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"Employing the degron system to understand transcription factor complexes involved in the response to interferons"

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Christopher Pree, BSc

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2 Zusammenfassung

Die durch Interferone (IFN) ausgelöste Signalübertragung über den Janus-Kinase (JAK)-Signalgeber und Aktivator der Transkription (STAT) spielt eine essenzielle Rolle in der Abwehr von Krankheitserregern. Das Binden von IFN an ihre Rezeptoren löst eine rasche Signaltransduktion von der Zelloberfläche bis zum Zellkern aus, wodurch latente STATs und IRFs aktiviert werden und Transkriptionskomplexe bilden, die die Genexpression vorantreiben. Der IFN-stimulierte Genfaktor 3 (ISGF3), zusammengesetzt aus STAT1, STAT2 und IRF9, dominiert die Reaktion auf Typ I IFN, während der Gamma-aktivierte Faktor (GAF), ein STAT1-Homodimer, die Reaktion auf Typ II IFN steuert. Neue Studien, die die Beteiligung nicht-kanonischer Komplexe wie STAT2-IRF9 oder STAT1-IRF9 an der Regulierung der Expression interferonstimulierter Gene (ISG) belegen, stellten dieses Konzept jedoch in Frage. Knockout-Experimente zeigten, dass der Verlust einer der Komponenten teilweise durch nicht-kanonische Komplexe kompensiert werden kann, wodurch die Expression einer Untergruppe von ISGs wiederhergestellt wird. Da diese Kompensation in den Knockouts eine Adaption an den permanenten Verlust eines Gens darstellen könnte, stellten wir die Frage, wie sich ein plötzlicher Verlust von STAT1, STAT2 oder IRF9 auf die ISG-Expression auswirken würde. Um diese Frage zu beantworten, setzten wir die AID-Technologie (Auxin-induziertes Degron) ein, um STAT1, STAT2 oder IRF9 akut abzubauen und so zu untersuchen, wie sich dies auf die Genexpression während verschiedener Phasen der IFN-Antwort auswirkt. Hier zeigen wir, wie das AID-System an die RAW264.7-Makrophagen-Zelllinie der Maus angepasst und zur Untersuchung der Rolle von Transkriptionsfaktoren verwendet werden kann. Das so entstandene AID-System bietet eine hervorragende Plattform für zukünftige Experimente, um die komplexe Regulierung von ISGs zu entschlüsseln.

3 Abstract

Interferon (IFN) mediated signaling by the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway plays an essential role in the defense against pathogens. The binding of IFNs to their receptors triggers a fast cell surface to nucleus signal transduction by activating latent STATs and IRFs to form transcriptional complexes that drive gene expression. The IFN stimulated gene factor 3 (ISGF3), composed of STAT1, STAT2 and IRF9, dominates the response to type I IFNs, while the gamma-activated factor (GAF), a STAT1 homodimer, regulates the type II IFN response. This concept has been challenged by recent studies demonstrating the involvement of non-canonical complexes, such as STAT2-IRF9 or STAT1-IRF9, in the regulation of interferon stimulated gene (ISG) expression. Knockout experiments showed that the loss of one of the components can be partially compensated by non-canonical complexes, rescuing the expression of a subset of ISGs. Since this compensation may result from adaptation to the permanent loss of a gene, we asked the question how a sudden loss of STAT1, STAT2 or IRF9 would affect ISG expression. To answer this, we employed the auxin-inducible degron (AID) technology to acutely degrade STAT1, STAT2 or IRF9 and thus study how this affects gene expression during different stages of the IFN response. Here we show how the AID system can be adapted in the murine RAW264.7 macrophage cell line and used to study the role of transcription factors. The created AID system provides an excellent platform for future experiments to unravel the complex regulation of ISGs.

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4 Introduction

4.1 The immune system

Years of evolution led to the formation of a complex network comprised of many different cell types, pathways and molecules that protect against a multitude of challenges caused by microbial or viral pathogens. If, when and how this immune system is activated must be carefully regulated and be specific to the type of attack. Failure or loss of components can have devastating effects, ranging from inability to defend against pathogens to hypersensitivity and attack of the host itself. The mammalian immune system has two major components: (i) innate immunity and (ii) adaptive immunity (Chaplin, 2010; Marshall et al., 2018; Paludan et al., 2021).

The innate immune system presents the first line of defense against pathogens. It relies on the recognition of distinct molecular patterns (pathogen-associated molecular patterns, PAMPs) by specialized receptors (pattern recognition receptors, PRRs) to mount a corresponding immune response. The receptors can be grouped into three major categories: (i) Toll-like receptors (TLRs), (ii) nucleotide oligomerization domain (NOD)-like receptors (NLRs), (iii) retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs). TLRs are found on cell surfaces and endosomes, whereas RLRs and NLRs reside inside the cytoplasm. PAMPs are very specific and conserved molecular structures that are essential for pathogen viability and survival. They are not present in host cells and two prominent examples are lipids, especially the lipopolysaccharides (LPS) from bacteria and foreign nucleic acids (RNA, DNA) from viruses. Besides PAMPs, the innate immune system can also sense “danger” through damage-associated molecular patterns (DAMPs), molecules that are only found during cell lysis and inflammation (Fensterl & Sen, 2009; D. Li & Wu, 2021; Mogensen, 2009; Paludan et al., 2021; Turvey & Broide, 2010).

Downstream of PRRs exist several signaling cascades with effects ranging from cytokine production and inflammation to the activation of the adaptive immune system and the expression of antipathogenic genes. The three major pathways are (i) the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), (ii) the mitogen-activated protein kinases (MAPKs), and (iii) interferon regulatory factor (IRF) pathway. NF-κB and MAPKs play crucial roles in regulating the inflammatory

responses, whereas IRF is involved in the production of IFNs (Coccia & Battistini, 2015; Mogensen, 2009).

The adaptive immune system is characterized by a slower, but more comprehensive defense against the invading pathogen. The response is primarily mediated by B- and T-lymphocytes that differentiate upon the recognition of pathogen specific antigens. This leads to the recruitment and activation of other immune cells and the production of pathogen-specific antibodies. A hallmark of the adaptive immunity is the capability of memorizing past encounters in specialized cells, which results in a faster adaptive response upon repeated exposure (Chaplin, 2010; Marshall et al., 2018).

The innate and adaptive immune systems play hand in hand in the fight against pathogens. In the beginning, the innate system is more prominent, as the adaptive response takes several days to come into full effect, due to lymphocyte differentiation and specialization. Furthermore, innate factors play an important role in the activation of the adaptive response, and once active, adaptive components amplify their response by recruiting innate factors as well. This interplay is the cornerstone of the immune system in mammals and the reason why it is so effective against a broad variety of pathogens (Chaplin, 2010; Marshall et al., 2018; Paludan et al., 2021).

4.2 The Interferon system

IFNs are small proteins which are expressed and secreted by cells upon pathogen invasion to mobilize the immune system. More than 60 years ago IFNs were discovered as a substance that interferes with viral replication in chick embryo cells (Isaacs & Lindenmann, 1957). Subsequent years of studying uncovered their importance in the immune system as signaling cytokines and their involvement, not only in the anti-viral defense, but also in the defense against microbes and other pathogens, their anti-tumor activity, and their role in the inflammatory response. In mammals, IFNs are categorized into three distinct families according to their specific receptor binding and molecular structure and induce the expression of antipathogenic genes through the JAK-STAT pathway (Decker et al., 2005; Fensterl & Sen, 2009; Majoros et al., 2017; Negishi et al., 2018; Parker et al., 2016; Schindler et al., 2007).

4.2.1 The JAK-STAT pathway

IFN mediated signaling by the JAK-STAT pathway plays an essential role in the immune system's defense against pathogens and is characterized by a fast cell surface to nucleus signal transduction to drive gene expression. The signaling cascade starts at the cellular membrane, where specialized receptors recognize and bind the IFNs. Upon binding, the receptor complexes form and trigger the activation of intracellular associated JAKs to phosphorylate themselves and the intracellular domain of the receptors. Once active, the receptor complexes activate latent STATs, which form transcriptional complexes and translocate to the nucleus where they drive the expression of the so-called interferon regulated genes (Schindler et al., 2007; Stark et al., 1998; Stark & Darnell, 2012).

4.2.2 Different types of Interferons

Type-I IFNs (IFN-I) represent the largest family of IFNs and are typically expressed by infected cells. They are associated with antiviral, antiproliferative and antitumor activity, but are also important for the onset of the adaptive immune system and the microbial defense. Depending on the mammalian species, IFN-I encompass about 20 members, including IFN-α, IFN-β, IFN-ω, IFN-ε, IFN-κ, IFN- ζ (mice), IFN-τ (ruminants), and IFN-δ (pigs). The most important and well-studied members are IFN-α and IFN-β, which are the main type of IFN-I expressed during bacterial and viral challenges (Decker et al., 2005; Fensterl & Sen, 2009; Negishi et al., 2018). IFN-I gene expression is induced by the recognition of PAMPS by PRRs that ultimately lead to the activation of key transcription factors, such as IRF family members. The initial IFN-I production relies on the phosphorylation of IRF3, which associates with NF-κB and activator protein 1 (AP1) family members, to form the IFN-β enhanceosome, for stimulating the IFN-β production. This in turn leads to the induction and phosphorylation of IRF7, which together with IRF3, forms a positive feedback loop that drives the second wave of IFN-I expression through IFN-α (McNab et al., 2015). During this study, IFN-β was used as a representative for IFN-I signaling.

Type-II IFNs (IFN-II) have only one member, IFN-γ, and target activated immune cells like macrophages, natural killer (NK), and T cells. While IFN-Is are more correlated with the response against viruses, IFN-IIs strengthen cell-mediated immunity against different types of intracellular pathogens. They are also linked to

inflammatory responses and antigen processing and presentation. IFN-II production is not induced by PAMPs or PRR signaling, but through cytokines released by activated macrophages. The factors and pathways that drive this expression in the different cells are not entirely understood, but production is mostly stimulated by interleukin (IL)-12, IL-15, IL-18, and IFN-I (Castro et al., 2018; Schoenborn & Wilson, 2007; Schroder et al., 2004).

Type-III IFNs (IFN-III), are the most recently identified family of IFNs and were discovered in 2003 by two independent teams (Kotenko et al., 2003; Sheppard et al., 2003). They family consists of IFN-λ1 (IL-29), IFN-λ2 (IL-28A), IFN-λ3 (IL-28B) and IFN-λ4. Their role and function is very similar to IFN-I, with the key difference, that IFN-III target only epithelial cells in mucosal and other barrier surfaces, such as the blood brain barrier (Wack et al., 2015). Like IFN-I, IFN-III are induced by PRR-mediated activation of transcription factors like IRF3, IRF7, NF-κB and AP1 upon recognition of PAMPs. Recent studies also showed the importance of IFN-III signaling in the placenta, were they might play an essential role in the antiviral defense of the fetus by the mother during pregnancy (Wells & Coyne, 2018).

4.2.3 The JAKs

The JAK family are non-receptor tyrosine protein kinases and consist of the four members in vertebrates: JAK1, JAK2, JAK3, and Tyrosine kinase 2 (TYK2). They are composed of about 1000 amino acids and are characterized by their four structural domains (Figure 1). (i) The C-terminal kinase domain, with catalytic activity to phosphorylates substrates. (ii) The pseudo kinase domain, with no catalytic activity, but regulating the catalytic activity. (iii) The Src homology 2 (SH2) domain, and (iv) the 4.1, ezrin, radixin, moesin (FERM) domain are both critical for receptor association (Banerjee et al., 2017; Hu et al., 2021; Morris et al., 2018; Schindler et al., 2007). The JAKs associate non-covalently with the cytosolic domains of the IFN receptor complexes and play a crucial part in the signaling cascade that leads to interferon-stimulated gene (ISG) expression (Darnell et al., 1994; Schindler et al., 2007).

4.2.4 The STATs

There are seven mammalian members of the STAT family: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6. They range in size from 750 – 900 amino acid residues and share several conserved regions (Figure 1). (i) The amino-terminal domain is important for STAT dimer formation and nuclear translocation. (ii) The coiled coil domain facilitates inactive STAT dimerization but is protruded outwards upon activation to serve as a docking platform for other proteins. (iii) The DNA-binding domain (DBD) is essential for role of STATs as transcription factors, as it allows for the binding to the gamma-interferon-activated sequence (GAS) on the DNA. (iv) The short linker domain that connects the DBD to the (v) SH2 domain, which recognizes phosphorylated residues and plays and an important role in receptor association and STAT dimerization. (vi) The C-terminal transcriptional activation domain (TAD), where a single conserved tyrosine residue (pY) is located, that is phosphorylated by JAKs and essential for STAT activation by dimerization. During homeostasis, STATs reside in the cytoplasm as inactive monomers or preformed dimers that are structurally different compared to their active forms. Upon their activation through phosphorylation, their conformation is changed and either STAT homodimers or complexes in conjunction with non-STAT proteins are formed. They translocate to the nucleus to act as transcription factors to drive ISG expression. (Banerjee et al., 2017; Hu et al., 2021; Morris et al., 2018; Platanitis & Decker, 2018; Schindler et al., 2007). The two relevant STATs for this study are STAT1 and STAT2 which play crucial roles in the expression of ISGs.

