; Cataog #12400-1).

487

488 **Generation of monoclonal mouse IRF9 antibody**

489 The murine monoclonal anti-IRF9 antibody was generated in collaboration with Egon Ogris,

490 Stefan Schüchner and Florian Martys from the MFPL monoclonal antibody facility. Full

491 length murine IRF9 was cloned into a pET-Duet1 (Novagen; Catalog # 71146) vector and

492 expressed in E. coli Rosetta pLysS strain and purified using Ni-sepharose beads.

493 Hybridomas from antibody-producing B cells and myeloma cells for the production of

494 monoclonal IRF9 antibodies were generated. The best signal-to-noise ratio in ChIP and

495 western blot analysis was obtained with the single clone 6F1-H5 which was used for this

496 study. The purified antibody can now be purchased from Sigma (Anti-IRF-9, clone 6F1-H5,

497 Cat. No. MABS1920, Emd Millipore).

498

499 **RNA isolation, cDNA synthesis and Q-PCR**

500 Total RNA was extracted from mouse bone-marrow-derived macrophages using the

501 NucleoSpin RNA II kit (Macherey-Nagel; Catalog #: 740955). The cDNAs was prepared

502 using Oligo (dT18) Primer and the RevertAid Reverse Transcriptase (Thermo Fisher

503 Scientific). Real-time qPCR experiments were run on the Mastercycler (Eppendorf) to

504 amplify the Gapdh (housekeeping gene), using SybrGreen (Promega). Primers for qPCR

505 and ChIP PCR listed in supplementary table 3.

506

507 **Western Blot**

508 Cells were lysed in Laemmli buffer (120 mM Tris HCl pH 8, 2% SDS, 10% glycerol). Protein

509 concentration was determined (Pierce BCA Protein Assay Kit). 30µg of protein were mixed

510 with β-mercaptoethanol and bromophenol blue, boiled and loaded on a 10% SDS

511 polyacrylamide gel.

512 Proteins were blotted on a PVDF membrane at 4 °C for 16 hours at 200 mA and for 2 hours

513 at 400 mA in carbonate transfer buffer (3 mM Na2CO3, 10 mM NaHCO3, 20% Ethanol).

514   The membrane was blocked in 5% milk powder in TBS-T for 1 hour at room temperature.

515   The membrane was washed three times with TBS-T and then incubated with the primary

516   antibody over night at 4°C while shaking (Stat1 (Cell Signaling, Catalog #9172); Stat2 (Cell

517   Signaling, Catalog #72604); Lamin B (Santa Cruz, Catalog #sc-6217); α-Tubulin (Sigma,

518   Catalog #T9026); Phospho-Stat1 (Tyr701) (Cell Signaling, Catalog #9167); Phospho-

519   STAT2 (Tyr689) (Cell Signaling Catalog #07-224); Lamin A/C (Santa Cruz, Catalog #sc-

520   376248); GAPDH(Millipore, Catalog #ABS16); IRF9 (6F1)). The next day the membrane

521   was washed three times again with TBS-T and incubated with an appropriate HRP-coupled

522   secondary antibodies for 1h at room temperature (Jackson ImmunoResearch Inc. Code #

523   111-035-003, Code # 115-035-144). The membrane was analyzed with the ChemiDoc™

524   Imaging System from Bio-Rad.

525

Nuclear and Cytoplasmic extraction

527   1×10^6 bone marrow derived macrophages or Raw 264.7 cells were seeded in a 10 cm dish.

528   The next day cells were treated for three hours with 500 nM staurosporine (Staurosporine

529   solution from Streptomyces sp., Sigma S6942) or 15µM JAK Inhibitor (Pyridone 6, biovision,

530   #2534) and afterwards stimulated for 30 minutes either with IFN-β or with IFN-γ. Extraction

531   was carried out according to manufacturer's instructions (NE-PER™ Nuclear and

532   Cytoplasmic Extraction Reagents, Thermo Fischer, #78833). The whole nuclear fraction was

533   loaded, whereas only 30% of the cytoplasmic fraction were loaded on a 10% SDS gel.

534

Immunofluorescence

536   2×10^5 BMDMs were seeded on glass cover slides, treated with IFN-β for 30 minutes and

537   fixed with 3% paraformaldehyde for 20 minutes at room temperature. Cells were

538   permeabilized with 0.1 % saponin in 0.5 M NaCl PBS. Blocking and all the stainings were

539   carried out in 0.1 % saponin and 1 % BSA in 0.5 M NaCl PBS. The IRF9 antibody was used

540   as a 1:10 dilution over night at 4 °C. Secondary goat anti-mouse Alexa Fluor® 488 igGH+L

541 (1:500; Catalog # A-11001) was purchased from Invitrogen. Samples were mounted in DAPI

542 (ProLong™ Diamond Antifade Mountant with DAPI, Invitrogen, # P36962). Images were

543 acquired using Zeiss Axio Imager Z2 with 63X oil objectives. Images were processed and

544 analyzed using the ImageJ software. The background range in all images for DAPI was

545 adjusted to 1500-16383 and for GFP 3000-16383. The images were changed to 14bit. A

546 composite picture was made DAPI was set to to magenta and GFP to green. The type of

547 picture was converted to RGB and a scale bar displaying 10 µm was inserted.

