SUMO2 and SUMO3 redundantly prevent a non-canonical type I interferon response

John Tyler Crowl

A dissertation
submitted in partial fulfillment of the
requirements for the degree of

Doctor of Philosophy

University of Washington
2018

Reading Committee:
Daniel Stetson, Chair
Andrew Oberst
Jessica Hamerman

Program authorized to Offer Degree:
Immunology

© Copyright 2018
John Tyler Crowl

University of Washington

Abstract

SUMO2 and SUMO3 redundantly prevent a non-canonical type I interferon response

John Tyler Crowl

Chair of the Supervisory Committee:

Daniel Stetson

Department of Immunology

Detection of nucleic acids by innate immune sensors triggers the production of type I interferons (IFNs). While IFNs are essential for host defense against viral infection, dysregulated production of IFNs underlies numerous autoinflammatory diseases. We have found that loss of sumoylation results in a potent, spontaneous IFN response. Vertebrates possess three small ubiquitin-like modifiers (SUMOs) that can be conjugated onto target proteins and alter protein function in diverse but still poorly characterized ways. We demonstrate that regulation of IFN by sumoylation is redundantly mediated by both SUMO2 and SUMO3, but not SUMO, revealing a previously unknown function of SUMO2/3. Remarkably, this IFN response is independent of all known IFN-inducing pathways and does not require either of the canonical IFN-associated transcription factors IRF3 or IRF7. Taken together, our findings demonstrate that SUMO2 and SUMO3 are specific and essential negative regulators of a non-canonical mechanism of IFN induction.

Table of Contents

Acknowledgements

Six years ago, I moved from Kansas City to Seattle to start my PhD at the University of Washington. I had just finished my undergraduate education, and I had never lived outside the Midwest. Without the help and support of many people along the way, I would not have been able to succeed in this program.

The Department of Immunology at UW has been an incredible place to be trained as a scientist. It is a supportive and collaborative environment, and I have been fortunate to receive scientific feedback, questions, and encouragement from many different people in the department. Each of the members of my thesis committee have provided valuable input to my project and has been instrumental in my scientific and professional development.

In particular, I am grateful to Dan Stetson, my mentor and the chair of my committee, for the support he has given me throughout graduate school. Dan’s enthusiasm about science and his continual excitement over the next big idea are characteristics that I aspire to in my own career. In the Stetson lab, Dan has created a scientific environment that encourages both independence and collaboration, and it has been and excellent place to learn and explore. Each member of the Stetson lab has at various points set aside their time to discuss experimental ideas, edit manuscripts, or go get a cup of coffee.

I have been fortunate to be supported by a number of people outside of my work and science. In particular, my friends Luke, Nicole, and Lauren became my Seattle family. They encouraged me, they fed me, and they reminded me to go have fun when I forgot to. Finally, I’d like to thank my parents and my family. I have been blessed with a large family who have supported me at every step along this journey. They are an invaluable source of inspiration and guidance.

Chapter 1: Introduction

Detection of viral nucleic acids is an ancient form of host defense

The ability to distinguish self from non-self is a fundamental feature of the immune system essential for protective immunity against pathogens. In vertebrates, the immune system is divided into two branches, the innate and adaptive immunity, which have developed unique approaches to the challenge of self/non-self discrimination. The innate immune response relies on germline encoded receptors to rapidly mobilize an effective defense against invading pathogens. By necessity, the germline encoded receptors of the innate immune system detect evolutionarily conserved features common to broad classes of pathogens, known as pathogen associated molecular patterns (PAMPs). In contrast, adaptive immunity relies on somatic recombination in a small subset of immune cells to generate a diverse repertoire of pathogen specific receptors. While this robust, pathogen-specific immune response results in long lasting protective immunity, it takes time to generate and is dependent on additional signals provided by the innate immune system that establish the existence and context of the infection.

The innate immune response provides broad protection against invading pathogens and initiates the development of pathogen specific adaptive immune responses through the recognition of evolutionarily conserved PAMPs. Pathogenic bacteria and fungi possess unique proteins, lipids, and carbohydrates that comprise a robust source of foreign ligands detectable by host encoded pattern recognition receptors (PRRs). Viruses present a unique challenge for pathogen recognition as they recruit host proteins and machinery for replication, and viral assembly by these host processes renders much of the virus indistinguishable from self. Detection of viral infection must therefore rely on a compromise and the recognition of a ligand that isn’t explicitly non-self. Nearly all recognition of viral infection relies on the detection of foreign nucleic acids, and this is an ancient form of antiviral defense.

In bacteria, self/non-self discrimination is facilitated through sequence specific recognition and cleavage of foreign nucleic acids by restriction enzymes[1] and the CRISPR-Cas9 system[2]. In early eukaryotes argonaute proteins and RNA interference provide a similar system of sequence specific recognition and antagonism of foreign RNA[3]. Early in the vertebrate lineage a shift in the mechanism of nucleic acid recognition occurred that coincided with the development of the adaptive immune system. Rather than differentiating self from non-self through sequence recognition, vertebrate receptors recognize structures and subcellular localizations unique to foreign nucleic acids. The detection of foreign nucleic acids in vertebrates activates the production of type I interferons (IFNs) and the initiation of an antiviral gene program. In mammals, there are two distinct sets of receptors that recognize foreign nucleic acids in two distinct compartments: the toll-like receptors and the cytosolic sensors.

Exogenous nucleic acids are recognized by Toll-like receptors

Charles Janeway proposed the concept of pattern recognition in 1989, and over the next several years a series of experiments focusing on the toll-like receptors (TLRs) would prove the validity of this hypothesis[4]. Toll was first identified in *Drosophila* as an essential mediator of anti-fungal immunity[5], and subsequent studies in mammals identified Toll-like receptor 4 (TLR4) as the specific sensor of lipopolysaccharide, a bacterial PAMP[6-8]. Since that time additional TLRs have been identified and their ligands characterized. The TLRs can be divided into two groups based on their subcellular localization and their ligands. One group of TLRs is found on the surface of the plasma membrane and scans the extracellular space for unique PAMPs on the surface of bacteria and fungi. Whereas TLR3, TLR7, TLR8, and TLR9 are restricted to endosomal compartments and recognize double-stranded RNA (dsRNA), single-stranded RNA (ssRNA), and double-stranded DNA (dsDNA). The localization of nucleic acid sensing TLRs within endosomes prevents their activation by both extracellular nucleic acids released by dying cells and intracellular nucleic acids part of normal biological processes. Further enforcing the restricted activation of these receptors, endosomal TLRs must be proteolytically cleaved to be activated, and this only occurs once they have entered the endosomal compartment[9].

In mammals, TLR expression is primarily restricted to phagocytic sentinel cells, and recognition of their specific ligand activates an inflammatory gene program. Detection of nucleic acids by endosomal TLRs turns on an additional antiviral program through the induction of type I IFNs. Toll-like receptors 3 and 4 use the adaptor protein TRIF to activate the kinase TBK1 and the transcription factor IRF3[10]. The induction of type I IFNs by TLRs 7, 8, and 9 is specific to plasmacytoid dendritic cells, and dependent on the adaptor protein MyD88, the kinase IKKα, and the transcription factor IRF7[11]. Thus, self/non-self discrimination by the endosomal TLRs relies largely on restricted expression to sentinel cells and their restricted subcellular localization within those cells.

Cytosolic nucleic acid sensors recognize viral nucleic acids in infected cells

A second set of nucleic acid sensors is located within the cytosol and detects foreign nucleic acids within infected cells. In contrast to the TLRs, these sensors are ubiquitously expressed and can be found in nearly all cell types. cGAS is the cellular sensor of cytosolic DNA and recognizes dsDNA independent of sequence[12]. The sequestration of cellular DNA within the nucleus physically separates cGAS from endogenous DNA. In contrast, the sensors of cytosolic RNA, the RIG-I Like receptors (RLRs), are located in the cytosol alongside host RNA[13]. The RNA sensors detect structural features enriched on viral RNA that are not commonly found within host RNA in order to provide specific detection of foreign nucleic acids. MDA5 and RIG-I bind to long, dsRNA and 5’ triphosphate RNA, respectively. Both of these nucleic acid sensing pathways signal through unique adaptor proteins. cGAS signals through the adaptor protein STING whereas the RLRs signal through the adaptor protein MAVS. These two pathways converge at the kinase TBK1, which phosphorylates and activates the transcription factor IRF3.

Detection of endogenous nucleic acids results in autoinflammatory/autoimmune disease

The detection of cytosolic and endosomal nucleic acids by the TLRs, RLRs, and cGAS is essential for host defense against viral infection. However, these responses must be tightly regulated to prevent the development of autoimmune and autoinflammatory disease. The activation of any of these pathways triggers the production of type I IFNs, a family of potent antiviral cytokines. Type I interferons signal through the interferon alpha receptor (IFNAR), a ubiquitously expressed surface receptor, and induce the expression of interferon stimulated genes (ISGs) in both an autocrine and paracrine fashion. It is therefore important that the activation of a type I IFN response be tightly controlled both at the level of nucleic acid detection and signaling through IFNAR.