4.2.5 The IRFs

The family of IRF proteins are composed of nine members in mammals, IRF1-9 and are involved in the regulation of gene expression during both innate and adaptive immunity upon reaction to a plethora of stimuli (Honda et al., 2006). They are defined by two conserved domains (Figure 1). (i) The N-terminal DBD, for binding to the interferon-stimulated response element (ISRE) on the DNA and (ii) the C-terminal IRF-association domain (IAD), which is important for protein-protein interactions and complex formation (Negishi et al., 2018; Platanitis & Decker, 2018). In contrast to STATs only IRF3, 5, and 7 require phosphorylation of serine residues of the IAD, whereas IRF2, 4 and 8 can function without any modification (Sharf et al., 1997). IRF3

and IRF7 were mentioned earlier for their importance in IFN-I and IFN-III production. IRF9, differs from the other IRFs in two ways, it is not transcriptionally active on its own and the IAD contains a special binding site for STAT2 (Rengachari et al., 2018). In this study, the focus lies on IRF9 as it is a key a factor for the assembly of the ISGF3 complex and the regulation of ISG expression.

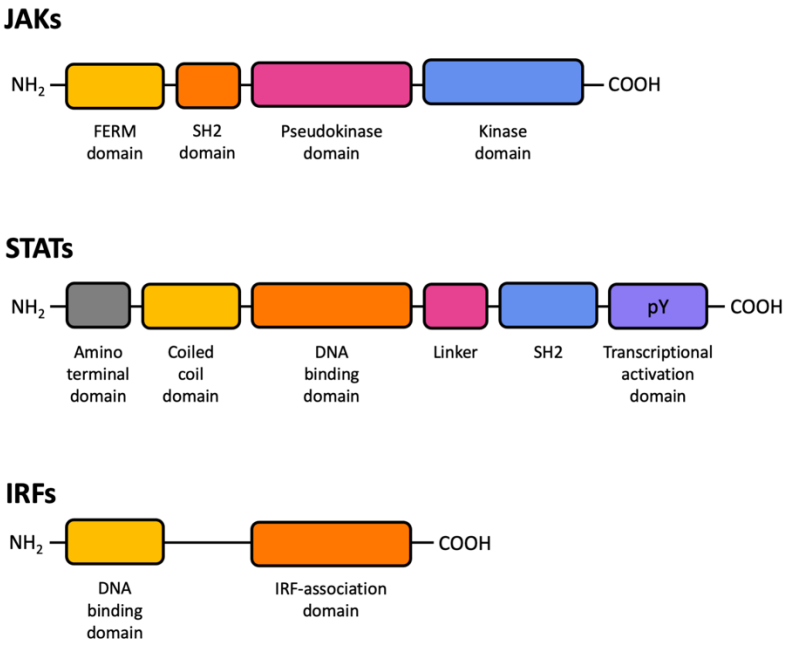


Figure 1. Structure of JAK-STAT pathway components.
Schematic representation of JAK, STAT and IRF molecules and their domains.

4.2.6 The Interferon signaling cascade

IFN signaling is mediated by the JAK-STAT pathway (Figure 2) and relies on the signal transduction from receptor complexes on the cellular membrane to intracellular associated JAKs that ultimately activate transcription factors that drive the expression of specific genes (Schindler et al., 2007).

IFN-I signaling is mediated by the heterodimeric transmembrane IFN-α receptor (IFNAR), formed by the IFNAR1 and IFNAR2 subunits. (Coccia & Battistini, 2015; Decker et al., 2005). The binding of IFN-I to IFNAR causes the associated JAKs (JAK1 and TYK2) in the cytoplasm, to auto-phosphorylate and to phosphorylate the intracellular domain of the receptors. This in turn causes the phosphorylation and activation of STAT1 and STAT2. Together with IRF9, STAT1 and STAT2 form the ISGF3 transcriptional complex in the nucleus, and bind the ISRE elements on the DNA

to drive ISG expression (Majoros et al., 2017; Negishi et al., 2018; Platanitis et al., 2019; Walter, 2020).

IFN-II signals through the heterodimeric IFN-γ receptor (IFNGR), composed of two IFNGR1 and two IFNGR2 subunits (Trinchieri, 2010; Young & Bream, 2007). The associated JAKs are JAK1 and JAK2 and their activation ultimately leads to the phosphorylation of STAT1, which dimerizes and forms the gamma interferon-activated factor (GAF). This factor then translocates to the nucleus and binds to gamma interferon-activated sites (GAS) for ISG expression (Majoros et al., 2017; Negishi et al., 2018; Walter, 2020).

The IFN-λ receptor (IFNLR) is formed by the IFNλR1 and IL-10R2 subunits and recognizes IFN-III. Even though the receptors are different, the associated JAKs are the same as in IFN-I and therefore resembles the signal transduction which ultimately leads to ISGF3 complex formation (Wack et al., 2015).

4.2.7 Non-canonical signaling complexes

IFN signaling through the JAK-STAT pathways is not as simple and straightforward as the canonical pathway may suggest. It was shown that the ISGF3 complex is involved in the response to all three types of IFN and that the loss or inhibition of either STAT1 or STAT2 can be compensated by the formation of non-canonical complexes (Figure 2). Furthermore, the characteristic tyrosine phosphorylation of the STATs is not as crucial as believed, as unphosphorylated complexes are also able to drive ISG expression (Majoros et al., 2017).

The response to IFN-γ is typically driven by GAF formation and subsequent binding of the STAT1 homodimers to GAS sites on the DNA. It was shown that expression of some ISRE-driven genes (*Ifit2* and *Cxcl19*) was facilitated by a non-canonical STAT1-IRF9 complex during early response to IFN-γ. Although, STAT2 was not needed in this context, it is believed that STAT2 is important for the late or delayed response to IFN-γ that was observed in some genes. In these cases, either the non-canonical ISGF3^{II} complex, comprised of phosphorylated STAT1, unphosphorylated STAT2 and IRF9, or the canonical ISGF3 complex is formed and binds to the ISRE element for gene expression. In conclusion, STAT2 can influence IFN-γ stimulated expression of a subset of genes in a positive or negative way. It can enhance gene expression by forming the ISGF3^{II} and ISGF3 complexes and repress

it by binding to phosphorylated STAT1 and thereby prevent GAF formation (Majoros et al., 2017).

STAT1 has a crucial role in the response to IFNs, as it is an essential component of the GAF and ISGF3 complexes. Surprisingly, IFN-I stimulated resistance to several viral and bacterial pathogens could still be retained to some extent in the absence of STAT1. Later studies identified STAT2 as a critical component, which lead to the formation of the hypothesis that an non-canonical complex between STAT2 and IRF9 forms, that can rescue IFN-I stimulated ISG expression in some cases (Majoros et al., 2017). Evidence supporting this hypothesis also came from an experiment studying the expression of *Mx1*, a classical ISRE-driven gene, in the absence of STAT1, STAT2 or IRF9. Only in *Stat1*$^{-/-}$ macrophages and during prolonged IFN-I stimulation gene expression could be rescued (Abdul-Sater et al., 2015). This suggests that there is a fallback system for the defense against pathogens in place that can compensate the loss of STAT1.

The canonical JAK-STAT pathway depends on the tyrosine-phosphorylation of the STATs as a prerequisite for the translocation to the nucleus where they act as transcription factors. In contrast to this, the concept of the U-STATs ascribes activity to STATs lacking this phosphorylation. U-STATs are known to be involved in organelle metabolism and function. It has been reported that they are also involved in the regulation of specific genes and are able to shuttle between the nucleus and the cytoplasm, even though they are unphosphorylated. This mechanism is not entirely understood yet as U-STATs are usually exported out of the nucleus under steady-state conditions. According to the current hypothesis, early response to IFN-I causes the canonical ISGF3 complex formation. Prolonged IFN-I exposure causes the accumulation of STAT1, STAT2 and IRF9 and enables the formation of the unphosphorylated ISGF3 (U-ISGF3) complex, that can sustain the expression of a specific subset of ISGs (Majoros et al., 2017; Platanitis et al., 2019).

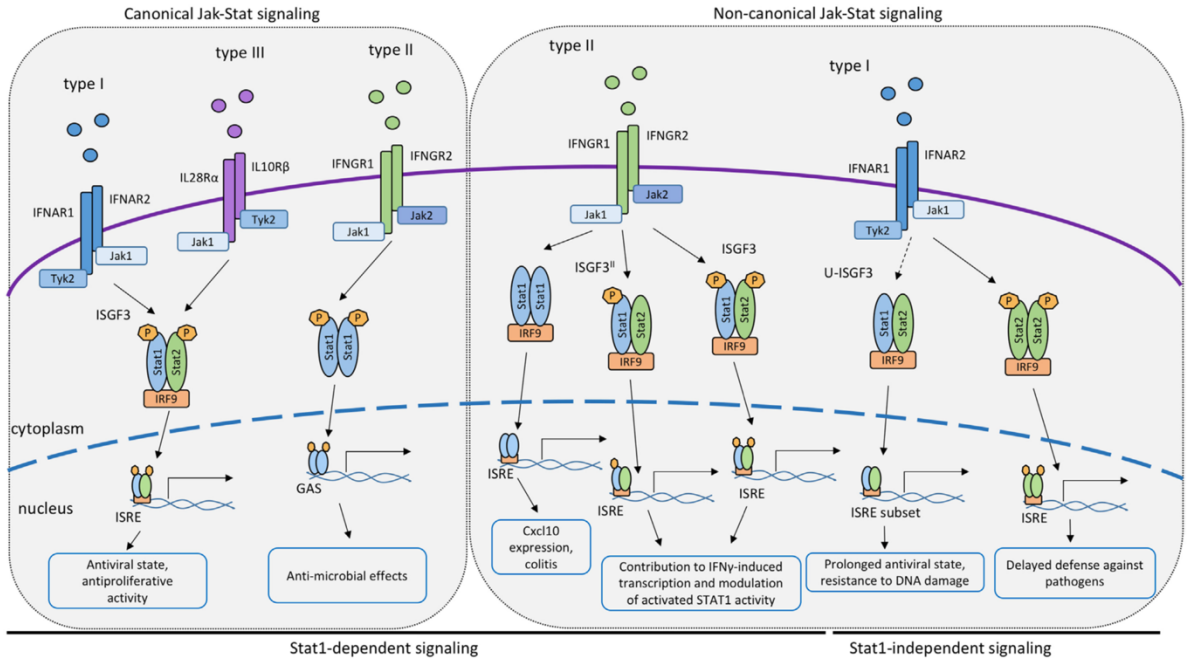


Figure 2. Canonical and non-canonical JAK-STAT signaling.
Schematic representation of the canonical and non-canonical complexes formed upon IFN stimulation. IFN-I (type I, in blue) is bound by IFNAR1 and IFNAR2, while IFN-III (type III, in purple) binds to the IL28Rα and IL10Rβ, resulting in the activation of the ISGF3 complex and its subsequent translocation to the nucleus and binding to the ISRE to drive gene expression. IFN-II (type II, in green) signals through IFNGR1 and IFNGR2, which promotes STAT1 homodimer formation (GAF) and translocation into the nucleus to bind to GAS elements on the DNA for gene expression. Besides the canonical pathway and complexes, it has been shown that a variety of non-canonical complexes exist, also capable of inducing ISG expression, for example STAT1-IRF9, STAT2-IRF9, $ISGF3^{II}$ and U-ISGF3. Figure adapted from Majoros et al. (2017).

4.2.8 Interferon signaling during homeostasis

The immune system must be ready at any given time to arm a rapid response against an invading pathogen, which requires a fast switch form a homeostatic to an activated state that drives the expression of anti-pathogenic genes. This is achieved by priming the ISG expression prior to the IFN-mediated response to pathogens. So far, two models have emerged to explain this phenomenon: (i) the “autocrine loop” model of basal IFN-I production and signaling (Gough et al., 2012; Mostafavi et al., 2016), and (ii) the model independent of IFN signaling, through preformed STAT2-IRF9 complexes (Platanitis et al., 2019).

According to the “autocrine loop” model, IFN-I are constantly expressed at low levels through ongoing environmental exposure and signal through the IFNAR receptors to drive basal expression of ISGs. This includes also JAK-STAT components and disruptions in this system can shift the predominant expression of STAT1 and STAT2 to other STAT members, therefore impeding the ISGF3 complex formation.

This basal ISG expression is enhanced through the canonical PRR-mediated signaling and increased IFN production (Gough et al., 2012; Mostafavi et al., 2016).

It was shown by Platanitis et al. (2019) that STAT2-IRF9 complexes drive ISG expression during homeostasis, without requiring IFNAR-mediated activation. Upon IFN-mediated signaling, the STAT2-IRF9 complex is rapidly replaced by the canonical ISGF3 complex directly on the DNA, due to its higher affinity.

Although, a complete picture is still missing, these findings display the complexity that controls ISG expression during homeostasis and offer explanations on how cells facilitate the rapid switch to a full response.