548

549 **RNA-seq**

550 1.5×10^7 cells were seeded on 15 cm dishes. The next day cells were stimulated for 2h

551 either with IFN-β or with IFN-γ. 7 ml Qiazol Lysis Reagent (Qiagen) were added per 15 cm

552 dish. Cells were scraped and vortexed for 20 seconds. 1 ml of the RNA samples in Trizol

553 were used for the RNA prep and 200 µl of Chloroform were added. Samples were vortexed

554 for 15 seconds and centrifuged for 5 minutes at full speed at room temperature. Supernatant

555 was mixed with 1 volume Isopropanol, as well as 1/10 volume 5 M NaCl were added.

556 Samples were incubated for 10 minutes at room temperature and centrifuged at 16.000g for

557 30 minutes at 4 °C. The pellets were washed twice with 75 % EtOH, dried and resuspended

558 in 30 µl of dH_2O.

559 For DNase treatment and clean up the RNase-free DNase Set (Qiagen, #79254) and

560 RNeasy Mini Kit (Qiagen, #74104) were used. For library preparation, the NEBNext®

561 Poly(A) mRNA Magnetic Isolation Module together with the NEBNext Ultra II RNA Library

562 Prep Kit from NEB #E7770L was used according to the manufacturer's protocol. The

563 samples were quality checked and sequenced at the Vienna Biocenter Core Facilities NGS

564 Unit. The RNA-seq experiment was carried out as three independent biological replicates.

565

566 **Cloning**

567 Full length STAT2 and IRF9 mouse cDNA were cloned into the pcDNA3.1 mycBioID vector

568 (provided by Kyle Roux) and further subcloned into the pCW57.1 (Catalog # 41393) or

569 pLVX-TRE3G-ZsGreen1 (Catalog #631350) and used for lentiviral transduction of Raw264.7

570 cells.

571

572 **BioID**

573 BioID was performed according to a published protocol [4]. mycBioID was a gift from Kyle

574 Roux (Addgene plasmid 35700).

575 5×10^6 stable Raw 264.7 cells were seeded on 15 cm dishes and treated with 0,2 µg/ml

576 doxycycline for 24h. 50 µM biotin were added for 18 additional hours. Cells were stimulated

577 for 1.5h either with IFN-β or with IFN-γ. Cells were washed and lysed at room temperature

578 (lysis Buffer: 50mMTris pH7.4; NaCL 500mM; 0,2% SDS; EDTA 5mM + 1x protease

579 inhibitors). Triton X-100 and 50mMTris pH7.4 were added and the protein lysates were

580 sonicated 2x for 30 seconds. Lysates were centrifuged for 5 minutes at full speed and

581 supernatant was transferred to a new tube. Magnetic Pierce Streptavidin beads #88817

582 were washed 3x with lysis buffer. 105 µl beads were incubated with 1,3 mg of protein lysate

583 over night at 4°C. 21 µl beads were kept for western blot analysis, the rest of the beads was

584 used for the analysis with liquid chromatography mass spectrometry. Beads were washed at

585 room temperature with wash buffer 1(2% SDS in H_2O), wash buffer 2 (0,1% deoxycholic

586 acid; 1% TritonX-100, 1mM EDTA, 500mM NaCl, 50mM HEPES; H_2O) and wash buffer 3

587 (0,5% deoxycholic acid; 0,5% NP-40; 1mM EDTA; 250mM LiCl, 10mM Tris pH7.4;H_2O).

588 Beads were washed 5 times with 50mM Tris pH 7.4 and another 5 times with 50 mM

589 ammonium bicarbonate (ABC) and then resuspended in 30 µL of 1 M urea in 50 mM ABC.

590 10 mM dithiothreitol was added and the samples incubated for 30 min at room temperature

591 before adding 20 mM iodoacetamide and incubating for another 30 min at room temperature

592 in the dark. Remaining iodoacetamide was quenched by adding 5 mM DTT and the proteins

593 were digested with 300 ng trypsin (Trypsin Gold, Promega) at 37 °C overnight. After

594 stopping the digest by addition of 1% trifluoroacetic acid (TFA), the supernatant was

595 transferred to a new tube. The beads were washed with 30 µL 0.1% TFA, the supernatants

596 were combined, and the peptides were desalted using C18 Stagetips [5].