One key mechanism that sets thresholds for activation of the intracellular nucleic acid sensors involves the activity of enzymes that modify or metabolize self nucleic acids. One example of this form of regulation is three-prime repair exonuclease 1 (TREX1), a DNA exonuclease that prevents the activation of cGAS by endogenous DNA [14-16]. In humans, loss of function mutations in the *TREX1* gene cause Aicardi-Goutieres Syndrome (AGS), a rare and severe autoimmune disease that presents with symptoms similar to those of a congenital viral infection [17]. Several additional AGS genes have been identified, all of which encode proteins important for nucleic acid metabolism or sensing [18]. Moreover, numerous other monogenic autoinflammatory diseases are characterized by a type I IFN signature that can be identified in peripheral blood cells, collectively referred to as “interferonopathies” [18]. The underlying causes of these rare interferonopathies can be classified into three categories: those that cause excessive activation of cytosolic nucleic acid sensors, those that impact type I IFN receptor signaling, and those that exert their effects through currently unknown mechanisms [18-20]. Surprisingly, while activation of TLRs has been associated with complex autoimmune disease, TLR activation has yet to be associated with a single monogenic interferonopathy. Importantly, the genetic definition of these rare diseases and their underlying mechanisms has provided insights into a number of more common human autoimmune disorders that share a type I IFN signature as a defining feature, including systemic lupus erythematosus (SLE), systemic sclerosis, and Sjogren's syndrome. In order to understand the dysregulation underlying the autoinflammatory and autoimmune diseases associated with a type I IFN signature, it is important to understand the full breadth of mechanisms that regulate type I IFN responses.

Figures

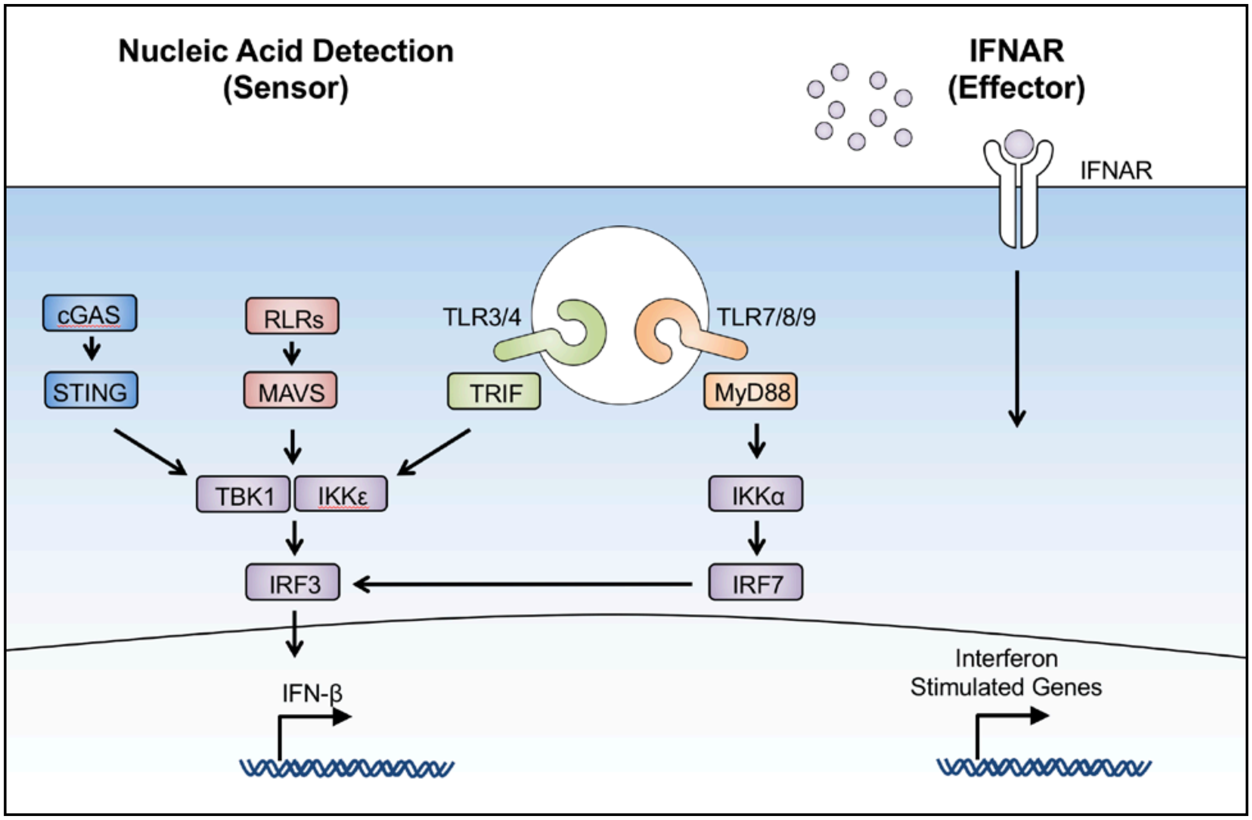


Figure 1: Nucleic acid sensing pathways activate type I interferon responses

Chapter 2: The role of Trex1-interacting proteins in the regulation of type I IFN responses

Introduction

Detection of foreign nucleic acids by innate immune receptors triggers an antiviral response through the production of type I interferons (IFNs). While these pathways are essential for antiviral defense, they must be tightly regulated to avoid inappropriate activation by endogenous nucleic acids. One essential mechanism that prevents the chronic activation of the cGAS-STING pathway is the degradation of endogenous DNA by the DNase Trex1[14]. In humans, loss of function mutations in Trex1 result in severe autoimmune and autoinflammatory diseases including Aicardi-Goutieres syndrome (AGS)[17], systemic lupus erythematosus (SLE)[21], and familial chilblain lupus (FCL)[22,23]. The autoimmune pathology observed in human patients with loss of function mutations in Trex1 is recapitulated in *Trex1*$^{-/-}$ mice, which have elevated IFN production, chronic inflammation in multiple tissues, and die of lethal inflammatory myocarditis[14,24,25].

Genetic crosses have broadly elucidated how autoimmune disease progresses in *Trex1*$^{-/-}$ mice. Inflammation and mortality are dependent on the cGAS-STING pathway, as crossing *Trex1*$^{-/-}$ mice onto an *Ifnar1*$^{-/-}$, *Irf3*$^{-/-}$, *Sting*$^{-/-}$, or *Cgas*$^{-/-}$ background abrogates tissue inflammation and mortality[14,16,25]. Furthermore, crossing *Trex1*$^{-/-}$ mice onto a *Tcra*$^{-/-}$ or *Ighm*$^{-/-}$ background, which lack functional T and B cells respectively, rescues mortality[25]. These genetic crosses have highlighted a model for autoimmune progression in *Trex1*$^{-/-}$ mice where chronic activation of the cGAS/STING pathway by endogenous DNA generates autoreactive B and T cells responsible for mortality.

While genetic crosses have revealed the pathological consequences of chronic cGAS activation by endogenous DNA, the identity of the DNA substrates degraded by Trex1 and the requirements for their degradation remain unclear. Trex1 contains a C terminal domain essential for its localization on the endoplasmic reticulum and a cytoplasmic DNase domain [14,26]. Trex1 degrades cytosolic DNA with accessible, single-stranded overhangs, preventing the accumulation of cytosolic DNA and the chronic activation of cGAS[27]. Multiple sources of endogenous DNA have been proposed as Trex1 substrates including DNA replication intermediates, damaged DNA, and DNA derived from endogenous retroelement replication. How these substrates would enter the cytosol and become accessible to Trex1 remains unclear.

In order to identify novel regulators of type I IFN responses and Trex1 function, we performed a yeast-2-hybrid screen to identify Trex1-interacting proteins. In this screen we identified 6 unique proteins that interact with Trex1: Bromodomain Containing 7 (BRD7), Basic Transcription Factor 3 like-4 (BTF3L4), Chromatin Assembly Factor 1, subunit a (CAF1A), Replication Factor C, subunit 2 (RFC2), Protein Inhibitor of Activated STAT (PIAS1), and SUMO Activating Enzyme 2 (SAE2). Several of these proteins co-immunoprecipiate with Trex1, confirming their interaction in mammalian cells. To our surprise, we found that each of the six Trex1-interacting proteins localize to the nucleus, raising the question of how and when these nuclear proteins colocalize with Trex1 on the endoplasmic reticulum[28]. Several of the proteins identified in this screen play critical roles in DNA replication. Both RFC2 and CHAF1A associate with PCNA, an essential processivity factor for DNA replication. RFC2 loads PCNA onto replicating DNA and CHAF1A deposits histones onto the DNA afterwards[29,30]. Two of the six proteins identified in this screen, SAE2 and PIAS1, are small ubiquitin like modifiers (SUMO) ligases that post-translationally conjugate SUMO onto target proteins. SUMO is an important regulator of chromatin structure and is important for the coordination of DNA replication[31]. Less is known about the functional roles of BRD7 and BTF3L4, though BRD7 does bind to acetylated histones[32]. In addition to their established roles proteins important in DNA replication and repair, some of the yeast-2-hybrid proteins are important regulators of antiviral responses. *Pias1*$^{-/-}$ mice are hyperresponsive to interferon signaling, and this occurs through modification of both STAT1[33] and IRF3[34]. Additionally, *Brd7*$^{-/-}$ mice are hyperresponsive to inflammatory stimuli[35].