4.3 The auxin-inducible degron system

One way to study the function and importance of a gene is to remove it from the genome or in other words, knock the gene out, and study how this loss affects the system. A gene knock-out is created by modifying the protein coding region on the DNA in a way that it disrupts the expression of the target gene. This can be achieved with many different molecular techniques, with the most prominent being the CRISPR/Cas9 system, which was also used in this study (Jinek et al., 2012; Khalil, 2020; H. Li et al., 2020). Besides the many advantages this system offers, one of the drawbacks is the fact, that the knocked-out gene is irreversible missing from the beginning of the development and the possibility that other genes may compensate for this loss. This is especially true for the components of the JAK-STAT pathway as there are several ways how the loss of one component can be compensated by the others and thereby rescue gene expression (Abdul-Sater et al., 2015; Majoros et al., 2017). Searching for possible solutions to overcome this hurdle, we employed a system developed by Nishimura et al. (2009) that, instead of targeting the gene directly, focusses on the product, the protein, and uses the cellular machinery to target it for degradation instead. This has the big advantage, that the protein can execute its usual functions, but can be removed very quickly to study how its absence affects the system.

4.3.1 Protein degradation pathways

There are thousands of proteins expressed constantly, with most of them requiring further processing, while others are needed only in certain quantities and at specific times. The mastermind controlling this system is the proteostasis network (PN), which maintains protein homeostasis by watching over the synthesis of new proteins, the processing or folding for their correct function, and the degradation of not needed or misfolded proteins. Two pathways control protein degradation in eukaryotic cells: (i) the ubiquitin-proteasome system (UPS) that targets individual proteins that are damaged, misfolded, or not needed for degradation, and the (ii) autophagy-lysosome system, responsible for clearing larger protein complexes, cellular compartments, or membrane associated proteins (Klaips et al., 2018).

At its core, the UPS relies on the attachment of ubiquitin to proteins, which in turn are then recognized by the proteasome and degraded (Figure 3). Ubiquitin is a highly conserved protein and can be attached to one or more lysine residues on a target protein. Since ubiquitin possesses lysine residues as well, it can be attached to itself to form poly-ubiquitin chains, which are a strong signal for degradation by the proteasome. This post-translational modification (PTM) is facilitated by three enzymes: ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2), and ubiquitin ligase (E3). In general, E1 binds to ubiquitin and transfers it to E2 and E3 catalyzes the transfers of ubiquitin from E2 to the substrate (Hanna et al., 2019; Zhao et al., 2022). There exist more than 600 E3 enzymes, each specific for another substrate, represented by four main groups based on their binding motif of the E2 enzyme: (i) homologous to the E6-AP carboxyl terminus (HECT)-type, (ii) really interesting new gene (RING)-finger type, (iii) U-box type, and (iv) plant homeo domain (PHD)-finger type (Thompson et al., 2021).

Important for this study is the biggest subfamily of the RING-finger ligases, the Cullin-based subfamily. The E3 ligase complex that is formed by this family is the SKP1, CUL1, F-box protein (SCF) complex. It consists of RING-box 1 (RBX1), which is important for E2 recruitment, cullin 1 (CUL1), the backbone of the complex, followed by S-phase kinase-associated protein 1 (SKP1), that binds the variable F-box protein, which is in turn bound to a specific substrate (Thompson et al., 2021). Nishimura et al. (2009) used this machinery to target specific proteins for degradation.

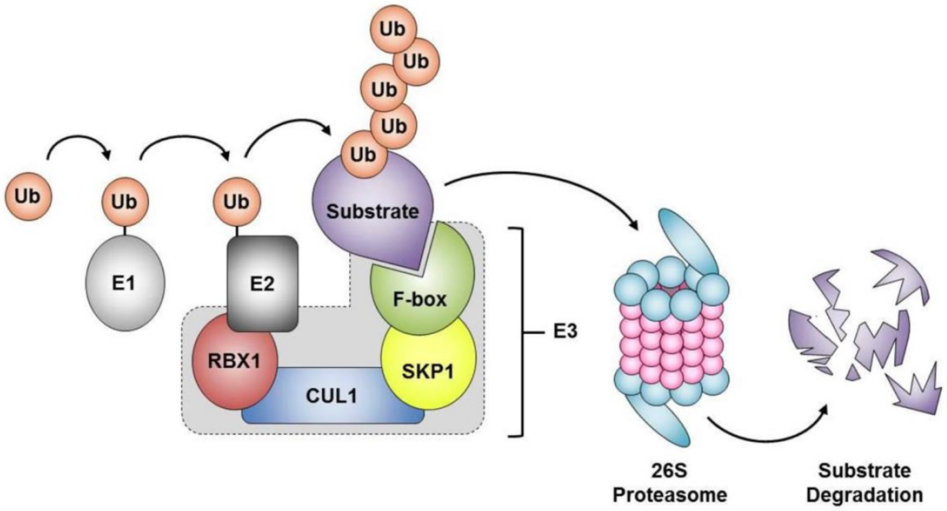


Figure 3. Ubiquitin mediated protein degradation by the proteasome.
Protein degradation through the ubiquitin-proteasome system relies on the attachment of ubiquitin (Ub) to a target substrate, thereby marking it for degradation by the proteasome. The reaction is catalyzed by three enzymes E1, E2 and E3, where ubiquitin is activated, transferred, and finally attached covalently to the target. There exist many E3 ligases, each specific for a distinct substrate, one of them being the SCF complex, comprised of the RING-box 1 (RBX1), cullin 1 (CUL1), S-phase kinase-associated protein 1 (SKP1) and a variable F-box protein. Figure adapted from Thompson et al. (2021).

4.3.2 Principles of the degron system

The auxin-inducible degron (AID) system was developed by Nishimura et al. (2009) and is based on the auxin-mediated protein degradation pathway found in plants. The auxin (aux) family of plant hormones consists of small molecules that play an important role during multiple phases of plant development. Auxin response factors (ARF) are transcription factors that drive the expression of auxin-responsive genes. The Aux/IAA family of early auxin-response genes inhibits ARFs and blocks gene expression. In the presence of auxin, the E3 ligase F-box protein transport inhibitor response 1 (TIR1) binds the inhibitory Aux/IAA and promotes their E3-mediated proteosomal degradation. This, in turn, restores ARF-mediated gene expression (Teale et al., 2006).

To transfer this system from plant to eukaryotic cells, two things are needed, the F-box protein TIR1 and a sequence or motif that is recognized by TIR1 and targets the protein for degradation (degron) in the presence of auxin (Figure 4). For the AID technology, Nishimura et al. (2009) used the F-box protein TIR1 from *Oryza sativa* (OsTIR1), which was able to form a functioning SCF E3 ligase complex with endogenous SKP1, CUL1, and RBX1. The *Arabidopsis* Aux/IAA gene IAA17 (Kubota et al., 2013) served as a template to create a 7-kD small degron tag, termed mini-AID (mAID). It was shown that mAID-fused proteins could be the degraded rapidly in

human cells expressing OsTIR1 during the presence of indole-3-acetic acid (IAA), an auxin family member (Nishimura et al., 2009).

Although this system has many advantages, the two major drawbacks are: (i) the leakiness of the degradation is quite high, as mAID tagged proteins were degraded even in the absence of auxin and (ii) the amount of auxin needed for degradation is very high, which could lead to toxicity in some cell lines during prolonged exposure. To address these issues, Yesbolatova et al. (2020) improved the technology and created the AID version 2 (AID2), which was also used in this study. The specificity of the system was improved through a bump-and-hole approach (Islam, 2018): the F74G mutation in OsTIR1 (OsTIR1^{F74G}) introduced a hole in the auxin-binding site, which is filled by the bumped-IAA analogue, 5-Ph-IAA. This eliminated the leakiness of the AID system and allowed for much lower concentrations of auxin to achieve an even fast degradation of the target proteins (Yesbolatova et al., 2020).

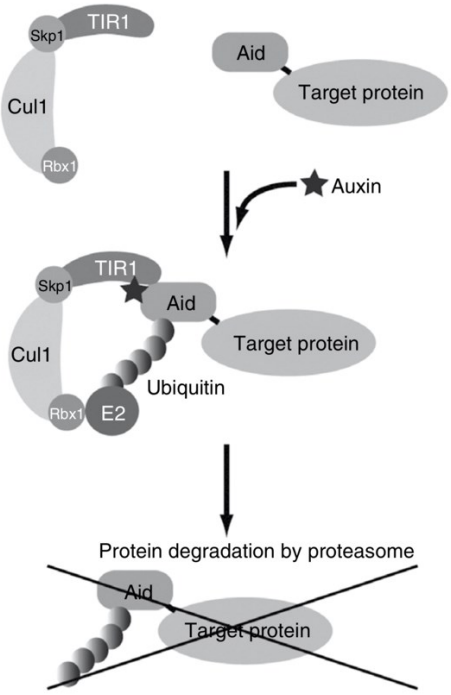


Figure 4. Protein degradation by the AID system.
The AID technology needs two components to work, the F-box protein TIR1 and the protein with a degron tag (Aid). TIR1 is part of the SCF E3 ligase complex, recognizes and binds the degron sequence in the presence of auxin. This leads to the attachment of ubiquitin to the Aid-tag, which targets the whole protein for degradation by the proteasome. Figure adapted form Nishimura et al. (2009).

5 Hypothesis and Aims

Multiple studies proved the existence of non-canonical transcriptional complexes during the IFN-mediated response, although their exact role is still poorly understood. They have been linked to the basal expression of ISGs independent of IFN stimulation and to the ability to rescue a subset of ISG expression upon loss of individual STATs from the ISGF3 complex. Based on this, we hypothesize that some of these non-canonical complexes exist as a primary mechanism to ensure the expression of a core set of ISGs during pathological challenges. We also hypothesize that during the interferon stimulation they exist side-by-side to the canonical complexes but are outcompeted due to their lower affinity and stability. Once the ISGF3 complex is unable to form, complexes like STAT1-IRF9 or STAT2-IRF9 take over and rescue gene expression. We suspect that this process takes some time as gene expression cannot be immediately compensated by non-canonical complexes.

With this study we aim to provide further insights to the role of non-canonical JAK-STAT signaling by employing the AID2 system to rapidly deplete STAT1, STAT2 or IRF9. With this tool we aspire to investigate how a sudden loss of these transcription factors affect ISG expression during different stages of the IFN response.

6 Results

6.1 STAT1 is crucial for ISG expression

To generate a baseline and understand how the complete loss of JAK-STAT pathway components affects the ISG expression in immortalized murine RAW264.7 macrophages (Taciak et al., 2018), we knocked-out *Stat1*, *Stat2*, and *Irf9* (Supplementary Figure 1). The cell lines were then stimulated with IFN-β to induce an IFN-I response, or IFN-γ for the IFN-II response, and the expression levels of a subset of ISGs were analyzed (Figure 5).

The loss of STAT1 resulted in significant loss of expression for all the tested ISGs (Figure 5), indicating the crucial role of STAT1 in ISGF3 and GAF complexes. The deficiency of STAT2 or IRF9 significantly affected the expression of the *Batf2*, *Gbp2*, *Gbp3,* and *Gbp6* genes during IFN-β stimulation, as a delayed and weaker response was measured. Although, the same genes remained largely unaffected by the loss of STAT2 or IRF9 during IFN-γ stimulation, *Batf2* and *Gbp3* expression was reduced during the early response (Figure 5). This correlates with the canonical understanding, that IFN-I response is driven by the ISGF3 complex and the loss of either STAT1, STAT2, or IRF9 completely shuts down expression. As IFN-II signaling largely depends on GAF, the loss of either STAT2 or IRF9 had no effect on the expression of these ISGs during IFN-γ stimulation. In contrast, *Ifit3* was heavily affected by the loss of STAT2 and IRF9 as there was no response to IFN-β stimulation in either case. Even during IFN-γ stimulation, expression levels were unable to rise above their untreated values (Figure 5). For *Mx2*, STAT1 and STAT2 are essential for its expression during IFN-β and IFN-γ stimulation, as the gene could only be expressed very weakly at later response stages in the absence of IRF9 (Figure 5).

Taken together, we were able to set a reference of the transcriptional dependencies of JAK-STAT pathway components and identify two subsets of ISGs. The first one made up of *Batf2*, *Gbp2*, *Gbp3*, and *Gbp6*, following the canonical way in the response to IFN-γ. The other set, *Ifit3* and *Mx2*, showed not only dependency on STAT1, but relied also heavily on STAT2 and IRF9, indicating that their IFN-II response also relies on the ISGF3 complex.