597 Peptides were separated on an Ultimate 3000 RSLC nano-flow chromatography system

598 (Thermo-Fisher), using a pre-column for sample loading (Acclaim PepMap C18, 2 cm × 0.1

599 mm, 5 µm, Thermo-Fisher), and a C18 analytical column (Acclaim PepMap C18, 50 cm ×

600 0.75 mm, 2 µm, Thermo-Fisher), applying a segmented linear gradient from 2% to 80%

601 solvent B (80% acetonitrile, 0.1% formic acid; solvent A 0.1% formic acid) at a flow rate of

602 230 nL/min over 120 min. Eluting peptides were analyzed on a Q Exactive HF Orbitrap mass

603 spectrometer (Thermo Fisher), which was coupled to the column with a nano-spray Flex ion-

604 source (Thermo Fisher) using coated emitter tips (New Objective).

605

606 **Shotgun mass spectrometry data acquisition and processing**

607 The mass spectrometer was operated in data-dependent mode, survey scans were obtained

608 in a mass range of 380-1650 m/z with lock mass activated, at a resolution of 120k at 200

609 m/z and an AGC target value of 3E6. The 10 most intense ions were selected with an

610 isolation width of 2 Da, fragmented in the HCD cell at 27% collision energy and the spectra

611 recorded at a target value of 1E5 and a resolution of 30k. Peptides with a charge of +1 or >

612 +6 were excluded from fragmentation, the peptide match feature was set to preferred, the

613 exclude isotope feature was enabled, and selected precursors were dynamically excluded

614 from repeated sampling for 30 seconds.

615 Raw data were processed using the MaxQuant software package (version 1.5.5.1; [6] and the

616 Uniprot mouse reference proteome (www.uniprot.org) as well as a database of most

617 common contaminants. The search was performed with full trypsin specificity and a

618 maximum of two missed cleavages at a protein and peptide spectrum match false discovery

619 rate of 1%. Carbamidomethylation of cysteine residues were set as fixed, oxidation of

620 methionine, phosphorylation of serine, threonine and tyrosine, acetylation of lysine, and N-

621 terminal acetylation as variable modifications. For label-free quantification the "match

622   between runs" feature and the LFQ function were activated [7] - all other parameters were left

623   at default.

624   MaxQuant search results were further processed using the Perseus software package

625   (version 1.5.5.3, [8]. Contaminants, reverse hits, and proteins identified only by site were

626   removed and the log2 transformed LFQ values were used for protein quantification. Mean

627   LFQ intensities of biological replicate samples were calculated and missing values were

628   replaced with a fixed value close to the detection limit. Only proteins that were quantified in

629   both biological replicates in the samples of interest and displayed at least a 3-fold

630   enrichment compared to the MYC-BirA control were considered to be enriched and

631   represented using heat maps.

632

633   **Targeted mass spectrometry data acquisition and processing**

634   PRM assays were generated based on the shotgun measurements, selecting up to 10 high

635   intensity proteotypic peptides for STAT1, STAT2, and IRF9, with no missed cleavages, no

636   methionine, and a good distribution over the chromatographic gradient. PRM assay

637   generation was performed using Skyline [9]. After a test run with a pooled sample showing

638   high target protein expression, we selected 6 or 7 peptides with a single charge state per

639   protein according to their signal-to-noise ratio and their distribution over the gradient and

640   designed a scheduled PRM assay with 6 min windows. For PRM data acquisition we

641   operated the same instrument type as for shotgun MS, applying a 60 min gradient for

642   separation and with the following MS parameters: survey scan with 30k resolution, AGC

643   1E6, 30 ms IT, over a range of 400 to 1300 m/z, PRM scan with 60 k resolution, AGC 1E5,

644   400 ms IT, isolation window of 1.2 m/z with 0.4 m/z offset, and NCE of 28%. In between

645   samples, we acquired wash runs with the same method to monitor potential carry-over.

646   The data analysis, manual validation of all transitions (based on retention time, relative ion

647   intensities, and mass accuracy), and relative quantification was performed in Skyline. Carry-

648   over was negligible, all selected peptides eluted within the 6 minute time windows. The four

649   to six most intense transitions were selected for each peptide and their peak areas were

650   summed up for peptide quantification (total peak area). Peptide intensities were summed up

651   to protein intensities. As quality control we extracted ion chromatograms (MS1) of eight

652   peptides from three highly abundant background carboxylases which showed a LFQ ratio of

653   roughly 1:1 in the shot-gun measurements and calculated normalization factors based on

654   these peptides for the PRM dataset. Unnormalized and normalized protein ratios (log2) of

655   IRF9, STAT1 and STAT2 were similar indicating good reproducibility over all measurements.

656

657   **Visualization of mass spectrometry data**

658   The network diagram was generated with Cytoscape software[10]. Only proteins that displayed

659   a three-fold enrichment in the samples of interest compared to the ligase only control were

660   considered enriched and are displayed in the network. Spatial arrangement of nodes and

661   colour code reflect grouping depending on whether proteins were already pre-associated in

662   the absence of any signal or where specifically enriched upon 1.5h of IFN-β or IFN-γ.