Based on these observations we propose that Trex1-interacting proteins may play important roles in the regulation of endogenous nucleic acid detection.

Results

Loss of SAE2 results in a spontaneous type I interferon response

There are two main ways in which Trex1-interacting proteins could be involved in the regulation of nucleic acid sensing. First, these proteins may directly regulate Trex1 or affect its ability to associate with and degrade DNA substrates. If this hypothesis is true, we would expect a cGAS-dependent IFN response to occur in the absence of these proteins, phenocopying *Trex1*$^{-/-}$ cells. A second possibility is that these proteins may govern biological processes, such as DNA replication and repair, that generate Trex1 substrates. In this case, loss of these proteins may not result in an IFN response, as Trex1 would still be functional and able to degrade endogenous DNA.

To determine whether any of the Trex1-interacting proteins are negative regulators of type I IFN production, we used a lentiCRISPR approach to target each unique protein. We transduced THP-1 cells, a human monocytic cell line, with lentiviral constructs encoding the endonuclease Cas9 and a guide RNA (gRNA) specific to each gene. As controls, we transduced THP-1s with constructs encoding a non-targeting gRNA and a gRNA specific to *TREX1*. When possible, successful targeting was verified by a restriction fragment length polymorphism assay (RFLP). Each gRNA target site overlapped with a restriction enzyme digest site, allowing us to validate the extent of targeting using a using a restriction digest assay that evaluates disruption of the CRISPR-targeted sequence (Fig. 1.1A). For *BTF3L4*, where this was not feasible, we evaluated gene targeting by Sanger sequencing (Fig. 1.1B) After differentiation of THP-1 cells with PMA, we used quantitative RT-PCR to evaluate expression of *IFNB1* and the interferon stimulated genes (ISGs) *IFI27* and *ISG15* (Fig. 1.1C). Disruption of *TREX1* resulted in a spontaneous IFN response, as expected. In addition, lentiCRISPR targeting of one of the proteins identified in the yeast-2-hybrid screen, the SUMO ligase SAE2, resulted in a spontaneous type I IFN response.

Targeting of RFC2 disrupts cell cycle progression

One prevailing hypothesis for the identify of Trex1 substrates is that Trex1 degrades replication intermediates and damaged DNA arising from normal DNA replication and repair processes. To test whether any of the Trex1-interacting proteins are essential regulators of cell cycle progression or DNA repair, we assessed cell cycle state and looked for markers of DNA damage in lentiCRISPR-targeted cells. Targeted cells were stained with DAPI and an antibody specific to gamma H2A.X to determine cell cycle state and to quantify the percent of cells with double-stranded DNA breaks. (Fig. 1.2). In contrast to previous data, the cell cycle distribution of *TREX1*-targeted cells was identical to the non-targeting control. We did however observe a slight, but statistically insignificant increase in the percentage of gamma-H2A.X positive cells. Of the Trex1-interacting proteins, only *RFC2*-targeted cells showed significant differences in cell cycle state, which arose predominantly due to an increase of cells in the S phase. In addition, an increased percentage of *RFC2*-targeted cells were gamma-H2AX positive compared to the non-targeting control. Thus, RFC2 is an important regulator of cell cycle progression and prevents DNA damage.

Discussion

We had hypothesized that the Trex1-interacting proteins identified in the yeast-2-hybrid screen were important regulators of type I IFN production, either through the direct regulation of Trex1 or through their involvement in the generation of endogenous DNA substrates. Using lentiCRISPR-targeting we have identified SAE2, a Trex1-interacting protein and SUMO ligase, as an important negative regulator of type I IFN production. Sumoylation is a post-translational modification similar to ubiquitination and can affect protein function in a variety of different ways. Sumoylation of multiple proteins essential for IFN signaling has been observed, however the functional consequences of sumoylation vary based on the identity of the sumoylated protein. To our surprise, we did not observe a spontaneous IFN response in cells targeted for PIAS1, a SUMO ligase previously characterized as a negative regulator of IFNAR signaling[33,34]. One possible explanation for this discrepancy is that PIAS1 may be an important negative regulator of IFNAR signaling but is not essential for preventing a spontaneous IFN response in the absence of stimulation.

Given the strong association between the Trex1-interacting proteins and DNA replication and repair processes, a putative source of endogenous DNA, we investigated whether the loss of Trex1-interacting proteins would result in disruption of cell cycle progression and DNA damage. SAE2 was the only protein identified in the yeast-2-hybrid screen that prevented a spontaneous IFN response, but we did not observe any alterations in cell cycle state or markers of DNA damage. In contrast to published data, we did not observe differences in cell cycle state or DNA damage in Trex1-targeted cells compared to the non-targeting control[36]. These differences could be due to cell type or species-specific differences, but a more robust assessment of cell cycle markers would be informative. Of the Trex1-interacting proteins, only *RFC2*-targeted had significant alterations in cell cycle progression and DNA damage compared to the non-targeting control. An increased percentage of *RFC2*-targeted cells were in the S phase of the cell cycle and these cells also had increased DNA damage as assessed by gamma-H2A.X staining. The lack of an IFN response in these cells is informative, as it demonstrates that a breakdown in the cell cycle and DNA repair is insufficient to trigger a type I IFN response. This may be due to the presence of functional Trex1, which could degrade any additional DNA ligands caused by disruption of these processes. It would be interesting to determine whether the IFN response in *TREX1*-targeted cells would increase if they were also deficient in RFC2, due to an additional increase in endogenous DNA ligands. However, from these data we conclude that SAE2 is an important negative regulator of type I IFN responses, and RFC2 is an essential regulator of cell cycle progression.

Figures

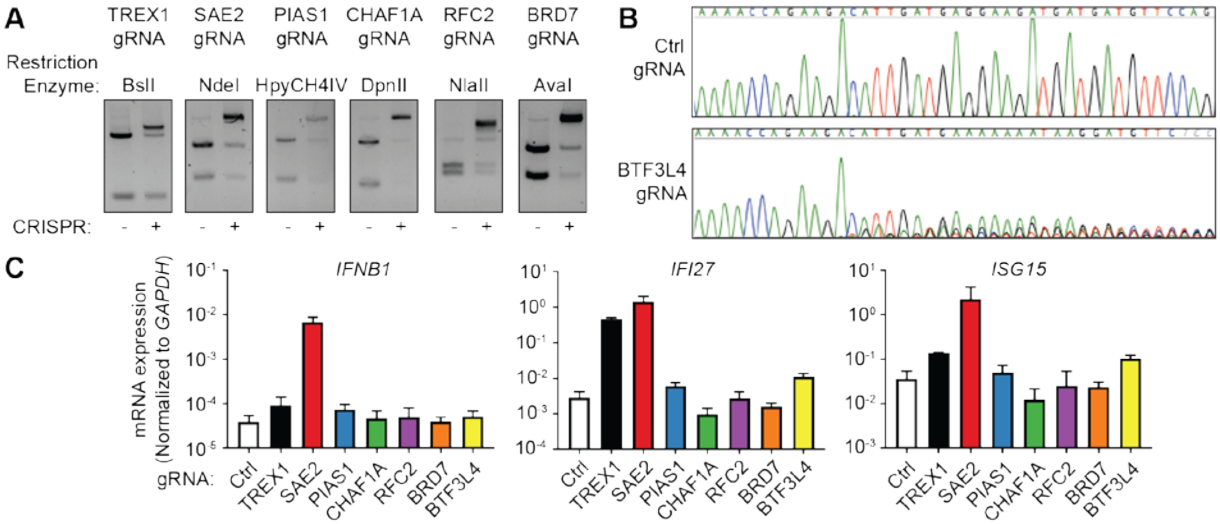


Figure 2.1: Loss of SAE2 induces a spontaneous type I IFN response. THP-1 monocytes were transduced with a lentiCRISPR lentivirus encoding Cas9 and the indicated guide RNAs. (A) Restriction fragment length polymorphism (RFLP) assay assessing lenti-CRISPR targeting of SAE2, PIAS1, PIAS2, PIAS3, and PIAS4. (B) Sanger sequencing of *BTF3L4* exon 4 targeted with the indicated guide RNAs. (C) Evaluation of IFNB1, IFI27, and ISG15 mRNA expression in PMA-differentiated THP-1 cells by quantitative RT-PCR.