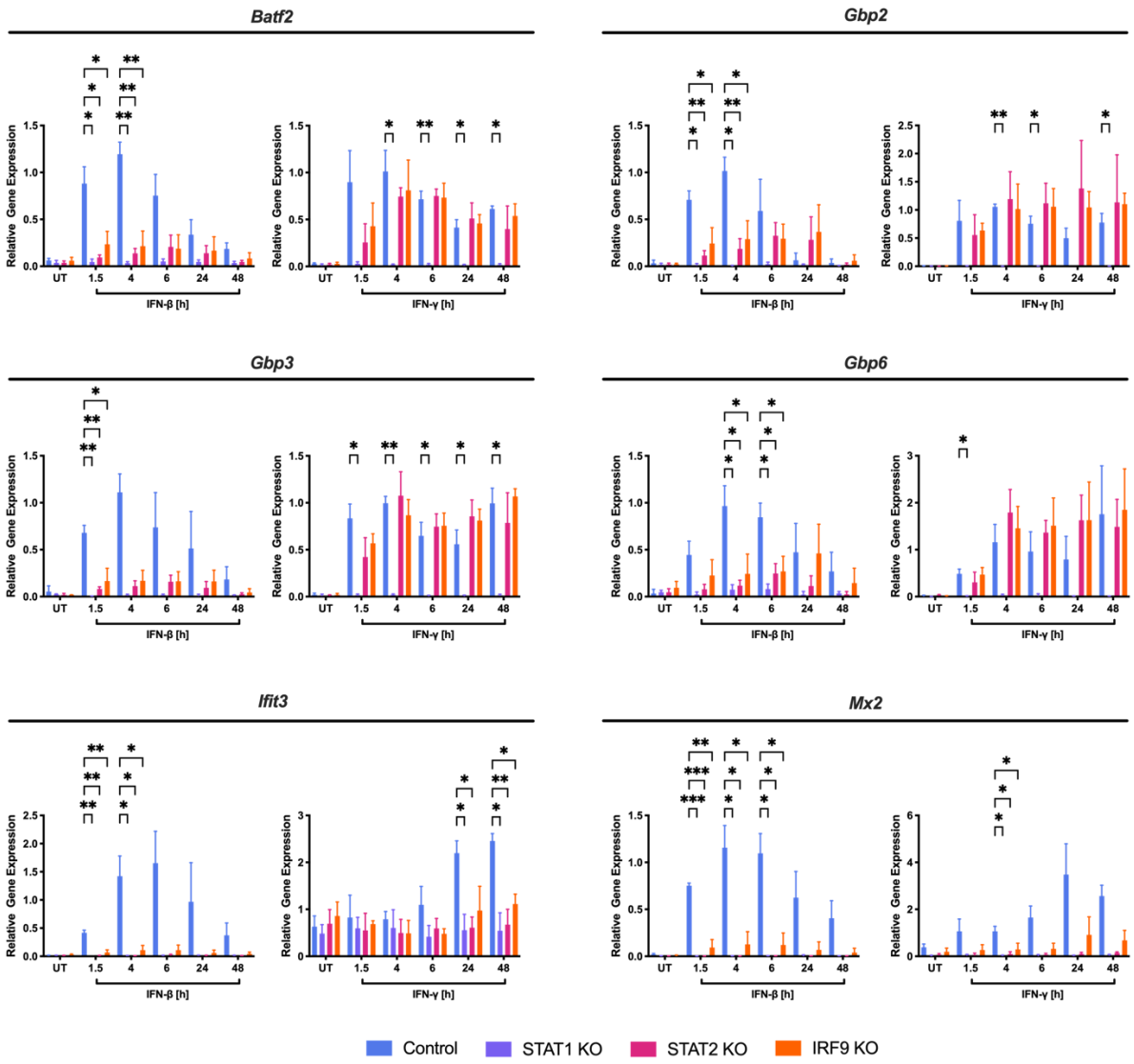


Figure 5. ISG expression is impaired by the loss of transcriptional components.
Quantitative real-time polymerase chain reaction (qPCR) showing pre-mRNA expression of the indicated genes in STAT1 KO, STAT2 KO or IRF9 KO RAW264.7 macrophages expressing OsTIR1^{F74G} relative to the housekeeping gene *Gapdh*. Cells were seeded at 80.000 cells/well in a 6-well cluster and stimulated with either IFN-β or IFN-γ for 1.5, 4, 6, 24 or 48 hours. The *Rosa*$^{-/-}$ RAW264.7 macrophage monoclonal cell line was used as a positive control. The data shown represents the mean and standard deviation of three biological replicates with up to three technical replicates each. P-values (*P ≤ 0.05; **P ≤ 0.01)

6.2 Establishing the AID2 system in the RAW264.7 cell line

The AID system needs two components to work, the F-box protein TIR1 and a mAID tagged protein, that can be targeted for degradation. The KO cells lines that were already generated (Figure 5), were transduced with the improved OsTIR1^{F74G}. To complete the AID system, mAID-fused variants of STAT1, STAT2, and IRF9 were transduced into the respective KO cell line, reconstituting the missing protein. To verify the protein expression levels of the mAID tagged variants and OsTIR1^{F74G}, we performed Western blot with corresponding antibodies (Figure 6).

We could successfully reconstitute the knocked-out genes and express mAID tagged variants of STAT1 (Figure 6A), STAT2 (Figure 6B), IRF9 (Figure 6C), and OsTIR1^{F74G}, thereby creating RAW264.7 macrophage degron cell lines for the subsequent experiments.

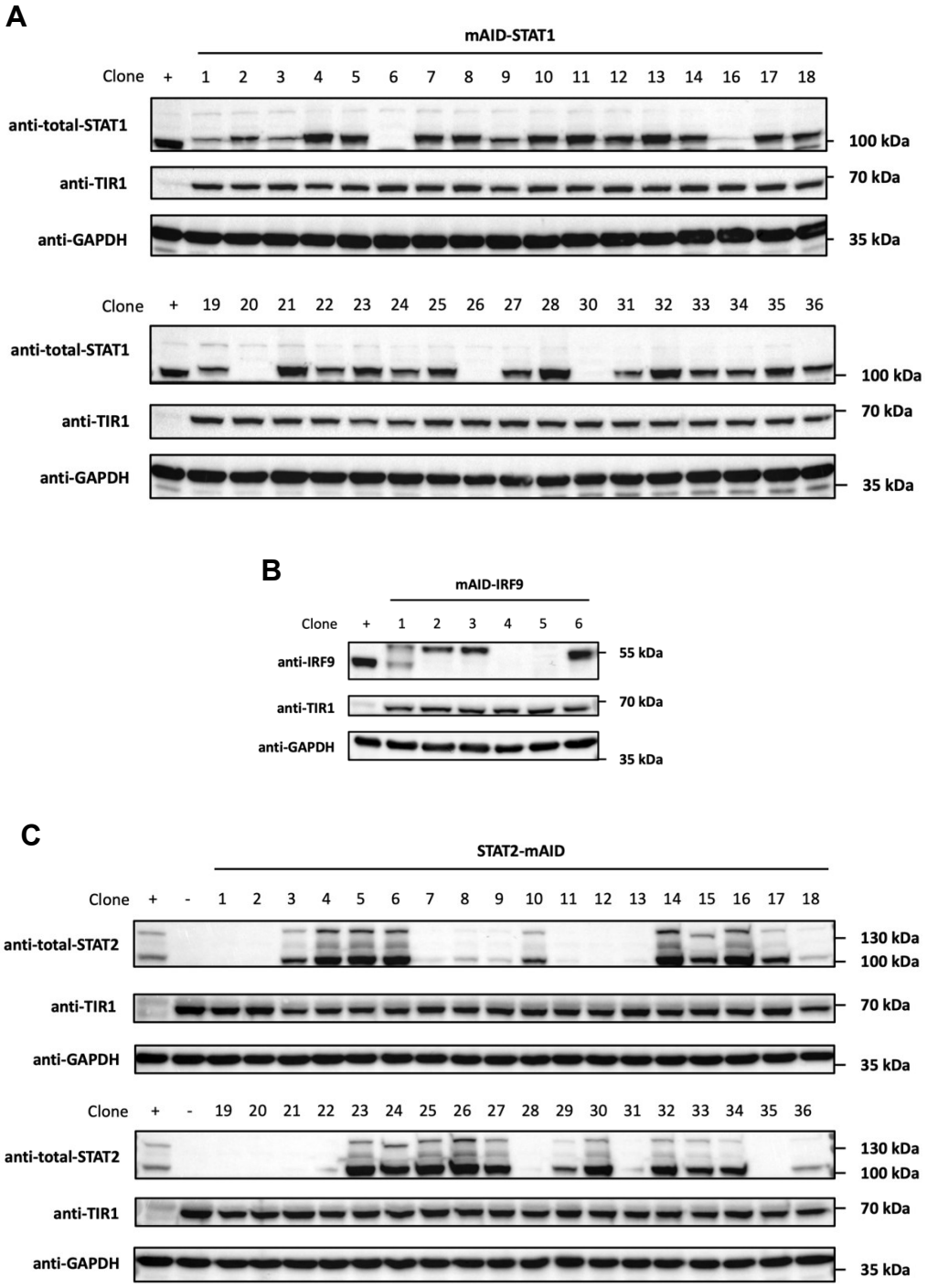


Figure 6. mAID tagged STAT1, STAT2 and IRF9 are successfully expressed in KO cell lines. Western blots showing protein expression of TIR1, the housekeeping gene GAPDH and (A) total-STAT1 in *Stat1*$^{-/-}$ RAW264.7 macrophages expressing OsTIR1^{F74G} and STAT1 with a N-terminal mAID tag, or (B) IRF9 in *Irf9*$^{-/-}$ RAW264.7 macrophages expressing OsTIR1^{F74G} and IRF9 with a N-terminal mAID tag, or (D) total-STAT2 in *Stat2*$^{-/-}$ RAW264.7 macrophages expressing OsTIR1^{F74G} and STAT2 with a C-terminal mAID tag. (A-C) Cells were stimulated with doxycycline (dox) for 48 hours. The *Rosa*$^{-/-}$ RAW264.7 macrophage cell line was stimulated with IFN-β for 2 hours and used as a positive control (+).

6.3 mAID tagged proteins are degraded in an auxin-dependent manner

With the successful establishment of STAT1, STAT2, and IRF9 degron cell lines, the next step was to test if the AID system works as intended. We aimed to establish a system with a fully functional protein, that behaves like the original one, with the ability to control its expression and degradation with doxycycline and auxin respectively by utilizing the AID2 system. The following experiments were carried out using the STAT2 degron cell line.

To test the doxycycline inducible expression and to see if STAT2 can be phosphorylated as well, three different single cell-derived clones were tested. Cells were stimulated with doxycycline for 24 hours, IFN-β for four hours and their protein expression was determined (Figure 7A). From the tested single cells clones, only the single cell clone 14 showed the most robust expression upon doxycycline stimulation when compared to clones 10 and 33. STAT2 was only phosphorylated upon IFN-β treatment and not with doxycycline alone (Figure 7A). This phosphorylation is crucial for STAT2 function as a transcription factor in the ISGF3 complex formation and its nuclear localization to drive ISG expression (Steen & Gamero, 2013).

To test the auxin-dependent E3-mediated protein degradation, cells were stimulated with doxycycline for six hours, IFN-β for four hours to check for phosphorylation and different concentrations of auxin to determine optimal conditions for protein degradation (Figure 7B). Again, only upon doxycycline and IFN-β stimulation, STAT2 was expressed and phosphorylated. The degradation through the AID system is dependent on the concentration of auxin, if it is too low, tagged proteins were not degraded completely. Only in cells treated with 75 µM auxin for four hours, the protein signal returned to the levels of cells not treated with IFN-β and doxycycline (Figure 7B). It is important to note, that these cells are still stimulated with doxycycline, therefore STAT2-mAID is still expressed, but immediately degraded to a point where it is not detectable anymore.

To determine if protein expression can be gradually controlled, cells were stimulated with different doxycycline concentrations for six to 48 hours (Figure 7C). We were unable to observe any visible differences between the stimulation durations and concentrations, as STAT2 was expressed during all conditions. This means, that

cells can safely be stimulated for shorter and longer periods with doxycycline, without impacting STAT2-mAID levels in a significant way.

In conclusion, we were able to induce mAID tagged STAT2 in a doxycycline dependent manner, show that can be phosphorylated upon IFN-β treatment, and degraded in the presence of auxin. In other words, we were successful in establishing the AID2 system in the RAW264.7 macrophage cell line.