663   treatment. The ClueGO app [11] was used to find overrepresented GO processes and a

664   network of connected GO terms was created.

665

666   **ChIP and ChIP-seq**

667    $1,5 \times 10^7$ bone marrow derived macrophages were seeded on a 15cm dish. The next day

668   cells were stimulated for 1.5h either with IFN-β or with IFN-γ. Cells were crosslinked for 10

669   minutes at room temperature in 1% formaldehyde PBS (thermos fischer #28906). Cells were

670   quenched with 0.125 M glycine for 10 min at RT. Cells were harvested and washed twice

671   with ice cold PBS.   Cells were centrifuged for 5 min at 1350 g at 4°C. Pellets were snap

672   frozen in liquid nitrogen and stored at 80° C over night. Frozen pellets were thawn on ice for

673   60 minutes.   Pellets were resuspended in 5 mL LB1 (50mM Hepes, 140mM NaCl, 1mM

674   EDTA, 10% glycerol, 0,5% NP40, 0,25% TritonX100) by pipetting and rotated at 4°C for 10

675   min.   Samples were centrifuged for 5 minutes at 1350 x g at 4 °C. Pellets were resuspend in

676   5 mL LB2 (10mM Tris, 200mM NaCl, 1mM EDTA, 0,5mM EGTA) by pipetting and rotated at

677 4 °C for 10 min. Samples were centrifuged for 5 minutes at 1350 x g at 4°C. Pellets were

678 resuspend in 3 mL LB3 (10mM Tris, 100mM NaCl, 1mM EDTA, 0,5mM EGTA, 0,1%

679 deoxycholate, 0,5% N-lauroylsarcosine). Samples were split into 2 x 1.5 mL in 15 mL

680 polypropylene tubes suitable for the Bioruptor® Pico (Diagenode). BioRuptor Sonicator

681 settings: power = high, "on" interval = 30 seconds, "off" interval = 45 seconds, 6 cycles.

682 Sonicated samples were centrifuged for 10 minutes at 16000 x g at 4 °C to pellet cellular

683 debris. Chromatin concentration was measured by NanoDrop and 25 µg of chromatin were

684 used for each IP. 300 µl 10% Triton X-100 were added to each 3 mL sonicated lysate. 25

685 µg of chromatin were stored at 4°C which served later on as an input control. Antibody of

686 interest was added to sonicated chromatin aliquot and mixed (anti-STAT1 Santa Cruz sc-

687 346; anti-STAT2 Santa Cruz Catalog # 07-140, IRF9 6F1-H5). All samples were filled up to

688 1ml with dilution buffer (16.5 mM Tris pH 8, 165 mM NaCl, 1.2 mM EDTA, 1% Triton X-100,

689 0.1% SDS, 0.1 mM PMSF, and cOmplete EDTA-free protease inhibitor cocktail (Sigma

690 Aldrich)). Samples were rotated at 4 °C over night. 50 µl of magnetic beads (Dynabeads

691 protein G, Life technologies, 10003D) per sample were blocked overnight in dilution buffer

692 containing 1 % BSA at 4°C. The next day 50 µl of the beads were added to each sample

693 and incubated at 4°C while rotating. Afterwards the beads were washed with 1 ml RIPA

694 buffer (50 mM Tris HCL pH 8, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium

695 deoxycholate, 1 mM DTT), 2x High Salt buffer (50 Mm Tris pH 8, 500 mM NaCl, 0.1% SDS,

696 1% NP-40), 2x LiCl buffer (50 mM Tris pH 8, 250 mM LiCl, 0.5% sodium deoxycholate, 1%

697 NP-40) and TE buffer (10 mM Tris pH 8, 1 mM EDTA) for 10 minutes at 4 °C. The samples

698 were eluted in freshly prepared elution buffer (2% SDS, 100 mM NaHCO$_3$, 10 mM DTT). The

699 crosslink between proteins and DNA was reversed by adding 200 mM NaCl to each sample

700 and incubation at 65 °C at 300 rpm for 12 hours. Proteinase K, 40 mM Tris pH 8 and 10 mM

701 EDTA were added to each sample and incubated for 1 hour at 55 °C and 850 rpm. Each

702 sample was transferred to a phase lock tube (5Prime), mixed 1:1 with phenol-chloroform-

703 isoamylalcohol (PCI) and centrifuged for 5 minutes at 12000 × g. Supernatant was

704 transferred and mixed with 800 µl 96% Ethanol, 40 µl 3M CH$_3$COONa pH 5.3 and 1 µl

705 Glycogen and stored for at overnight at -20 °C. Samples were centrifuged for 45 minutes at

706 4 °C and 16000 × g. Pellets were washed in ice cold 70% ethanol and dried at 65 °C, before

707 diluting the DNA in H$_2$O.