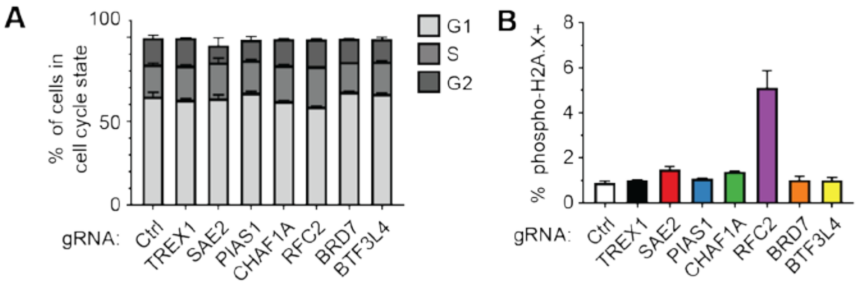


Figure 2.2: Loss of RFC2 increases phospho-H2A.X. THP-1 monocytes were transduced with a lentiCRISPR lentivirus encoding Cas9 and the indicated guide RNAs. Targeted THP-1 cells were stained and analyzed by flow cytometry for (A) DAPI and assessed for cell cycle state and (B) phospho-H2A.X to assess DNA damage.

Chapter 3: Sumoylation prevents a spontaneous type I IFN response

Introduction

In a yeast 2-hybrid screen for proteins that interact with Trex1, we identified the small ubiquitin like modifier (SUMO) ligase SAE2. SAE2, together with its partner SAE1, forms the only human E1 SUMO ligase. Conjugation of SUMO to target proteins proceeds in a manner similar to ubiquitination. The E1 ligase first becomes charged with the SUMO protein via a thioester bond between the c-terminal glycine of SUMO and a catalytic cysteine reside of the E1 ligase. Next, SUMO is transferred to the sole E2 enzyme, UBC9. Finally, in concert with one of several E3 SUMO ligases, SUMO is conjugated by an isopeptide bond to a lysine residue on a specific target protein[37]. Unlike ubiquitination, where E3 ligases are essential for ubiquitin conjugation, SUMO E3 ligases are not required for the conjugation of SUMO onto target substrates[38]. Rather, SUMO E3 ligases facilitate the interaction between the SUMO E2 ligase and a particular substrate in vivo and enhance its sumoylation.

How might SUMOylation prevent a spontaneous IFN response? Like ubiquitination, conjugation of proteins with SUMO can modify their functions in diverse ways. However, the functional consequences of SUMOylation are less understood than those of ubiquitination. This is in part because there are three SUMO genes that encode three SUMO proteins. SUMO2 and SUMO3 are 97% identical to each other, whereas SUMO1 is only 50% identical to SUMO2/3. However, *Sumo2*$^{-/-}$ mice are embryonic lethal at 10.5 days of gestation, whereas *Sumo1*$^{-/-}$ mice and *Sumo3*$^{-/-}$ mice are viable. At the mRNA level, SUMO2 is the most abundant, accounting for ~80% of total SUMO mRNA in both embryonic and adult murine tissues[39]. An individual SUMOylation site in a specific target protein can be modified by more than one SUMO protein [40], suggesting redundancy of SUMO conjugation that complicates efforts to assign specific functions to each SUMO protein.

One important functional distinction between SUMO1 and SUMO2/3 is the presence of a canonical sumoylation motif on SUMO2/3 at lysine 11, which allows for polymeric chain formation[41]. These chains can enhance interactions with SUMO-interacting motifs and are essential for the recruitment of SUMO targeted ubiquitin ligases (STUbLs), which target proteins for proteasomal degradation through the conjugation of polymeric ubiquitin chains[42]. Until recently, lysine 11 was considered the only site where SUMOs could be conjugated to each other. The conjugation of SUMO2/3 elongates the chain, and the conjugation of SUMO1, which lacks the canonical sumoylation site, would effectively terminate it. A recent meta-analysis of SUMO proteomic studies revealed additional non-canonical sumoylation sites on SUMO1 and SUMO2/3, which allow for increased complexity and the formation of branched chains[43]. No functional role has yet been ascribed to these branched chains, and it remains possible that they are incidental or nonfunctional events. We therefore set out to identify the unique contributions of the three vertebrate SUMOs and their ligases in the regulation of type I IFN production.

Results:

Loss of SUMO ligases triggers a spontaneous type I IFN response

We have observed that loss of SAE2, an essential SUMO ligase, results in a spontaneous IFN response and therefore hypothesize that sumoylation broadly prevents IFN production. To determine whether sumoylation limits type I IFN responses, we employed a lentiCRISPR approach to target the human genes that encode the E1 and E2 SUMO ligases: *SAE1, UBA2* (SAE2), and *UBE2I* (UBC9). We transduced THP-1 cells, a human monocytic line, and mouse embryonic fibroblasts (MEFs) with lentiviral constructs encoding the endonuclease Cas9 and a guide RNA (gRNA) specific to each gene. As controls, we transduced THP-1s with constructs encoding a non-targeting gRNA and a gRNA specific to *TREX1*, a known regulator of the cytosolic DNA sensing pathway. Loss of each SUMO ligase was verified by western blot (Fig. 3.1A and C). Interestingly, we found that disruption of *SAE1* resulted in decreased expression of SAE2 protein and vice versa, demonstrating that each component of the E1 SUMO ligase is essential for the stability of its partner. After differentiation of THP-1 cells with PMA, we used quantitative RT-PCR to evaluate expression of *IFNB1* and the interferon stimulated genes (ISGs) *IFI27* and *ISG15* (Fig. 3.1B). Disruption of *SAE1*, *UBA2* (SAE2), or *UBE2I* (UBC9) resulted in potently elevated expression of *IFNB1*, *IFI27*, and *ISG15* mRNA compared to the non-targeting control. To our surprise, we found that SUMO ligase targeted cells had dramatically elevated *IFNB1* expression in comparison to *TREX1*-targeted cells. Therefore, disruption of SUMO ligases in THP-1 cells drives an aberrant and potent type I IFN response in the absence of exogenous stimulation. We also observed an increase in type I IFN responses in sumoylation-deficient MEFs, though the magnitude of this response was less that what we observed in THP-1 cells (Fig. 3.1D). These data corroborate the recent report of enhanced IFN and ISG responses caused by inducible deletion of *Ube2i* (UBC9) in mouse cells[44] and reveal that each of the essential components of the sumoylation pathway are required to prevent a spontaneous IFN response.

The catalytic activity of SAE2 is required for the negative regulation of type I IFN

To confirm that the elevated IFN response in SUMO ligase deficient cells is due to loss of sumoylation and not due to an unanticipated function of these enzymes, we tested whether the catalytic activity of SAE2 was required to prevent the IFN response. SAE2 possesses a cysteine residue at position 173 that is essential for the formation of a thioester bond between SAE2 and SUMO, and the subsequent transfer of SUMO via an isopeptide bond to a lysine residue on a target protein[38]. We generated constructs encoding N-terminus HA tagged wild-type or C173S SAE2, rendered CRISPR resistant by silent mutations in the *UBA2* (SAE2) gRNA targeting site. We transduced THP-1 cells with lentiviral constructs encoding a GFP control, wild-type SAE2 (WT), or C173S SAE2, and then subsequently transduced these cells with lentiCRISPR constructs encoding Cas9 and either a control or SAE2-targeting gRNA. Successful reconstitution and subsequent targeting after puromycin selection was verified by western blot (Fig. 3.2A). Expression of wild-type SAE2, but not GFP or the C173S catalytic mutant, prevented the expression of *IFNB1* (Fig. 3.2B) and the ISGs *IFI27* and *ISG15* (Fig. 3.2C) in SAE2-targeted cells. Thus, the catalytic activity of SAE2 is essential for the regulation of type I IFN by sumo ligases.

Individual loss of PIAS proteins does not trigger a spontaneous IFN response

The E1 and E2 SUMO ligase are essential for conjugation of SUMO onto target proteins, but they frequently act in concert with one of several E3 SUMO ligases. However, and unlike the E1 and E2 ligases, the E3 ligases are not essential for SUMO conjugation onto all substrates but are instead thought to enhance sumoylation of specific substrates[38]. Previous research indicates that the E3 ligase protein inhibitor of activated STAT1 (PIAS1) dampens inflammatory and IFN responses to exogenous ligands through regulation of both IRF3 and STAT1[34,45].To determine whether PIAS1 or other well characterized E3 SUMO ligases in the PIAS family prevent a spontaneous IFN response, we used lentiCRISPR to disrupt the genes encoding PIAS1, PIAS2, PIAS3, and PIAS4. THP-1 cells were transduced with lentiviral constructs encoding Cas9 and a gRNA specific to each *PIAS* gene. Each gRNA target site overlapped with a restriction enzyme digest site, allowing us to validate the extent of targeting using a using a restriction digest assay that evaluates disruption of the CRISPR-targeted sequence (Fig. 3.3A). We found that disruption of each PIAS gene did not result in increased expression of *IFNB1, IFI27*, or *ISG15* compared to the non-targeting control (Fig. 3.3B), indicating that no individual PIAS gene is essential for the regulation of IFN by sumoylation.