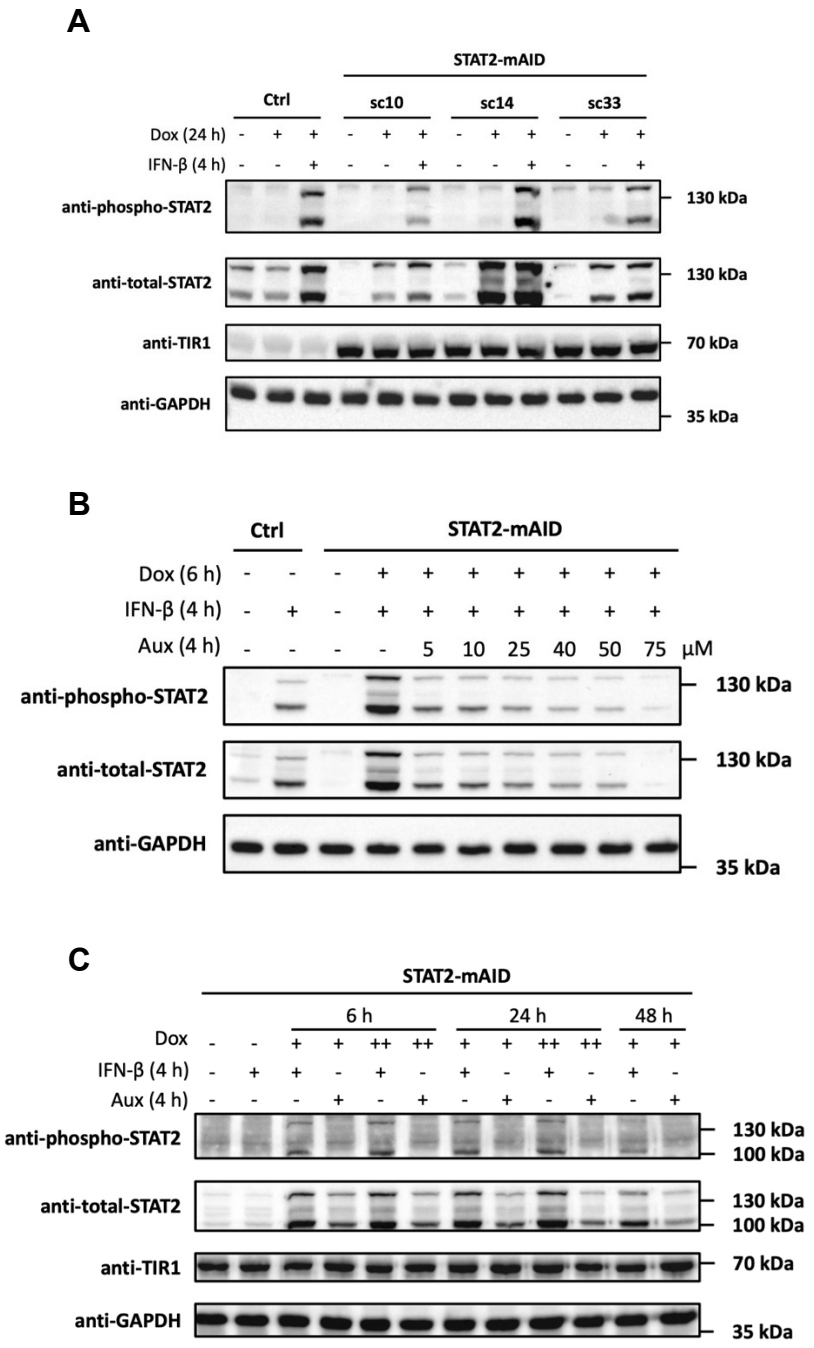


Figure 7. mAID tagged STAT2 can be degraded in an auxin-dependent manner.
(A) Western blot showing protein expression of phospho-STAT2, total-STAT2, TIR1 and the housekeeping gene GAPDH in different single cell clones (sc) 10, 14 and 33 of *Stat2*$^{-/-}$ RAW264.7 macrophages expressing OsTIR1^{F74G} and STAT2 with a C-terminal mAID-tag. Cells were seeded at 300.000 cells/well in a 6-well cluster and stimulated with doxycycline (dox) for 24 hours and IFN-β for 4 hours. (B) Western blot showing protein expression of phospho-STAT2, total-STAT2 and the housekeeping gene GAPDH in the single cell clone 14 of *Stat2*$^{-/-}$ RAW264.7 macrophages expressing OsTIR1^{F74G} and STAT2 with a C-terminal mAID-tag. Cells were seeded at 300.000 cells/well in a 6-well cluster and stimulated with doxycycline (dox) for 6 hours, IFN-β for 4 hours and auxin (aux) for 4 hours with the indicated concentrations. (C) Western blot showing protein expression of phospho-STAT2, total-STAT2, TIR1 and the housekeeping gene GAPDH in the single cell clone 14 of *Stat2*$^{-/-}$ RAW264.7 macrophages expressing OsTIR1^{F74G} and STAT2 with a C-terminal mAID-tag. Cells were seeded at 150.000 cells/well in a 6-well cluster and stimulated with 1000 ng/mL (+) or 2000 ng/mL (++) doxycycline (dox) for 6, 24 or 48 hours and IFN-β for 4 hours. (A-C) The *Rosa*$^{-/-}$ RAW264.7 macrophage cell line was used as a positive control (Ctrl).

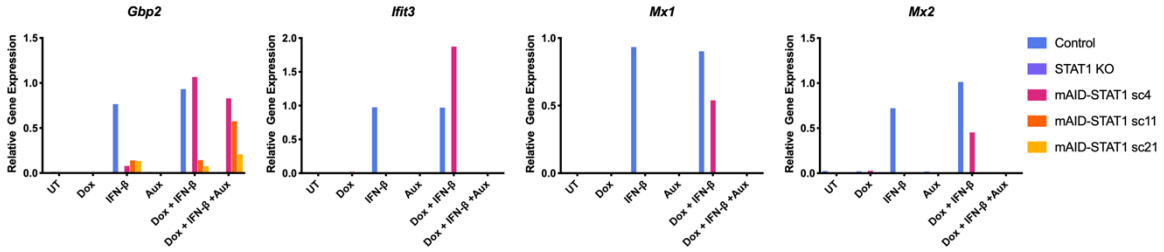
6.4 ISG expression is induced by mAID tagged proteins

The addition of a tag to a protein can impair the interaction with other proteins or the binding to DNA, both are important core functions of the ISGF3 and GAF complexes. To verify that the addition of the mAID-tag to STAT1, STAT2 and IRF9 did not compromise their role as transcription factors, the expression of a subset of ISGs were tested.

Multiple single cell-derived clones of mAID tagged STAT1 (Figure 8A) and STAT2 (Figure 8B) were treated with doxycycline to induce protein expression, IFN-β for phosphorylation and auxin for the E3-mediated degradation. Gene expression levels varied drastically between the different tested single cell-derived clones and only single cell clone 4 of mAID-STAT1 (Figure 8A) and single cell clone 14 of STAT2-mAID (Figure 8B) showed the most robust pre-mRNA expression of *Gbp2*, *Ifit3, Mx1* and *Mx2* upon doxycycline and IFN-β stimulation. Surprisingly, *Gbp2* expression showed less dependencies on STAT1 as the other ISGs did and the reason for this is not entirely understood yet. Treatment with doxycycline or IFN-β alone was not sufficient to induce gene expression. Upon treatment with auxin for four hours, *Ifit3*, *Mx1* and *Mx2* signals were lost, while *Gbp2* levels remained unchanged (Figure 8A) or were increased (Figure 8B).

This shows that mAID tagged STAT1 and STAT2 are capable of inducing ISG expression upon IFN-β treatment, which could be reverted through their auxin-induced degradation, confirming a fully functioning mAID tagged STAT1 and STAT2.

A



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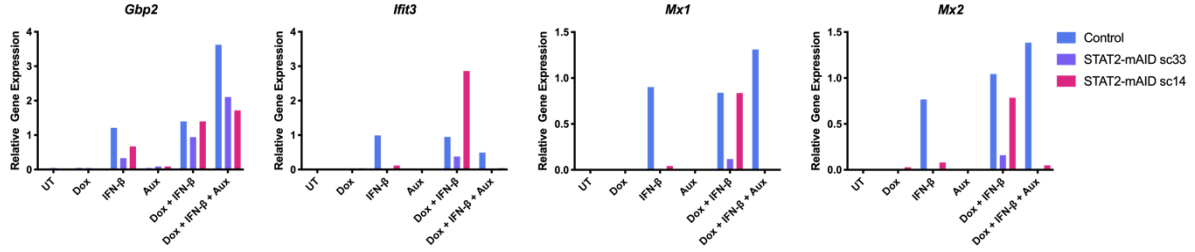

Figure 8. mAID tagged STAT1 and STAT2 are able to induce ISGs
(A) Quantitative real-time polymerase chain reaction (RT-qPCR) showing pre-mRNA expression of the indicated genes of different single cell clones (sc) 4, 11 and 21 of *Stat1*$^{-/-}$ RAW264.7 macrophages expressing OsTIR1^{F74G} and STAT1 with a N-terminal mAID-tag (mAIDI-STAT1) relative to the housekeeping gene *Gapdh*. Cells were seeded at 300.000 cells/well in a 6-well cluster and stimulated with doxycycline (dox) for 6 hours, IFN-β for 4 hours and auxin (aux) for 4 hours before lysing as indicated in the respective labelling. The single cell clone (sc) of *Rosa*$^{-/-}$ RAW264.7 macrophages (Control) and *Stat1*$^{-/-}$ RAW264.7 macrophages expressing only OsTIR1^{F74G} (STAT1 KO) were used as a positive and negative control respectively. (B) Quantitative real-time polymerase chain reaction (qPCR) showing pre-mRNA expression of the indicated genes in different single cell clones (sc) 14 and 33 of *Stat2*$^{-/-}$ RAW264.7 macrophages expressing OsTIR1^{F74G} and STAT2 with a C-terminal mAID-tag (STAT2-mAID) relative to the housekeeping gene *Gapdh*. Cells were seeded at 300.000 cells/well in a 6-well cluster and stimulated with 1000 ng/mL doxycycline (dox) for 24 hours, IFN-β for 4 hours and auxin (aux) for 4 hours before lysing. The *Rosa*$^{-/-}$ RAW264.7 macrophage monoclonal cell line was used as a positive control. (A, B) The data shown was obtained from one biological replicate and represents the mean of up to three technical replicants.

6.5 The AID2 system allows to control ISG expression

Yesbolatova et al. (2020) showed that with the improved AID2 system mAID tagged proteins can be degraded within 30 minutes. As we were interested how the ISGF3 components shape the gene expression, we set out to investigate how fast the rapid protein loss translates to a reduction in ISG expression. To address this, STAT2-mAID cells were stimulated with dox and IFN-β to induce gene expression, followed by a decreasing duration of auxin treatment before measuring pre-mRNA expression levels of a subset of ISGs (Figure 9).

We were able to identify two categories that show distinct responses to the sudden loss of STAT2. The first one consists of *Mx1*, *Mx2* and *Ifit3.* The pre-mRNA expression of *Mx1* and *Mx2* declined rapidly with only 10 minutes of auxin treatment (Figure 9C). The expression of *Ifit3* was reduced substantially, although longer exposure with auxin was required for more reduction in gene expression compared to *Mx1* and *Mx2* (Figure 9C). The increased auxin exposure correlated with a reduction in the expression levels, until it was completely lost after two hours. In contrast, for the second category of genes, which includes *Gbp2*, longer auxin stimulation increased the expression levels relative to shorter ones (Figure 9B, C). This effect was also observed in the respective control samples and coincides with data shown earlier (Figure 8B). Similar to *Ifit3*, very short auxin treatments resulted only in a slight reduction of *Gbp2* expression, which gradually decreased with longer auxin stimulation until one hour (Figure 9C). Upon auxin stimulation longer than one hour, the expression levels started to increase (Figure 9B). This might be reflecting more STAT1 homodimer binding compensating the STAT2 loss as was also speculated in the experiments with complete KOs (Figure 5), as *Gpb2* possesses both ISRE and GAS sites at its promotor (Ramsauer et al., 2007).

To sum up, we were able to degrade STAT2 as early as 10 minutes after exposure to auxin and show that protein degradation can directly affect ISG expression levels. The degree on how strongly this affected pre-mRNA levels varied between genes during the early response to IFN-I, hinting at the involvement of different transcriptional complexes.