708 For library generation, the NEBNext Ultra II DNA Library Prep Kit for Illumina from NEB

709 (Catalog #E7645S) was used   according to the manufacturer's protocol. The samples were

710 quality checked and sequenced at the Vienna Biocenter Core Facilities NGS Unit.

711

712 **Immunoprecipitation**

713 1.5 x 10^7 bone marrow derived macrophages were stimulated for 1.5h with IFN-β. Cells were

714 lysed in 1ml lysis buffer (10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 30 mM NaPPi, 50 mM NaF,

715 2 mM EDTA, 1% Triton X-100, 1mM DTT, 0.1 mM PMSF and 1× protease inhibitor). Cells

716 were incubated for 5 minutes on ice and then centrifuged for 5 minutes at 4°C, 12000 rpm.

717 The supernatant was transferred to a new tube. 20 µl (10% of the lysate used for the IP)

718 were used as an input control. 4 µg of an anti-STAT1 antibody (sc-346; Santa Cruz) or anti-

719 STAT2 (catalog no. 07-140; Upstate) antibody, as well as 80µl of the IRF9 antibody were

720 added to 200µl of lysate and incubated for 30 minutes at room temperature, while rotating.

721 50 µl of magnetic beads (Dynabeads protein G, Life technologies, 10003D) per sample were

722 washed with Frackelton buffer.   Then, 50 µl of the beads were added to each sample and

723 incubated for 1h at room temperature while rotating. Afterwards the beads were washed

724 three times with 1ml frackelton buffer and proteins were eluted in SDS sample buffer (250

725 mM Tris-HCl pH 6.8, 20% Glycerol, 1.6% SDS, 20% β-Mercaptoethanol, 0.002%

726 Bromophenol blue).

727

728 **Affinity pulldown of biotinylated ISRE probes**

729 1.5 x 10^7 Raw 264.7 macrophages were stimulated for 1.5h with IFN-β. Cells were

730 harvested, washed and lysed in 1ml lysis buffer (10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 30

731   mM NaPPi, 50 mM NaF, 2 mM EDTA, 1% Triton X-100, 1mM DTT, 0.1 mM PMSF, 1mM

732   Na3VO4 and 1× protease inhibitor). Cells were incubated for 5 minutes on ice and then

733   centrifuge for 5 minutes at 4 °C, 12000 rpm. 200 μl of each sample were incubated with 100

734   ng Oas1a (Fw Oas1a oligo 5' bioteg 5'TAGATTTTCAGTTTCCATTTCCCGAGAAGGGCA 3';

735   Rv Oas1a oligo 5'TGCCCTTCTCGGGAAATGGAAACTGAAAATCTA 3') and Isgf15 ISRE

736   probes (Fw ISG15 oligo 5' bioteg 5' TATTTTCTGTTTCGGTTTCCTTTTCCTAC 3'; Rv ISG15

737   oligo 5'GTAGGAAAAGGAAACCGAAACAGAAAATA3'). The reaction was carried out in the

738   presence of 0.1mM EGTA, 0,5mM DTT, 40mM KCL, 1mM MgCl2, 500ng competitor plasmid

739   and 2μl Poly dI:dC (#20148E, Thermo Scientific). Samples were incubated at room

740   temperature for 30 minutes while rotating. 50 μl of magnetic Pierce Streptavidin beads

741   #88817 were added to each sample and incubated for 10 minutes at room temperature while

742   rotating. Beads were washed three times with 1ml binding buffer and proteins were eluted in

743   SDS sample buffer (250 mM Tris-HCl pH 6.8, 20% Glycerol, 1.6% SDS, 20% β-

744   Mercaptoethanol, 0.002% Bromophenol blue).

745

746   **Statistical analysis**

747   mRNA expression data as well as ChIP data represent the mean values with standard error

748   of mean (SEM). Differences in mRNA expression data or % of input data were compared

749   using ratio t-test. All statistical analysis was performed using GraphPad Prism (Graphpad)

750   software. Asterisks denote statistical significance as follows: ns, $p>0.05$; *, $p \leq 0.05$; **, $p \leq$

751   0.01; ***, $p \leq 0.001$. In all experiments n=3, where n represents the number of biological

752   replicates.