Individual loss of STUbLs does not result in a spontaneous IFN response

Sumoylation can target proteins for proteasomal degradation through the recruitment of SUMO-targeted ubiquitin ligases (STUbLs). Multiple components of IFN signaling pathways are degraded by the proteasome after their activation, and loss of function mutations in proteasome subunits cause proteasome-associated autoinflammatory syndrome (PRAAS), which is associated with a chronic IFN signature[20,46]. To determine whether SUMO-targeted ubiquitin ligases could link the type I IFN response we observe in sumoylation-deficient cells to the proteasome, we used lentiCRISPR constructs to target the two known vertebrate STUbLs, RNF4 and RNF111[42,47]. Successful targeting was verified using a restriction fragment length polymorphism (RFLP) assay as described in Figure 1.1A (Fig. 3.4A). In both RNF4 and RNF111-targeted THP-1 cells we observed no increase in *IFNB1*, *IFI27*, or *ISG15* expression compared to the non-targeting control (Fig. 3.4B). While this does not rule out a role for the proteasome in the spontaneous IFN response caused by loss of sumoylation, it does indicate that neither of the two known STUbLs are individually important for this process.

SUMO2 and SUMO3 redundantly inhibit a spontaneous IFN response

The potent IFN induction caused by loss of SUMOylation provided an opportunity to test whether a particular SUMO protein was required for preventing this antiviral response. To determine which SUMOs are responsible for regulating IFN, we used lentiCRISPR to target each of the three SUMO genes, alone or in pairwise combinations. We confirmed targeting of SUMO proteins by western blot (Fig 3.5A) and evaluated gene targeting by Sanger sequencing (data not shown). To our surprise, deletion of any individual *SUMO* gene did not result in a spontaneous IFN response that recapitulated the effect of SAE2 targeting. However, the combined disruption of *SUMO2* and *SUMO3* yielded an IFN response that was even more potent than what we observed in SAE2-targeted cells (Fig. 3.5B). We noted a small increase in ISGs in cells doubly targeted for *SUMO1* and *SUMO2*, but the magnitude of this response was much less than what we observed in either *UBA2* (SAE2) or *SUMO2/3*-targeted cells. Our findings demonstrate a novel and redundant function for SUMO2 and SUMO3, but not SUMO1, as potent negative regulators of the type I IFN response.

Polymeric SUMO chains are not required for the negative regulation of type I IFN

SUMO2 and SUMO3 each contain a “canonical” sumoylation motif at lysine 11 to which additional SUMOs can be conjugated, resulting in the formation of poly-SUMO chains. Additionally, recent proteomics studies have revealed additional SUMO conjugation sites on four other lysines: 5, 7, 21, and 33[43]. Polymeric SUMO chains can enhance interactions with proteins containing SUMO-interacting motifs (SIMs), and they are known to play an important role in the recruitment of SUMO-targeted Ubiquitin ligases (STUbLs), which mediate the polyubiquitination of sumoylated proteins and link sumoylation to proteasomal degradation[42]. To further explore how SUMO2/3 regulates type I IFN responses, and to test whether polymeric SUMO chains are required for this regulation, we generated lentiviral expression vectors encoding N terminus HA tagged versions of wild-type SUMO2 (WT), an unconjugatable SUMO2 with a mutated diglycine motif (GG-AA), SUMO2 in which the canonical polysumoylation site possessed a conservative mutation (K11R), and SUMO2 in which all five known conjugatable lysines were mutated to arginines (5KR). Silent mutations were introduced into the gRNA target site of each SUMO2 expression vector to allow for CRISPR targeting of the endogenous gene only. THP-1 cells were transduced with lentiviral constructs encoding these SUMO2 proteins and then subsequently transduced with lentiCRISPR constructs targeting SUMO3 and SUMO2. Successful preconstitution and targeting were verified by western blot (Fig. 3.6A). As expected based on our previous findings with the SAE2 catalytic mutant (Fig. 3.2), expression of GFP or the GG-AA mutant failed to rescue the Type I IFN response in SUMO2/3-targeted cells. However, preconstitution of SUMO2/3-targeted cells with WT SUMO2 or either of the polysumoylation-deficient SUMO2 mutants dramatically reduced the expression of *IFNB1*, *IFI27*, and *ISG15* (Fig. 3.6B). These data corroborate the redundant functions of SUMO2/3 in IFN regulation and confirm that SUMO conjugation is essential for this regulation. Moreover, our findings implicate monosumoylation, not polysumoylation, as the key mechanism that regulates the IFN response.

SUMO2 and SUMO3 are required for proper regulation of the cell cycle

While performing these experiments, we observed that *SUMO2/3*-targeted cells appeared to grow slower and have increased cell death compared to the non-targeted cell lines, suggesting that SUMO2 and SUMO3 are essential for cell replication and survival. One possible explanation for the enhanced IFN response in SUMO2/3 deficient cells is that dysregulation of DNA replication and repair processes generates IFN-stimulatory DNA. In order to determine whether the cell cycle is altered in SUMO2/3-targeted cells, we stained each of the SUMO-targeted cell lines with a live/dead marker and used DAPI to distinguish cell cycle state. SUMO-targeted cells were analyzed by flow cytometry, and the distribution of live cells in G1, S, and G2 phase was quantified. To our surprise, in the SUMO2/3-targeted cells we observed a large increase in the percent of cells with a nuclear content greater than 4N, indicating that some of the SUMO2/3-targeted cells had failed to divide and had become polyploid (Fig. 3.7A). The slower cell growth we had observed can therefore likely be explained by a failure of these cells to divide after replication of their DNA. We next determined whether the negative regulation of type I IFN and cell cycle disruption could be separated through reconstitution with the polymeric chain deficient SUMO mutants. As expected, all non-targeted cells had a similar cell cycle profile regardless of which SUMO mutant they were reconstituted with, as did SUMO2/3-targeted cells reconstituted with wild-type SUMO2. An increased percent of the SUMO2/3-targeted cells reconstituted with GFP and the unconjugatable GG-AA mutant had become polyploid, as we would expect given our earlier findings. However, reconstitution with the two polymeric chain-deficient mutants had different effects on the cells, despite their identical capacity to prevent type I IFN production. Reconstitution with the K11 mutant rescued the polyploid phenotype, while reconstitution with the 5KR mutant was unable to do so (Fig. 3.7B). These data demonstrate that one or more non-canonical lysines play an important role in the regulation of cell division after DNA replication. We can therefore separate negative regulation of type I IFN production and regulation of cell division as non-canonical lysines are uniquely required for the regulation of cell division.

Discussion

We have demonstrated that sumoylation is an essential negative regulator of type I IFN production. The Dejean group recently demonstrated that *Ube2i*$^{-/-}$ (UBC9) cells are hyperresponsive to inflammatory ligands, and this is dependent on signaling through IFNAR[44]. Our work corroborates and extends these findings. We demonstrate that loss of any of the three proteins comprising the E1 and E2 SUMO ligases triggers a spontaneous type I IFN response. Furthermore, we show that the catalytic activity of these enzymes is essential to prevent spontaneous type I IFN production.

Sumoylation can proceed in the absence of SUMO E3 ligases in vitro, but E3 ligases are able to enhance sumoylation of specific substrates in vivo. Previous studies have identified the PIAS family of E3 ligases as important regulators of IFN signaling, and we had identified PIAS1 as a Trex1-interacting protein[34,48]. Based on these observations, we decided to investigate whether any PIAS ligates prevents the activation of type I IFN. However, targeting of each individual PIAS ligase did not result in a spontaneous IFN response. Given that SUMO E3 ligases are not required for conjugation of SUMO in vitro, it is possible that the regulation of IFN by sumoylation does not depend on an E3 ligase. Alternatively, several other E3 ligases have been identified and may mediate this function, though a role in the regulation of type I IFN responses has not been previously established. We therefore conclude that no individual PIAS protein is essential for IFN regulation by sumoylation, but we cannot rule out the possibility that other E3 ligases are essential for this function.

Human patients with loss of function mutations in proteasome subunits develop the autoinflammatory disease proteasome-associated autoinflammatory syndrome (PRAAS), which is associated with a chronic type I IFN response[46]. While sumoylation is not directly involved in proteasomal degradation, polymeric SUMO chains can recruit a class of ubiquitin ligases called SUMO-targeted Ubiquitin Ligases (STUbLs) that conjugate polymeric ubiquitin chains onto proteins and target them for proteasomal degradation. Two STUbLs have been identified in vertebrates: RNF4 and RNF111[42,47]. We observed that the loss of either of these STUbLs did not result in a spontaneous IFN response, and we therefore conclude that regulation of type I IFN by sumoylation does not depend on the proteasomal degradation mediated by STUbLs.