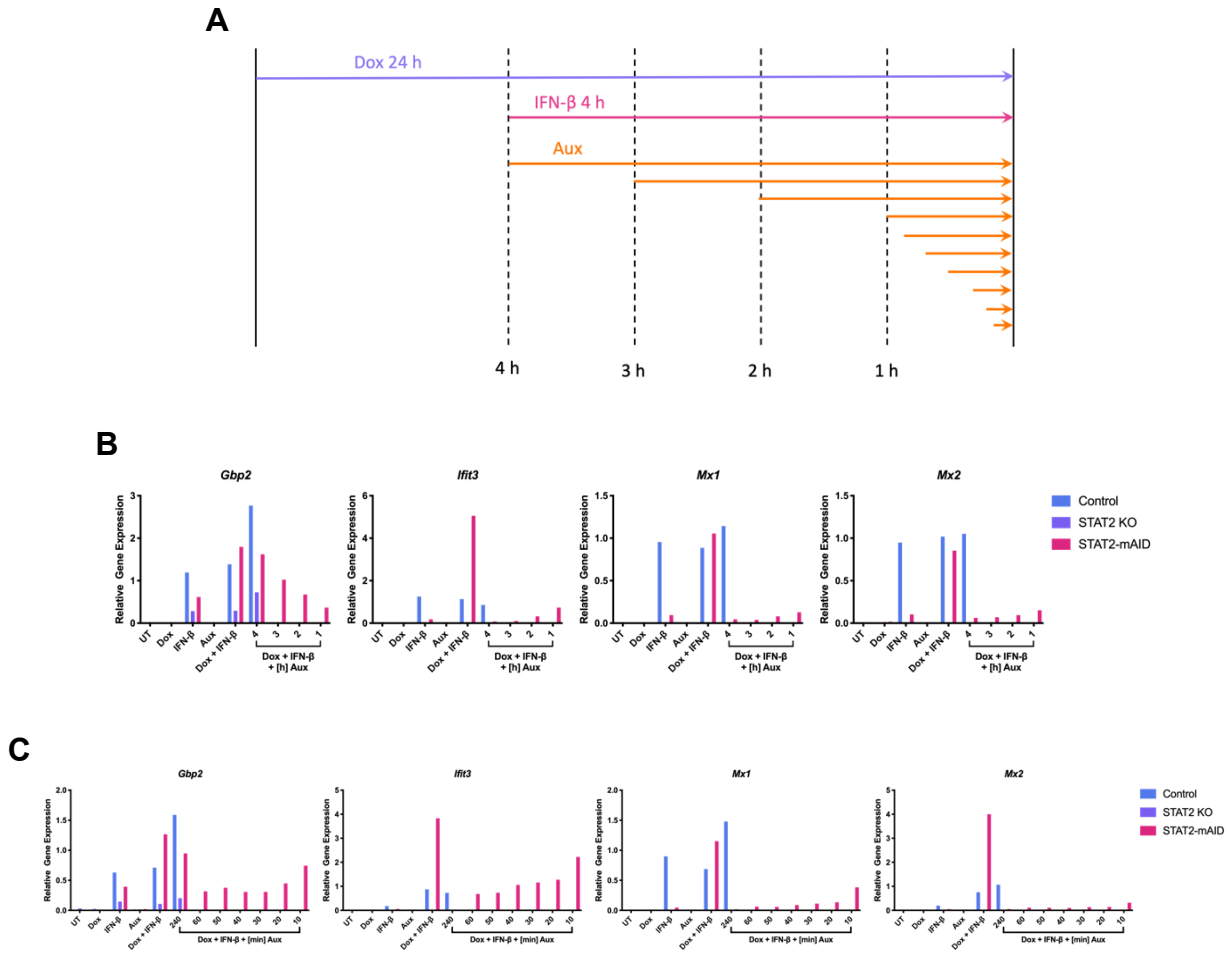

Figure 9. Auxin stimulation directly affects ISG expression levels during early IFN-I response. (A) Experimental overview showing the different stimulation conditions with their corresponding time frames. (B, C) Quantitative real-time polymerase chain reaction (qPCR) showing pre-mRNA expression of the indicated genes of the single cell clone (sc) 14 of *Stat2*$^{-/-}$ RAW264.7 macrophages expressing OsTIR1^{F74G} and STAT2 with a C-terminal mAID-tag (STAT2-mAID) relative to the housekeeping gene *Gapdh*. Cells were seeded at 300.000 cells/well in a 6-well cluster and stimulated with doxycycline (dox) for 24 hours, IFN-β for 4 hours and (B) auxin (aux) for 4, 3, 2 or 1 hour before lysing or (C) aux for 60, 50, 40, 30, 20 or 10 minutes before lysing. The *Rosa*$^{-/-}$ RAW264.7 macrophage monoclonal cell line and *Stat2*$^{-/-}$ RAW264.7 macrophages expressing only OsTIR1^{F74G} (STAT2 KO) were used as a positive and negative control respectively. The data shown was obtained from one biological replicate and represents the mean of up to three technical replicants.

6.6 STAT2 is crucial during the late IFN-I response

The previous experiments focused on the early responses to IFN-I and how the sudden loss of STAT2 affected the expression of a subset of ISGs. We were able to identify two categories, one relied completely on STAT2 for the gene expression, while the other one was able to compensate the loss of STAT2 very quickly. To investigate the role of STAT2 during the late IFN-I response, we repeated our previous experiment in STAT2 degron cells (Figure 9) and increased the IFN-β treatment to 18 hours and measured pre-mRNA levels of the same ISGs.

The expression of *Mx1*, *Mx2* and *Ifit3* during the late IFN-I response (Figure 10) was as dependent on STAT2 as the early response was (Figure 9), as even after one hour of auxin treatment, pre-mRNA levels were severely reduced. To our surprise, *Gbp2* expression levels were even less affected in the late IFN-I response (Figure 10), than in the early one (Figure 9).

With this, we were able to confirm not only the importance of STAT2 during the early and late response to IFN-I, but also the two categories of ISGs, with their distinct responses to the sudden loss of STAT2.

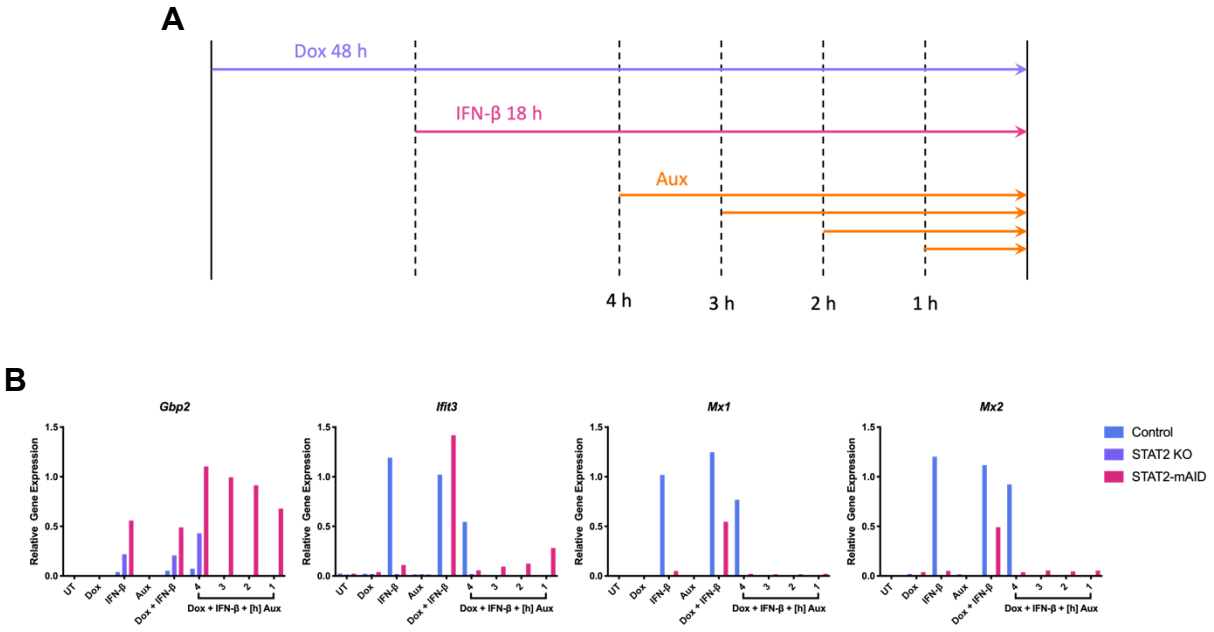

Figure 10. Auxin stimulation affects ISG expression levels during late IFN-β-mediated response (A) Experimental overview showing the different stimulation conditions with their corresponding time frames. (B) Quantitative real-time polymerase chain reaction (qPCR) showing pre-mRNA expression of the indicated genes of the single cell clone (sc) 14 of *Stat2*$^{-/-}$ RAW264.7 macrophages expressing OsTIR1^{F74G} and STAT2 with a C-terminal mAID-tag (STAT2-mAID) relative to the housekeeping gene *Gapdh*. Cells were seeded at 200.000 cells/well in a 6-well cluster and stimulated with doxycycline (dox) for 48 hours, IFN-β for 18 hours and auxin (aux) 4, 3, 2 or 1 hour before lysing as shown in (A). The *Rosa*$^{-/-}$ RAW264.7 macrophage monoclonal cell line and *Stat2*$^{-/-}$ RAW264.7 macrophages expressing only OsTIR1^{F74G} (STAT2 KO) were used as a positive and negative control respectively. The data shown was obtained from one biological replicate and represents the mean of up to three technical replicants.

7 Discussion

The regulation of gene expression by the JAK-STAT pathway is a hallmark of the innate responses to bacterial and viral challenges, characterized by a fast cell surface to nucleus signal transduction. Depending on the type of IFN, a specific transcriptional complex is formed, driving a different subset of ISGs (Schindler et al., 2007; Stark et al., 1998; Stark & Darnell, 2012). This idea has been challenged by the discovery of various non-canonical complexes that are also involved in the regulation of gene transcription (Abdul-Sater et al., 2015; Majoros et al., 2017). Here, we established an AID2 based system that can be used to study ISG regulation and provide further evidence for these non-canonical transcriptional complexes during the IFN response.

According to the canonical JAK-STAT signaling, IFN-I induces the assembly of the ISGF3 complex to express ISRE-driven genes, whereas IFN-II promotes GAF formation to regulate the transcription of GAS-driven genes. However, multiple studies were able to provide evidence for the existence of non-canonical complexes and their capability of regulating gene expression. Our laboratory has recently shown that IFN-II can also induce a large number of ISGs through the ISGF3 complex (Majoros et al., 2017; Platanitis et al., 2019). Multiple studies employing knockouts suggest that during different stages of IFN signaling, complexes containing IRF9 and either STAT1 or STAT2, but not both, have the potential to control ISG expression (Abdul-Sater et al., 2015; Rauch et al., 2015). However, a major limitation of experiments performed in KOs or with overexpression is that they create and environment which is most likely never encountered under physiological conditions. Based on this, we suspect that the main reason theses non-canonical complexes exist, is to rescue the expression of a core set of ISGs, if the main, canonical pathway fails to do so. Hence, we decided to use the AID2 technology developed by Yesbolatova et al. (2020). This system targets proteins with a degron tag for E3-mediated proteasomal degradation, in the presence of auxin. The two big advantages the AID system offers are the rapid protein degradation and the option to also recover protein levels again (Nishimura et al., 2009). This already allows for a fine control over protein availability within a cell, but we wanted to control it even more and eliminate as many interferences as possible. To achieve this, we decided to knock-out the genes of interest and replace them by mAID tagged variants. This eliminates any interference, the original versions might

cause, and reduces the background noise, as the effect we observe is only caused by the mAID tagged protein. We wanted to control the expression of the tagged protein as well and therefore utilized the Tet-On system (T. Das et al., 2016). This system allows for the activation of gene transcription only in the presence of tetracycline family derivatives like doxycycline (dox). With these modifications in place, we created a platform which allows for the control and observation of different conditions: (i) the KO state, no expression of mAID tagged variants, (ii) “normal” condition, controlled expression of tagged proteins, (iii) sudden loss of the target protein, (iv) prolonged loss and possible fallback mechanisms and (v) recovery of the mAID tagged protein.

We successfully expressed the mAID tagged STAT1, STAT2 and IRF9 (Figure 6) in the RAW264.7 macrophage cell line. Another critical component for a working AID system is the F-box protein TIR1, which was also successfully expressed in our cell lines (Figure 6). We optimized the system using mAID tagged STAT2 and confirmed that mAID tagged variants are expressed in the presence of doxycycline, are phosphorylated upon IFN-β stimulation (Figure 7B) and degraded in an auxin dependent manner (Figure 7C). The functionality of STAT1 and STAT2 were unaltered in the presence of the mAID tag since they were able to induce gene expression successfully upon dox and IFN-β stimulation as expected. The addition of auxin depleted STAT levels and rapidly affected ISG expression (Figure 7 and Figure 8). Taken together, we created a powerful tool, that enabled the temporal control over protein degradation and re-expression, which can be utilized to unravel the role of individual components of the ISGF3 complexes and address the formation of non-canonical complexes in a system more comparable to physiological conditions.

We specifically looked at pre-mRNA levels to exclude the effects of mRNA stability in the readout. Based on previous studies, one possibility is that non-canonical complexes, like STAT1-IRF9 or STAT2-IRF9, are formed alongside the iconic ISGF3 complex during the IFN-response, although at much lower levels. Due to their composition, they are less stable and thereby easily replaced by the ISGF3 complex. However, they only come into play if the canonical ISGF3 complex is unable to form, for example through the viral restriction of one of the STATs, inhibiting correct complex formation (Harrison & Moseley, 2020). This allows then that the non-canonical complexes are unfavored under normal conditions and act as a back-up mechanism to rescue the expression of specific ISGs. Another possibility is that the non-canonical complexes are formed de-novo exclusively in the absence of one of the STATs a priori.

The AID system provides a unique way of investigating this matter by removing the STATs very rapidly, leaving the cells no time to adjust to this loss, like in a KO. This might be then reflected by the delayed onset of back-up mechanisms that rescue gene expression.