753

754   **RNA-Seq Analysis**

755   Reads mapping to mouse rRNA transcripts were removed using bwa/0.7.12 alignment[12] and

756   samtools/1.3.1[13,14] Remaining reads were aligned to Mus musculus genome mm10 using

757   TopHat v2.1.1[15] (GTF annotation file mm10, RefSeq from UCSC, 2015/02). Reads in genes

758   were counted with htseq-count v0.6.1. [16] Differential expression analysis was carried out

759   using DESeq 2 v1.16.1[17], with an fdr threshold of 0.05.

760   Gene set enrichment analysis was performed using GSEA 3.0 against MsigDB v6.1

761   with gene abundance estimates in FPKM calculated using cufflinks v2.2.1. [18]

762   **ChIP-Seq Analysis**

763   Raw reads were processed using the AQUAS TF pipeline

764   (https://github.com/kundajelab/ChIP-Seq_pipeline;based off Encode phase-3;-idr_thresh

765   0.01), including alignment against the Mus musculus mm10 genome using BWA (v0.7.13[12]),

766   deduplication using Picard MarkDuplicates (v1.126), Peak Calling using macs2 (v2.1.1[19])

767   and spp (v1.13 [20]).

768

769   **Data availability**

770   Raw and analyzed data reported in this paper are available under accession number GEO:

771   GSE115435. Proteomics data will be uploaded to PRIDE and are available for reviewers from

772   the corresponding author upon request.

773

774   **Methods References**

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820
821

822 **Main Figure Legends**

823 **Figure 1. Conditions of basal ISG expression**

824 a, b) Bone marrow-derived macrophages (BMDM) isolated from wild-type (WT), Stat1$^{-/-}$,

825 Stat2$^{-/-}$, and Irf9$^{-/-}$ mice were treated with 250 IU/ml of IFN-β as indicated. Gapdh-

826 normalized gene expression was measured by RT-qPCR. Data represent the mean and

827 standard error of the mean (SEM) values of three independent experiments. *P*-values were

828 calculated using the paired ratio *t*-test (*$P \le 0.05$; **$P \le 0.01$, ***$P \le 0.001$).

829 c) Gene set enrichment analyses showing upregulation of an IFN and inflammatory

830 response signature of untreated WT compared to untreated Irf9-/- BMDM. The top correlated

831 genes for each biological triplicate are displayed in the corresponding heat maps. The total

832 height of the curve indicates the extent of enrichment (ES), with the normalized enrichment

833 score (NES), the false discovery rate (FDR) and the *P* value.

834

835 **Figure 2. IFN-induced changes in gene expression and chromatin-associated STAT**

836 **complexes.**

837 a-c) Scatterplot linking RNA-seq (n=3) with ChIP-seq (n=2) data. Significantly differentially

838 expressed genes between IRF9-/- and WT, WT untreated and WT IFN-β as well as WT

839 untreated and WT IFNγ are displayed in color for abs (logFC)>1.

840 ChIP-seq analysis was performed on untreated (a), IFN-β (b) or IFNγ (c) treated BMDMs of

841 wild-type (WT) and Irf9-/- (IRF9-/-) mice. Two independent biological replicates were used

842 for the analysis. Chromatin immunoprecipitation (ChIP) was performed using STAT1, STAT2

843 and IRF9 antibodies. Symbols indicate bound complexes as determined by peak

844 coincidence. d-f) Genomic analysis of STAT1 (S1) (scale 0-150), STAT2 (S2) (scale 0-200)

845 and IRF9 (scale 0-200) binding. Transcription factor binding at ISG and control loci (scale 0-

846 200). See also Supplementary Figure 1 and 2.

847

848

849 **Figure 3. Signal- and DNA dependence of complex formation from ISGF3 subunits.**

850 a) BMDMs from wild-type (WT) animals were treated for 1.5h with IFN-β and

851 immunoprecipitation was carried out using antibodies against STAT1, STAT2, IRF9 or an

852 IgG control. Immunoprecipitated complexes were analyzed by western blotting with

853 antibodies to STAT1, STAT2 and IRF9. Input controls represent 10% of the total lysate used

854 for the immunoprecipitation. b) Raw 264.7 cells were treated for 1.5h with IFN-β. Lysates

855 were either incubated with a biotinylated oligo harboring the Oas1a-ISRE or the Isg15-ISRE

856 sequence, or plasmid DNA. DNA bound protein complexes were isolated by streptavidin

857 affinity purification, followed by western blot analysis.

858

859 **Figure 4. Affinity labeling of IRF9 and STAT2 interactors.**

860 a), e) Analysis of IRF9 (a) and STAT2 (e) BioID PRM runs using Skyline software.