We initially began to study sumoylation because we uncovered SAE2 as a specific Trex1-interacting protein in a yeast 2-hybrid screen. This led us to hypothesize that sumoylation might influence the ability of Trex1 to regulate the cGAS-STING pathway of DNA sensing. Importantly, the DeJean group recently reported that mouse dendritic cells with conditional deletion of the *Ube2i* gene that encodes the UBC9 E2 SUMO ligase had increased basal IFN responses and dramatically enhanced inflammatory responses to innate immune stimuli [44]. They identified an enhancer region near the mouse *Ifnb1* locus that is bound by a SUMO1-conjugated protein under basal conditions. Upon LPS stimulation, SUMO1 disappears from this enhancer, leading to transcription of an enhancer-derived RNA that may influence *Ifnb1* transcription. While our data corroborate the role of sumoylation in suppressing the IFN response, we identify SUMO2/3, not SUMO1, as the essential SUMOs for IFN regulation. We therefore suggest that while this enhancer RNA may play an important role in influencing *Ifnb1* transcription, its potential repression by SUMO1 is insufficient to explain the elevated IFN response we observe in sumoylation-deficient cells. Importantly, our understanding of specific functions for individual SUMO proteins is not well developed, in large part because of the three distinct SUMO proteins and the fact that sumoylation sites in target proteins can be conjugated by multiple SUMO proteins. Our data reveal an essential function mediated by SUMO2 and SUMO3, but not by SUMO1, which will enable more mechanistic studies of these isoform-specific SUMO functions.

SUMO2/3, but not SUMO1, possess a canonical sumoylation motif that allows for the formation of polymeric chains. Since the negative regulation of type I IFN by sumoylation uniquely depends on SUMO2/3, we next investigated whether the formation of polymeric chains was necessary this regulation. Proteomic studies have recently revealed additional, non-canonical lysines on SUMO2/3 where SUMO can be conjugated. We therefore explored potential roles for the canonical and non-canonical lysines in the regulation of type I IFN production by reconstituting *SUMO2/3*-targeted cells with mutants of SUMO2. Mutation of either the canonical lysine individually or all sumoylatable lysines was able to rescue type I IFN production in *SUMO2/3*-targeted cells, demonstrating that polymeric chains are not required for type I IFN regulation by sumoylation.

While culturing *SUMO2/3*-targeted cells, we observed that they grew at a slower rate and appeared to have more cell death than non-targeted cells. Upon quantification of this difference, we were surprised to find that a significant percent of cells had become polyploid with a DNA content of greater than 4N. Because SAE2 was initially identified as a Trex1-interacting protein, we hypothesized that sumoylation was an important regulator of Trex1 and the cGAS-STING pathway. These defects in cell replication and the corresponding increase in cellular DNA suggested a putative increase in Trex1 ligands within the cell that could explain the spontaneous type I IFN response observed in *SUMO2/3*-targeted cells. In an attempt to separate the spontaneous type I IFN response from cell cycle disruption, we quantified the cell cycle state of mutant SUMO2 reconstituted *SUMO2/3*-targeted cells. The cell cycle defects were rescued in *SUMO2/3*-targeted cells reconstituted with the canonical lysine mutant K11R, but not those reconstituted with the 5KR mutant. These data indicate that formation of non-canonical sumo chains is uniquely required for DNA replication and cell division, and that dysregulation of the cell cycle is separable from the spontaneous IFN response we observe in the absence of sumoylation. Furthermore, this is the first observed example of a unique functional role for non-canonical polymeric SUMO chains.

Figures

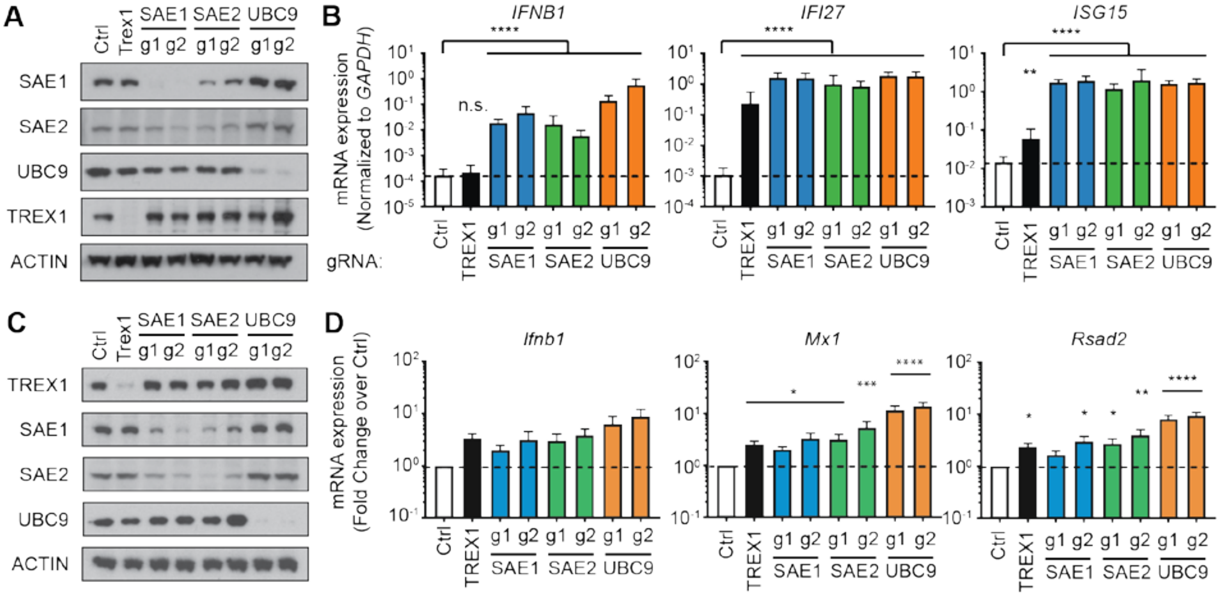


Figure 3.1: Loss of SAE1, SAE2, or UBC9 triggers a spontaneous IFN response in THP-1 cells. THP-1 monocytes (A and B) and mouse embryonic fibroblasts (C and D) were transduced with a lentiCRISPR lentivirus encoding Cas9 and the indicated guide RNAs. Western blot evaluation of SAE1, SAE2, UBC9, TREX1, and ACTIN protein in lentiCRISPR-targeted THP-1 cells (A) and MEFs (C). Evaluation of IFNB1, IFI27, and ISG15 mRNA expression in PMA-differentiated THP-1s (B) and MEFs (D) by quantitative RT-PCR. Statistical analysis was performed using a one-way anova and comparing control cells to SAE1, SAE2, UBC9, and TREX1-targeted cells, correcting for multiple comparisons using the Holm-Sidak method. N = 3-5 where N is the number of unique polyclonal cell lines generated. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$

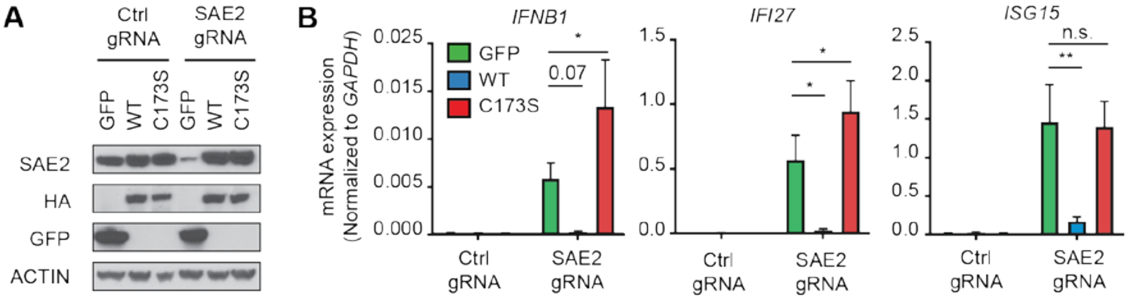


Figure 3.2: Regulation of IFN by SAE2 is dependent on its catalytic activity. THP-1 monocytes were transduced with lentiviruses encoding GFP, wildtype SAE2 (WT), and a catalytic mutant of SAE2 (C173S), and then transduced with a lentiCRISPR lentivirus encoding Cas9 and the indicated guide RNA. (A) Western blot evaluation of HA, GFP, SAE2, and ACTIN protein in the indicated THP-1 cell lines. (B and C) Evaluation of IFNB1, IFI27, and ISG15 mRNA expression in PMA-differentiated THP-1s by quantitative RT-PCR. Statistical analysis was performed using a two-way ANOVA and comparing GFP expressing THP-1s to WT and C173S reconstituted cells, correcting for multiple comparisons using the Holm-Sidak method. N = 4 where N is the number of unique polyclonal cell lines generated. * $P \leq 0.05$, ** $P \leq 0.01$