We were able to identify two unique responses to the sudden loss of the STATs during the different stages of IFN-I response. The first class is defined by behavior of *Mx1*, *Mx2* and *Ifit3*, were gene expression strikingly declined with the absence of the STAT1 or STAT2 (Figure 8, Figure 9 and Figure 10). Results from *Mx2* suggest that STAT2 is degraded very quickly once auxin is added and that this can be seen immediately on the transcriptional level, as expression was completely lost after 10 minutes of auxin stimulation (Figure 9). Why this is not observed in *Mx1* and *Ifit3* cannot be answered with this system. The second class is represented by *Gbp2*, where gene expression was rescued rapidly upon STAT1 or STAT2 loss (Figure 8, Figure 9 and Figure 10). To our surprise, when STAT2 was depleted for four hours, *Gbp2* pre-mRNA levels remained unchanged (Figure 8). Only when the duration of the auxin treatment was reduced, the expression levels declined, however they never were completely lost (Figure 9 and Figure 10). Based on this, we believe that *Gbp2* is one of the genes, that might be rescued by non-canonical complexes, like or STAT2-IRF9, if STAT2 is absent. The switch takes some time to come into effect, as the loss of STAT2 cannot be compensated immediately. This supports our idea of the side-by-side existence of non-canonical and canonical complexes with the goal to ensure the expression of crucial ISGs for the defense against pathogens. However, these effects could also be explained without the involvement of non-canonical complexes, as the *Gbp2* promoter contains a GAS element and therefore might be rescued by GAF in the absence of STAT2. Another possibility is that IRF1 plays a role in the regulation of *Gbp2* and can potentially rescue gene expression in the absence of STAT2 during the IFN-I response. Therefore, more replicates and experiments in STAT1, STAT2 and IRF9 will be necessary to confirm this theory.

Taken together, we were able to build a solid foundation for future experiments and give first insights to better understand what role non-canonical transcriptional complexes might have in the JAK/STAT pathway.

8 Materials and Methods

8.1 Molecular cloning

For the generation of the degron-plasmids, murine sequences of *Stat1* (NM_001205314.1, NCBI), *Stat2* (NM_019963.2, NCBI), *Irf9* (NM_001159417.1, NCBI) and the mAID (NCBI Reference Sequence NM_100306.4) sequence derived from the IAA17 protein of *Arabidopsis thaliana* (plasmid containing the sequence was a gift from Adrian Söderholm; Gijs Versteeg Lab, Max Perutz Labs, Vienna, Austria), were amplified via PCR, and cloned into a modified version of the pCW57.1 plasmid (Addgene plasmid #41393) using Gibson assembly (NEB, E2611). After the PCR, reactions were digested with DpnI (NEB, R0176) to remove the parental plasmid and then purified with the Monarch PCR & DNA Cleanup Kit (NEB, T1030) before continuing. The Gibson assembly was carried out by following the manufactures instructions, with one exception: the pmol of fragments smaller than 200 bp was increased to 2-3-fold and for fragments bigger than 200 bp increased to 5-fold in relation to the pmol of the backbone. The pCW57.1 plasmid was modified in the following way: the puromycin resistance cassette was exchanged for a blasticidin resistance cassette, derived from pLX303 (Addgene plasmid #25897) and the sequences between the TRE and hGPK promotor (including the chloramphenicol resistance cassette) were replaced by the target sequences of *Stat1, Stat2, Irf9* and mAID. The mAID sequence was either cloned before or after the *Stat1, Stat2 or Irf9* sequence and connected via a short glycine-serine-glycine linker. The start (ATG) and stop (TGA) codons were moved accordingly, to allow for proper protein expression. To avoid interference and a possible re-knock-out of *Stat1*, *Stat2* or *Irf9* by the doxycycline-inducible Cas9 protein, present in the target cell lines, the gRNA binding sites were mutated by using the Q5® Site-Directed Mutagenesis Kit (NEB, E0554) and Gibson assembly. This resulted in the following changes to the nucleotide sequences, without changing the protein code. For *Stat1* the region from G^{609} to W^{615} was mutated form “GGG GCC ATC ACA TTC ACA TGG” to “GGA GCA ATA ACG TTT ACG TGG”. For *Stat2* the region from G^{606} to V^{612} was mutated form “GGC ATT ACT TGT TCTT GGG TGG” to “GGA ATC ACC TGC TCC TGG GTT”. For *Irf9* the region from Q^{295} to R^{302} was mutated form “CAG GTG CAC AGC CTA GAC TGT CGG” to “CAA GTT CAT AGT CTG GAT TGC CGA”. Finished plasmids were transformed into chemically

competent *E. coli* Stbl3, isolated and sequenced. Verified plasmids were then transfected into HEK293T cells for lentiviral production and ultimately transduced into target RAW264.7 knock out cell lines.

8.2 Chemically competent *E. coli*

The *E. coli* Stbl3 strain was used for transformation, plasmid amplification and plasmid storage. Cells were made competent by the following protocol (Stefanie Toifl, Manuela Baccarini Lab, Max Perutz Labs, Vienna, Austria). An overnight culture was prepared from a single colony in 20 mL LB (for 1 L: 10 g Tryptone, 5 g Yeast Extract, 5 g NaCl, pH 7). On the next day, 200 µL of the culture were taken and inoculated in 20 mL fresh LB and grown at 37°C, 175 rpm until an OD of 0.45 to 0.50 was reached. Cells were then collected by centrifugation with 3.000 rcf for 5 min at 4°C. The supernatant was discarded, the pellet resuspended in 5 mL of TFBI buffer (30 mM KOAc, 50 mM $MnCl_2$, 100 mM KCl, 10 mM CaCl, 15% Glycerol, adjust pH to 5.5 with AcOH) and incubated on ice for 30 to 60 min. Afterwards, cells were pelleted with 2.000 rcf for 5 min at 4°C, the supernatant removed and resuspended in 2 mL of ice cold TFBII (10 mM H-MOPS, 75 mM $CaCl_2$, 10 mM KCl, 15% Glycerol, pH 7.0). Bacterial cells were than aliquoted to 100 µL aliquots in 1.5 mL microcentrifuge tubes, frozen in liquid nitrogen and stored at -80°C.

8.3 Transformation

All transformations were carried out by using the chemically competent *E. coli* Stbl3 and by following the same standard protocol: 100 µL of bacterial cells in 1.5 mL microcentrifuge tubes were thawed on ice, 5-10 µL of the plasmid of interest was added, mixed by flicking the tube and incubated for 5 min on ice. Bacteria were heat-shocked on a heating block for 1 min at 42°C, followed by a 2 min incubation on ice. For recovery, 900 µL of SOC medium (0.5% Yeast Extract, 2% Tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM Glucose) was added and incubated on a heating block at 37°C and 300 rpm shaking for 1 hour. Bacteria were plated on LB-Agar plates (1.5% agar) containing 100 µg/mL ampicillin (AppliChem, A0839) either directly by using 100-200 µL of the cells or pelleted at 11.000 rcf for 1 min, resuspended in 50-100 µL of SOC medium and then plated. Plates were

incubated for 1-2 days at 28°C until colonies were visible. The plasmids of successful transformants were isolated and verified by Sanger Sequencing (Microsynth).

8.4 Plasmid isolation and storage

Plasmids for sequencing were acquired by using the GeneJET™ Plasmid Miniprep Kit (Thermo Scientific, K0503) and verified sequences were stored as bacterial glycerol stocks (15% glycerol) at -80°C. Plasmids in bigger quantities needed for experiments were obtained with the Plasmid Maxi Kit (Qiagen, 12165) and stored at -20°C.

8.5 Cell culture and reagents

All cell lines were cultivated in high glucose Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich, D6429) supplemented with 10% Fetal Bovine Serum (FBS; Sigma-Aldrich, F7524), 100 U/mL penicillin (Sigma-Aldrich, P7794) and 100 ng/mL streptomycin (Sigma-Aldrich, S9137) in a humidified incubator at 37°C with 5% CO_2.

For storage, cells were grown until 80-100% confluent in 10 cm dishes. RAW264.7 cells were scraped, HEK293T cells were detached with Trypsin-EDTA and collected at 500 rcf for 5 min. The supernatant was removed, cells were resuspended in FBS containing 10% DMSO (Merck, 67-68-5) using 1/10 of the total amount of liquid the cells were cultured in, transferred into a cryovial, and stored at -80°C.

Frozen cells were thawed the following way: cells were resuspended in prewarmed DMEM to a final volume of 10 mL and centrifuged at 300 rcf for 5 min. The supernatant was removed, cells were resuspended in fresh DMEM and transferred into a cell culture dish.

For all experiments, cells were counted, and depending on how long they were kept in culture, 80.000 to 300.000 cells were seeded. First, cells were scraped and collected by centrifugation at 300 rcf for 5 min. The supernatant was aspirated, and they were resuspended in fresh DMEM. If cells were too dense, 1:5 or 1:10 dilutions for counting were prepared. Cells were then mixed 1:1 with 0.4% Trypan Blue Solution (Gibco, 15250061) for staining and counted manually by using a Neubauer chamber. Appropriated dilutions were made, cells were seeded with the desired amount in fresh cell culture dishes and incubated.

For the different experiments, cells were treated with the following reagents and concentrations (possible deviations are described in the figure legends): 1000 ng/mL doxycycline (Sigma-Aldrich, D9891), 250 U/mL murine IFN-β (PBL Assay Science, 12400-1), 10 ng/mL murine IFN-γ (a gift from Günther Adolf, Boehringer Ingelheim, Vienna), 75 µM 5-Ph-IAA (derivative of the natural auxin, indole-3-acetic acid; MedChemExpress, HY-134653).

8.6 Knock-out cell lines

To generate the knock-out (KO) cell lines, gRNA targeting either *Stat1, Stat2* or *Irf9* were cloned into the lentiviral 1358_sgRNA V2 U6-IT-mPgk-iRFP720 plasmid (gift from Johannes Zuber Lab, IMP, Vienna, Austria) by Ekaterini Platanitis and Mark Grannetia. The plasmids were then transduced into a RAW264.7 macrophage cell line featuring a doxycycline inducible Cas9 coupled with GFP (gift from Gijs Versteeg Lab, Max Perutz Labs, Vienna, Austria). To induce a KO, cells were treated with doxycycline for up to 48 hours. Successful KOs were verified by Western blot. The gRNAs are listed in Supplementary Table 1.

8.7 Transfection, transduction, and selection

The degron-plasmids of *Stat1*, *Stat2* and *Irf9* and the pRRL-SFFV-optOsTIR1F74G-T2A-eBFP2 plasmid (gift from Dea Slade Lab, Max Perutz Labs, Vienna, Austria) containing the OsTIR1^{F74G} were used to generate new cell lines by the following method. First, single cells of RAW264.7 *Stat1*$^{-/-}$, *Stat2*$^{-/-}$ and *Irf9*$^{-/-}$ were obtained. These single cells were then transduced with OsTIR1^{F74G} and selected for single cells. In the next step, the selected OsTIR1^{F74G} single cells of *Stat1*$^{-/-}$, *Stat2*$^{-/-}$ and *Irf9*$^{-/-}$ were transduced with their respective degron-plasmid, e.g., the plasmid coding for *Stat1* fused with a mAID-tag at the C- or N-terminus into the RAW264.7 *Stat1*$^{-/-}$ OsTIR1^{F74G} single cell line. This was repeated for *Stat2*$^{-/-}$ and *Irf9*$^{-/-}$ as well, in the end a total of six cell lines were created, expression the OsTIR1^{F74G} and C- or N-terminally mAID tagged STAT1, STAT2 or IRF9 (Supplementary Table 2).

Lentiviral particles were generated using a 2nd generation lentiviral system and 8 µg of DNA in a ratio of 5:1:5 (GAG/POL to VSV-G to plasmid of interest). HEK293T cells were seeded at 50-70% confluency in 10 cm cell culture dishes. The DNA was mixed with polyethylenimine (PEI; Sigma-Aldrich, 408727) in a ratio of 1:3 (µg DNA to

µg PEI) in DMEM without any supplements and incubated at room temperature (RT) for 20 min. Afterwards, the mixture was added dropwise to the cells and incubated for 48 hours in a humidified incubator at 37°C with 5% CO_2.

For transduction, RAW 264.7 target cell lines were seeded with 250.000 cells per well in a 6-well cluster the day before transduction. The supernatant, containing the lentiviral particles, was harvested from HEK293T cells, and filtered through a 45 µm filter. Target cells were transduced with a virus dilution of 1:2, 1:4, 1:10 or 1:20 or left untreated and incubated in a total of 2 mL fresh DMEM containing 6 µg/mL Polybrene (Sigma-Aldrich, TR-1003,) for 2-3 days. Then, the cells were washed twice with PBS (127 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO4, 1.8 mM KH_2PO_4, pH 7.4) and left in fresh DMEM. For selection of cells transduced with the degron-plasmids, 12 µg/mL blasticidin (Bsd; InvivoGen, ant-bl) was added and they were incubated for 2-3 days. Cells were then transferred into 10 cm dishes, grown until confluent, then used for further experiments and frozen for storage.

8.8 Cell sorting

The FACSAria™ Cell Sorter (BD) or FACSMelody™ Cell Sorter (BD) were used for obtaining single cells from a bulk culture. Cell were always sorted into 96-well clusters. The sorting parameters changed with respect to the used plasmid and signal. RAW264.7 macrophages containing only the gRNA and Cas9 for generating the KOs were sorted for high GFP and iRFP, as well as for live/dead. The RAW264.7 KOs harboring the $OsTIR1^{F74G}$ plasmid, were sorted for high BFP and live/dead. The RAW264.7 KOs with $OsTIR1^{F74G}$ and the degron-plasmids were selected with blasticidin and then sorted for live/dead. Sorted cells in 96-well clusters were checked regularly for contaminations and confluency and were ultimately transferred into 6-well clusters or 10 cm dishes for Western blot validation and storage.