861 RAW264.7 cells were treated with 0,2µg/ml doxycycline for 24h, followed by addition of

862 50µM biotin for 18 hours. Cells were lysed and protein complexes isolated by streptavidin

863 affinity purification, followed by analysis with LC-MS. Log2-transformed protein ratios were

864 calculated for two biological replicates from mycIRF9-BirA/mycBirA cells (a) or mycSTAT2-

865 BirA/mycBirA cells (e). b), f) Network diagram of IRF9 (b) or STAT2 interactors (f), generated

866 with Cytoscape software. Proteins that displayed a threefold enrichment in the experimental

867 samples compared to the ligase only controls were counted as hits and are displayed in the

868 network. Spatial arrangement of nodes and group color coding reflects proteins either pre-

869 associated in the absence of any signal or specifically enriched upon 1.5h of IFN-β or IFN-γ

870 treatment. Distance of connecting lines (edges) between proteins do not reflect the strength

871 of the interaction. c) ClueGO network analysis of the IRF9 interactome. The app was used to

872 find overrepresented GO processes and a network of connected GO terms was created.

873 Each node represents a GO biological process, and the colors represent the GO group. d)

874 Pie chart of ClueGO results. See also Supplementary Figure 1, 3 and 4.

875

876 **Figure 5. Localization of STAT complexes.**

877   a) IRF9 localization as determined by immunofluorescence. BMDMs of wild-type (WT) and

878   Irf9-/- (IRF9-/-) mice were left untreated or stimulated with 250 IU/ml of IFNβ for 30 min. The

879   cells were fixed and stained with anti-IRF9 antibody followed by Alexa Fluor® 488

880   conjugated secondary antibody (green). Nuclei were stained with DAPI (magenta). First Ab

881   (-) indicates control without the first antibody. The scale bars represent 10 µm. b,d, e) Raw

882   264.7 nuclear and cytoplasmic extracts were prepared from controls or after a 30-minute

883   treatment with IFN-β or IFN-γ and analyzed by western blot. The entire nuclear fraction and

884   30% of the cytoplasmic fraction are shown. b) STAT1, STAT2, IRF9 and α-Tubulin, Lamin

885   A/C controls are shown. 500nM staurosporine was added 3h prior to IFN treatment. c)

886   Phosphorylation of STAT1 at Y701, STAT2 at Y689, total STAT1, total STAT2, IRF9 and α-

887   Tubulin of total bone marrow derived macrophage lysates were determined. 15µM P6

888   inhibitor or DMSO were added for 3h prior to IFN treatment d) Phosphorylation of STAT1 at

889   Y701, total STAT1, α-Tubulin and Lamin A/C levels were determined. 15µM P6 inhibitor or

890   DMSO were added for 3h prior to IFN treatment. e) Phosphorylation of STAT2 at Y689, total

891   STAT2, IRF9, α-Tubulin and Lamin A/C. 15µM P6 inhibitor or DMSO were added for 3h prior

892   to IFN treatment. f) Nuclear and cytoplasmic extracts from control wt-BMDM or after 30-

893   minute treatment with IFNβ were analyzed for the phosphorylation of STAT2 at Y689 as well

894   as total STAT2, IRF9, α-Tubulin and Lamin B1. 15µM P6 inhibitor or DMSO were added for

895   3h prior to IFN treatment.

896

897   **Figure 6. Cross regulation of ISGF3 subunits.**

898   a) BMDMs isolated from WT, Stat1$^{-/-}$, Stat2$^{-/-}$, and Irf9$^{-/-}$ mice were left untreated or treated

899   with 15µM P6 inhibitor for 3h. Gapdh-normalized gene expression was measured by RT-

900   qPCR. Data represent relative expression in percentage, where WT untreated equals 100%.

901   Data represent the mean and standard error of the mean (SEM) values of three independent

902   experiments. *P*-values were calculated using the paired ratio *t*-test (*$P \leq 0.05$; **$P \leq 0.01$,

903   ***$P \leq 0.001$).

33

904   b) Genomic analysis of STAT1 (S1), STAT2 (S2) and IRF9 binding. Transcription factor

905   binding at Stat1, Stat2 and Irf9 transcriptional start site. Input, STAT2 and IRF9 (scale 0-

906   200). STAT1 (scale 0-150)

907   c) Whole-cell extracts from wild-type, Stat1$^{-/-}$, Stat2$^{-/-}$ and Irf9$^{-/-}$ BMDMs were tested by

908   western blot for total STAT1, STAT2 and IRF9 levels (representative of three independent

909   experiments).

910

911   **Figure 7.** Model depicting the molecular switch from resting to IFN-induced gene

912   expression. Whereas a preformed, signalling-independent STAT2-IRF9 complex drives the

913   basal expression of interferon-induced genes, both IFN-I and IFN-γ cause increased

914   transcription by replacing this complex with ISGF3. For detailed information see text.