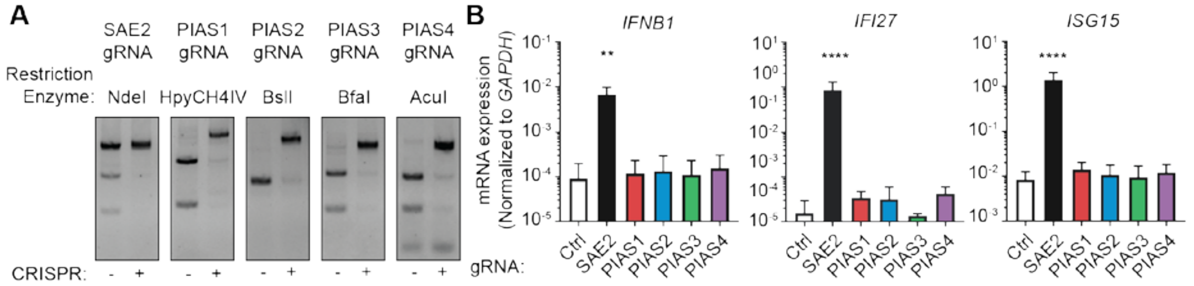


Figure 3.3: Loss of PIAS E3 ligases does not trigger a spontaneous IFN response. THP-1 monocytes were transduced with a lentiCRISPR lentivirus encoding Cas9 and the indicated guide RNAs. (A) Restriction fragment length polymorphism assay evaluation of SAE2, PIAS1, PIAS2, PIAS3, and PIAS4 lentiCRISPR-targeting. (B) Evaluation of IFNB1, IFI27, and ISG15 mRNA expression in PMA-differentiated THP-1 cells by quantitative RT-PCR. Statistical analysis was performed using a one-way ANOVA and comparing control cells to SAE1, SAE2, UBC9, and TREX1-targeted cells, correcting for multiple comparisons using the Holm-Sidak method. N = 3 where N is the number of unique polyclonal cell lines generated. ** P ≤ 0.01, **** P ≤ 0.0001

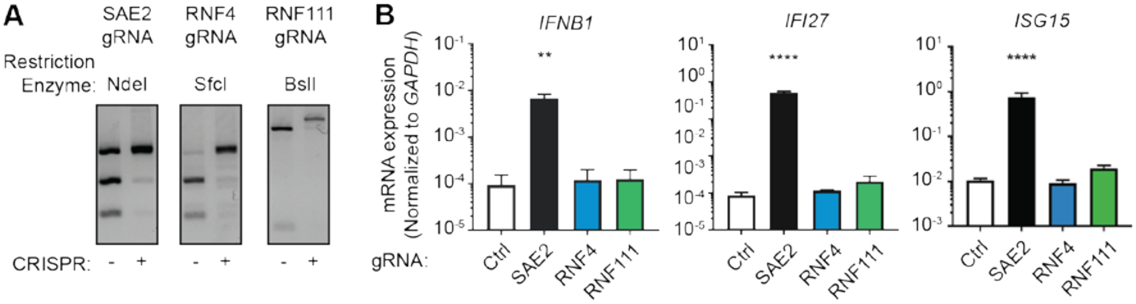


Figure 3.4: Loss of STUbLs does not trigger a spontaneous IFN response. THP-1 monocytes were transduced with lentiCRISPR lentiviruses encoding Cas9 and the indicated guide RNAs. (A) Restriction fragment length polymorphism assay evaluation of SAE2, RNF4, and RNF111 CRISPR-targeting in THP-1 cells. (B) Evaluation of IFNB1, IFI27, and ISG15 mRNA expression in PMA-differentiated THP1s by quantitative RT-PCR. Statistical analysis was performed using a one-way ANOVA and comparing SAE2, RNF4, and RNF111-targeted cells to the non-targeted control, correcting for multiple comparisons using the Holm-Sidak method. N = 3 where N is the number of unique polyclonal cell lines generated. ** P ≤ 0.01, **** P ≤ 0.0001

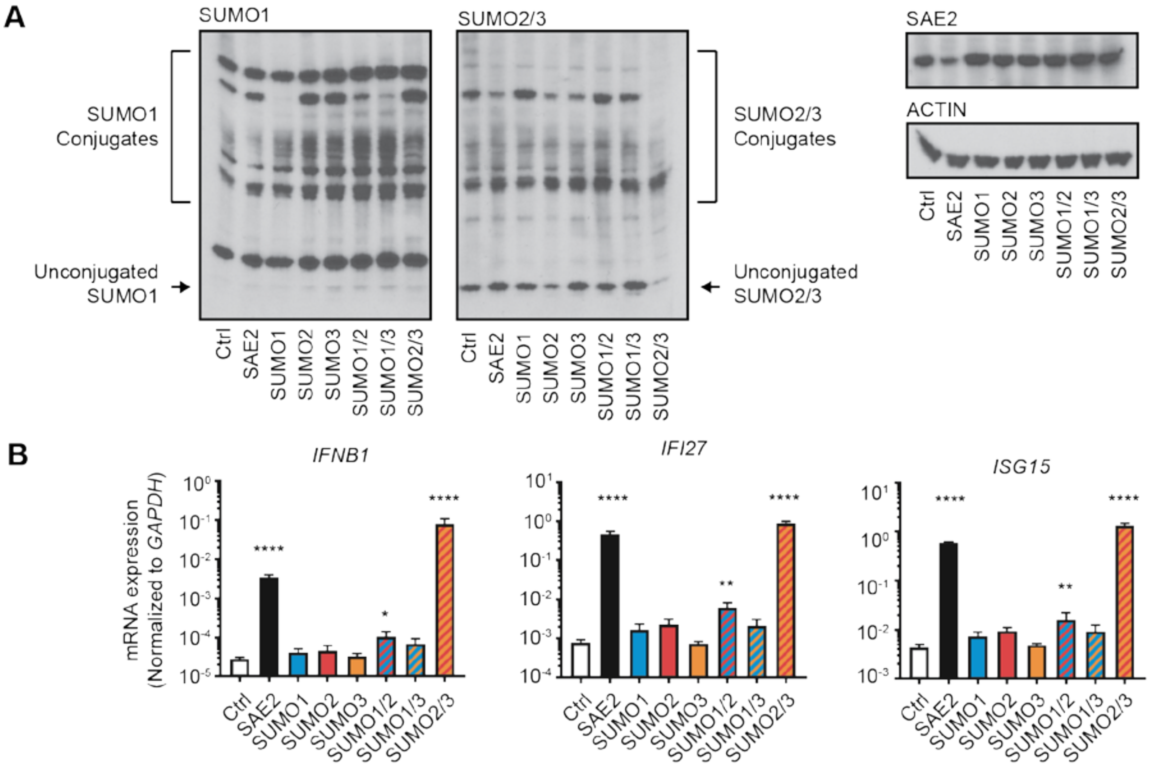


Figure 3.5: The combined loss of SUMO2 and SUMO3 triggers a spontaneous IFN response. THP-1 monocytes were transduced with lentiCRISPR lentiviral constructs encoding Cas9 and the indicated guide RNAs. (A) Western blot evaluation of SUMO1, SUMO2/3, SAE2, and ACTIN protein in lentiCRISPR-targeted THP-1 cells. (B) Evaluation of IFNB1, IFI27, and ISG15 mRNA expression in PMA-differentiated THP-1s by quantitative RT-PCR. Statistical analysis was performed using a one-way ANOVA and comparing all cell lines to the non-targeting control, correcting for multiple comparisons using the Holm-Sidak method. N = 3 where N is the number of unique polyclonal cell lines generated. * $P \leq 0.05$, ** $P \leq 0.01$, **** $P \leq 0.0001$