8.9 SDS-PAGE and Western blot

Successful KOs, transductions and degron experiments were verified via Western blot. For the experiments, cells were counted, depending on how long they were kept in culture, 150.000 to 300.000 cells were seeded, and treated in 6-well clusters. For selecting successful KOs or degron cell lines, cells were cultivated in 6-well clusters until they reached a confluency of 50-70%, before lysing. Additionally,

degron cell lines were also treated with dox 48 h prior to lysing. In general, cells were kept on ice and washed two times with ice-cold PBS before lysing them in Laemmli buffer (2% SDS, 10% Glycerol, 120 M Tris-HCl, pH 8). Cells were scraped, transferred into microcentrifuge tubes, and boiled for 5 min at 95°C in a heating block. The protein concentration was determined with the Pierce™ BCA Protein Assay Kit (ThermoScientific, 23227). Sample dilutions containing 10-20 µg of protein were made with Laemmli buffer and 10X SDS loading dye (50% β-Mercaptoethanol, 0.02% Bromphenolblue; 1X finial concentration) and boiled for 5 min at 95°C in a heating block before loading them on a 10% SDS polyacrylamide gel for PAGE.

The proteins were blotted onto a Nitrocellulose membrane (Merck, GE10600001) with 200 mA for 16 h and 400 mA for 2 hours in carbonate transfer buffer (3 mM Na_2CO_3, 10 mM $NaHCO_3$, 20% ethanol) at 4°C. The membrane was blocked in 5% milk powder in TBS-T (150 mM NaCl, 0.05% Tween-20, 10 mM Tris-HCl, pH 7.5) for 1 hour at RT with shaking, then washed three times with TBS-T for 10 min. The primary antibody was added and incubated overnight at 4°C while shaking. For the experiments, the following primary antibodies were diluted in TBS-T containing 2% BSA: total STAT1 (Cell Signaling, 14995; 1:1000), Phospho-STAT1 (Tyr701, Cell Signaling, 9167; 1:1000), total STAT2 (Cell Signaling, 72604; 1:1000), Phospho-STAT2 (Tyr689, Merck, 07-224; 1:1000), IRF9 (Platanitis et al., 2019); 1:20; can be purchased as Anti-IRF-9, clone 6F1-H5 from Sigma-Aldrich, MABS1920), OsTIR1 (MBLI, PD048; 1:1000), GAPDH (Millipore, ABS16, 1:3000). On the next day, the membrane was washed three times with TBS-T for 10 min and then incubated with the appropriate horseradish peroxidase-coupled secondary antibody (Jackson ImmunoResearch, 115-035-144 and 111-035-003; 1:6000) for 1 hour at RT with shaking. Afterwards, membranes were washed once with TBS-T for 10 min and incubated with SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Scientific, 34580). Images of the membranes were then acquired with the ChemiDoc™ Touch Imaging System (Bio-Rad).

8.10 RNA isolation, cDNA synthesis, and qPCR

The effect of the degron system on gene expression was measured by quantitative real-time polymerase chain reaction (qPCR). For the experiments, cells were counted, depending on how long they were kept in culture, 80.000 to 300.000

cells were seeded, and then treated in 6-well clusters. Total RNA was extracted by using the NucleoSpin RNA Mini kit for RNA purification (Macherey-Nagel, 740955). For lysing, cells were kept on ice and washed two times with ice-cold PBS before lysing them according to the manufacturer's instructions.

The cDNA was generated from pre-mRNA by using 400 ng of total RNA extract, Random Hexamer Primers (Thermo Scientific, SO142), dNTPs (Thermo Scientific, R0181), RevertAid Reverse Transcriptase (200 U/µL, Thermo Scientific, EP0442) and by following the manufacturer's instructions. The qPCR reaction was carried out on a Realplex 2 Mastercycler (Eppendorf) using the Luna Universal qPCR Master Mix (NEB, M3003). Primers for the RT-qPCR are listed in Supplementary Table 3.

8.11 Data analysis

Western blot images were analyzed with Image Lab (Bio-Rad) and figures were created with GIMP and PowerPoint (Microsoft). Data form qPCRs was analyzed in Excel (Microsoft) and Prism (Graphpad). The number of biological and technical replicates are described in the figure legend and mean values with standard error of mean (SEM) are shown.

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10 List of Figures

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12 List of Abbreviations

AID	auxin-inducible degron
ARF	auxin response factors
aux	auxin
CUL1	cullin 1
DAMPs	damage-associated molecular patterns
DBD	DNA-binding domain
degron	sequence or motif that targets a protein for degradation
dox	doxycycline
FERM	4.1, ezrin, radixin, moesin
GAF	gamma-activated factor
GAS	gamma-interferon-activated sequence
HECT	homologous to the E6-AP carboxyl terminus
IAA	indole-3-acetic acid
IAD	IRF-association domain
IFN	Interferon
IFN-I	Type-I IFNs
IFN-II	Type-II IFNs
IFN-III	Type-III IFNs
IFNAR	IFN-α receptor
IFNGR	IFN-γ receptor
IFNLR	IFN-λ receptor
IL	interleukin
IRF	interferon regulatory factor
ISG	interferon regulated gene
ISGF3	interferon stimulated gene factor 3
ISRE	interferon-stimulated response element
JAK	Janus kinase
LPS	lipopolysaccharide
mAID	mini-AID
MAPKs	mitogen-activated protein kinases
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NLRs	NOD-like receptors

NOD	nucleotide oligomerization domain
OsTIR1	TIR1 from *Oryza sativa*
PAMPs	pathogen-associated molecular patterns
PHD	plant homeo domain
PN	proteostasis network
PRRs	pattern recognition receptors
PTM	post-translational modification
qPCR	quantitative real-time polymerase chain reaction
RBX1	RING-box 1
RIG-I	retinoic acid-inducible gene-I
RING	really interesting new gene
RLRs	RIG-I-like receptors
sc	single cell clone
SCF	SKP1, CUL1, F-box protein
SH2	Src homology 2
SKP1	S-phase kinase-associated protein 1
STAT	signal transducer and activator of transcription
TAD	transcriptional activation domain
TIR1	transport inhibitor response 1
TLRs	Toll-like receptors
TYK	Tyrosine kinase
UPS	ubiquitin-proteasome system

13 Supplementary Data

Supplementary Table 1. gRNAs in this study (Platanitis et al., 2019).

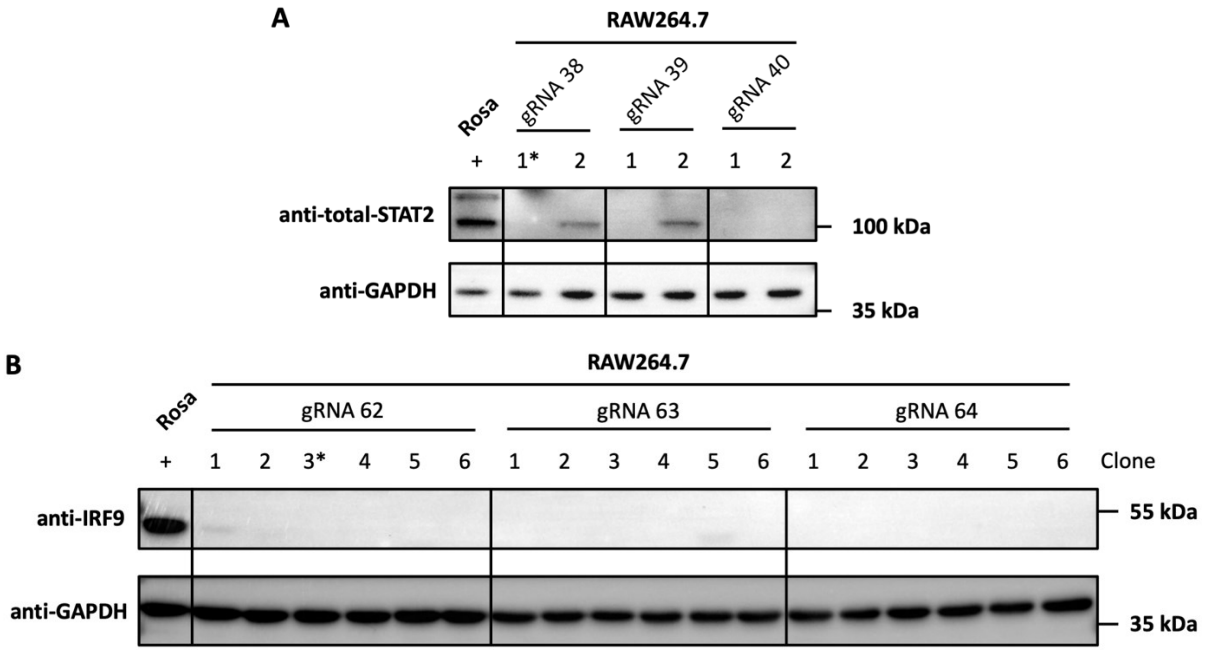
No.	Gene	sgRNA Sequence 5‘-3‘
38	*Stat2*	GGCATTACTTGTTCTTGGG
39	*Stat2*	GTTCTTGGTGAGATCCATC
40	*Stat2*	GATGCTGAAGTATAGCATGT
41	*Stat1*	GTGTCAATCCAAGGCCAGA
42	*Stat1*	GGGGCCATCACATTCACAT
43	*Stat1*	GTACTGTCTGATTTCCATG
62	*Irf9*	GGTGCACAGCCTAGACTGT
63	*Irf9*	GCAACCCTACATTTCTGGG
64	*Irf9*	GTATTCGATATACTTTGTA

Supplementary Table 2. Cell lines in this study.

Cell line origin	Gene KO	Description
RAW 264.7	*Stat1*	gRNA 42
RAW 264.7	*Stat1*	gRNA 42, OsTIR1^{F74G}
RAW 264.7	*Stat1*	gRNA 42, OsTIR1^{F74G}, STAT1-mAID (C-terminal)
RAW 264.7	*Stat1*	gRNA 42, OsTIR1^{F74G}, mAID-STAT1 (N-terminal)
RAW 264.7	*Stat2*	gRNA 38
RAW 264.7	*Stat2*	gRNA 38, OsTIR1^{F74G}
RAW 264.7	*Stat2*	gRNA 38, OsTIR1^{F74G}, STAT2-mAID (C-terminal)
RAW 264.7	*Stat2*	gRNA 38, OsTIR1^{F74G}, mAID-STAT2 (N-terminal)
RAW 264.7	*Irf9*	gRNA 62
RAW 264.7	*Irf9*	gRNA 62, OsTIR1^{F74G}
RAW 264.7	*Irf9*	gRNA 62, OsTIR1^{F74G}, IRF9-mAID (C-terminal)
RAW 264.7	*Irf9*	gRNA 62, OsTIR1^{F74G}, mAID-IRF9 (N-terminal)
RAW 264.7	*Rosa*	gRNA targeting the ROSA26 locus; used as control sample
HEK293T	-	Used for lentiviral production

Supplementary Table 3. RT-qPCR in this study.

Gene name	Forward Sequence 5’-3’	Reverse Sequence 3’-5’
Mx1 pre-mRNA	CGAGCAGCCTGTGTTCTTACT	GGAAGTGAAGTCGGATCAGGT
Mx2 pre-mRNA	CCTGACTTGACCTCATCGACT	ACTGGGAGGGGACCTGTTTC
Ifit3 pre-mRNA	GGCTCACATTGTCATGACTCC	TTCAGCTGTGGAAGGATCGC
Gbp2 pre-mRNA	TCCGCTAACTTTGTGGGGTT	TCCGCCATTTCAGAACATTTCAC
Gbp3 pre-mRNA	TTAAATGGTCGGCCCATCACA	GGAGCAAGGCTTAGCTGATT
Gbp6 pre-mRNA	AAGGGACATCATGCACACACT	TTCCTTCACATATTTTCTAGGGTGA
Oas2 pre-mRNA	TGGATGCAATCTGGGACCTG	CCCTTCCGGGAGTCATGCAG
Batf2 pre-mRNA	AAGGAGTGCAGTGTCTTCAGG	TGAGACCATCCCTAACCTGGTG
Gapdh pre-mRNA	CATGGCCTTCCGTGTTCCTA	GCGGCACGTCAGATCCA



Supplementary Figure 1. Successful STAT2 and IRF9 KOs in RAW264.7 macrophages.
(A) Western blot to very a successful *Stat2* knock-out (KO). The protein expression of total-STAT2 and the housekeeping gene GAPDH in RAW264.7 macrophages expressing a doxycycline inducible Cas9 protein and the guide RNA (gRNA) 38, 39 or 40 targeting *Stat2* is shown. (B) Western blot to very a successful *Irf9* KO. The protein expression of IRF9 and the housekeeping gene GAPDH in RAW264.7 macrophages expressing a doxycycline inducible Cas9 protein and the guide RNA (gRNA) 62, 63 or 64 targeting *Irf9* is shown. (A, B) Cells were and stimulated with doxycycline for 48 hours. The *Rosa*$^{-/-}$ RAW264.7 macrophage cell line was stimulated with IFN-β for 2 hours and acts as a positive control.