915

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918

Figure 1

a
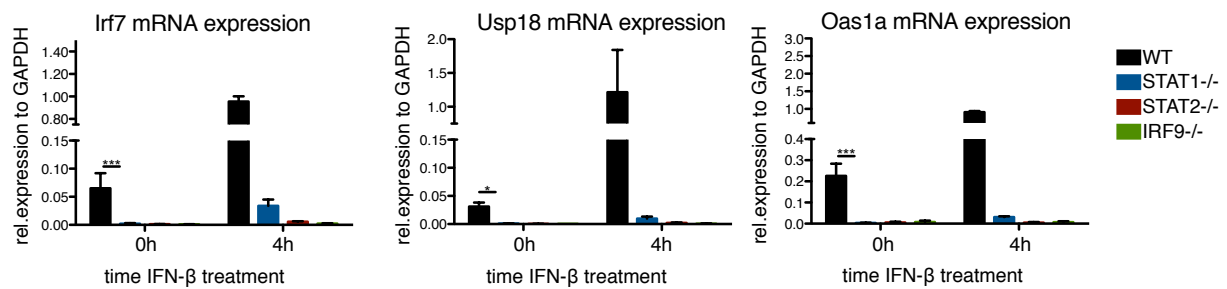
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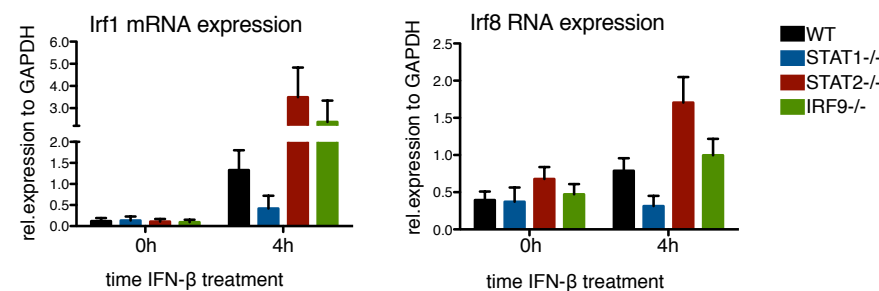
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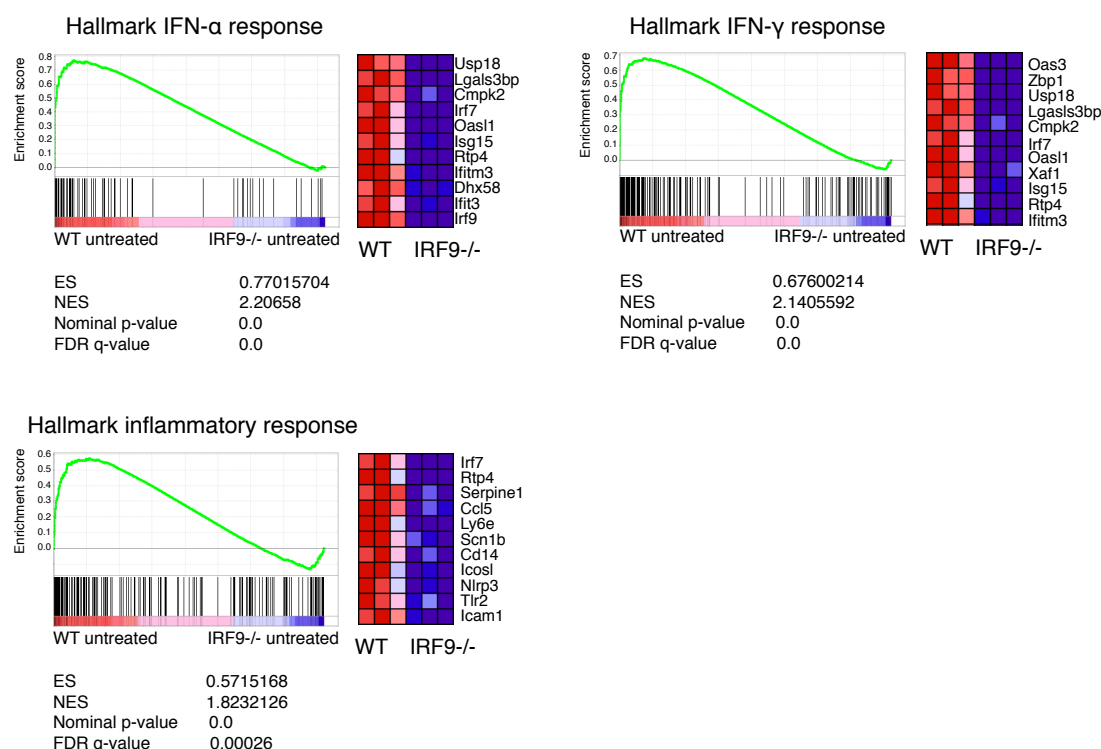
Figure 2

Figure 3

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Figure 5

Figure 6

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Figure 7