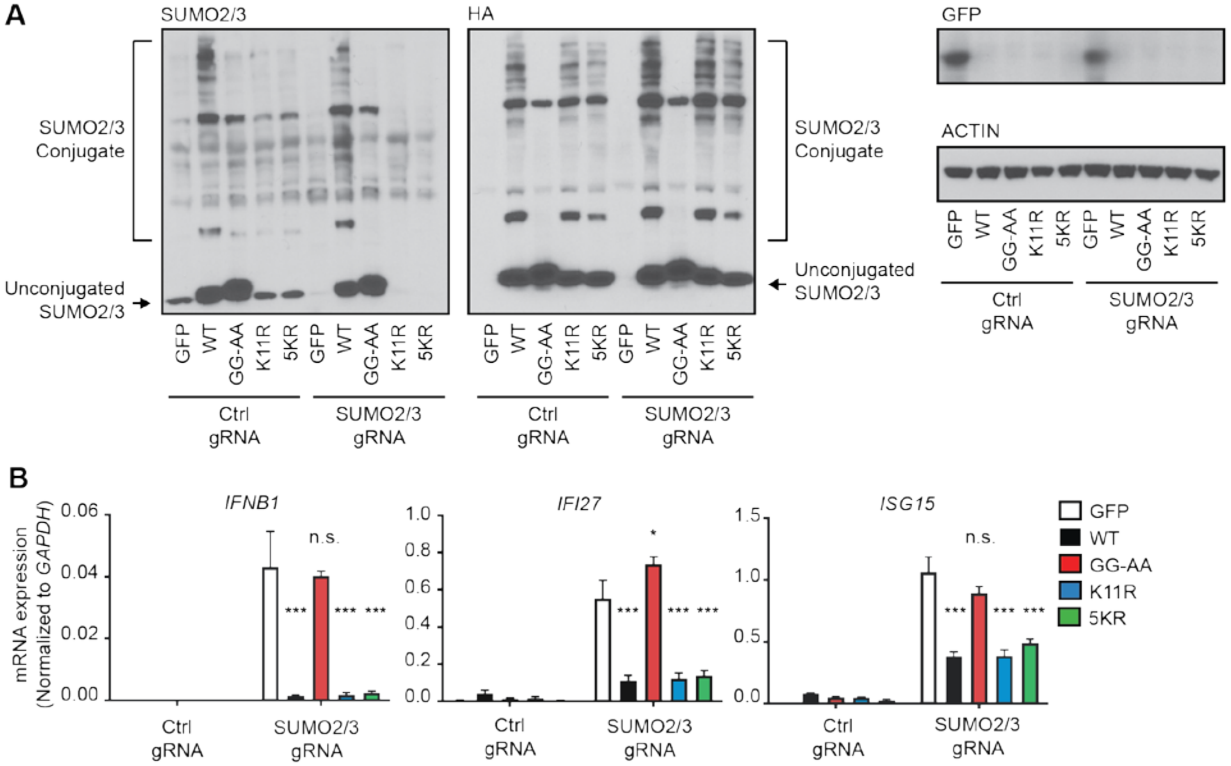


Figure 3.6: The formation of polymeric SUMO2 chains is not essential for the regulation of IFN by sumoylation. THP-1 monocytes were transduced with lentiviral constructs encoding GFP, wild-type SUMO2 (WT), an unconjugatable diglycine mutant (GG-AA), a lysine to arginine SUMO2 mutant at amino acid 11 (K11R), or a SUMO2 mutant in which all conjugatable lysine have been mutated to arginines (5KR). Each cell line was then subsequently transduced with the indicated guide RNAs. (A) Western blot evaluation of SUMO2/3, HA, GFP, and ACTIN protein in the indicated THP-1 cell lines. (B) Evaluation of *IFNB1*, *IFI27*, and *ISG15* mRNA expression by quantitative RT-PCR in PMA-differentiated THP-1 cells. Statistical analysis was performed using a two-way anova and comparing WT, GG-AA, K11R, and 5KR transduced cells to GFP transduced cells, correcting for multiple comparisons using the Holm-Sidak method. N = 3 where N is the number of unique polyclonal cell lines generated. * P ≤ 0.05, *** P ≤ 0.001

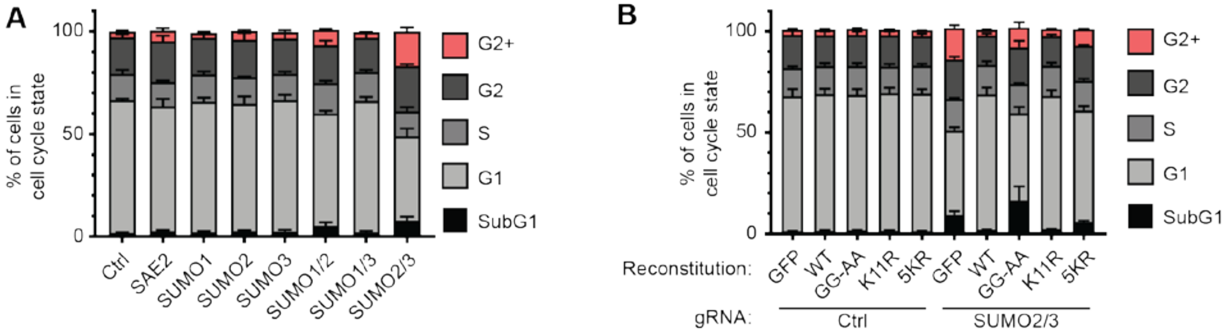


Figure 3.7: SUMO conjugation onto non-canonical lysines uniquely regulates cell cycle progression and cell division.
(A) THP-1 monocytes were transduced with a lentiCRISPR lentivirus encoding Cas9 and the indicated guide RNAs. Cells were stained with a live/dead marker and DAPI and assessed by flow cytometry. Cell cycle state was determined in live cells. (B) THP-1 monocytes were transduced with lentiviral constructs encoding GFP, wild-type SUMO2 (WT), an unconjugatable diglycine mutant (GG-AA), a lysine to arginine SUMO2 mutant at amino acid 11 (K11R), or a SUMO2 mutant in which all conjugatable lysine have been mutated to arginines (5KR). Each cell line was then subsequently transduced with the indicated guide RNAs. Cells were stained with a live/dead marker and DAPI and assessed by flow cytometry. Cell cycle state was determined in live cells.

Chapter 4: Sumoylation prevents a non-canonical type I IFN response

Introduction

There are four principal ways of triggering the IFN response in vertebrates. First, cyclic GMP-AMP synthase (cGAS) detects double-stranded intracellular DNA and signals through the adaptor protein STING to activate the kinase TBK1 and the transcription factor interferon regulatory factor 3 (IRF3), leading to the transcriptional activation of the *IFNB1* gene[12,49,50]. Second, the RIG-I-like receptors (RLRs) detect intracellular viral RNA and bind to the adaptor protein MAVS to trigger a similar TBK1-IRF3 response[51]. Third, toll-like receptors (TLRs) 3 and 4 use the adaptor protein TRIF to activate TBK1-IRF3[10]. Finally, TLRs 7 and 9 signal through MyD88 and IRF7, specifically in plasmacytoid dendritic cells, to activate a potent IFN response[11,52]. Importantly, all of the known IFN-inducing pathways require IRF3 and/or IRF7[11,53], revealing a conserved module that mediates the canonical IFN response[54].

Numerous components of the type I IFN signaling pathways are sumoylated. However, sumoylation of these proteins results in diverse functional outcomes depending on the identity of the sumoylated protein and the context in which sumoylation occurs. We initially identified SAE2 as a Trex1-interacting protein and therefore hypothesized that sumoylation is an important regulation of Trex1 and the cGAS-STING signaling pathway. Sumoylation of both cGAS and STING has been previously observed, however there are conflicting opinions about the functional consequences. For example, one group has shown that sumoylation of cGAS and STING promotes their stability and is essential for a robust response to DNA ligands. After their activation by DNA, desumoylation of cGAS and STING is essential for their degradation and the termination of the IFN response[55]. In this case, the coordinated conjugation and removal of SUMO from target proteins facilitates and regulates IFN production. However, it has also been demonstrated that sumoylation of cGAS prevents its activation by suppressing its DNA binding activity, a role distinct from promoting cGAS stability[56]. The conflicting observations in these two studies may be a result of unique functional consequences of sumoylation depending on the cellular context. Differences may also arise due to cell type specific differences or artifacts of SUMO overexpression. In addition to cGAS and STING, sumoylation has also been observed on numerous other components of IFN signaling pathways including RIG-I, MDA5, IRF3, IRF7, and STAT1, an important mediator of IFNAR signaling[34,48,57-59].

In order to understand the dysregulation underlying the autoinflammatory and autoimmune diseases associated with a type I IFN signature, it is important to understand the full breadth of mechanisms that regulate type I IFN responses. Using a combination of CRISPR-targeting and chemical inhibition, we assess the contributions of these signaling pathways and components to the spontaneous IFN production that occurs in sumoylation-deficient cells. In doing so we reveal the existence of a non-canonical type I IFN response prevented by sumoylation.

Results:

Neither STING nor MAVS is required for the spontaneous IFN response in sumoylation-deficient cells

We next sought to identify the pathway(s) responsible for triggering the IFN response in sumoylation-deficient cells. Since the majority of known mendelian interferonopathies are caused by mutations in regulators of nucleic acid sensing and metabolism, we first tested whether the cGAS-STING or RLR-MAVS pathways were responsible for driving the spontaneous type I IFN response in sumoylation-deficient cells. We generated lentiCRISPR constructs targeting either *TMEM173* (STING) or *MAVS* in a blasticidin resistant vector. These cells were then transduced with non-targeting control, *TREX1*-, or *UBA2*-targeting gRNAs. Successful targeting was verified by western blot (Fig. 4.1A), and functional disruption of STING and MAVS was evaluated by transfection of specific nucleic acid ligands. In response to calf thymus DNA, a cGAS ligand, *TMEM173* (STING)-targeted THP-1 cells had decreased expression of *IFNB1* compared to both non-targeted and *MAVS*-targeted THP-1 cells (Fig. 4.1B). Similarly, *MAVS*-targeted cells had a reduced *IFNB1* response to transfection with a triphosphate RNA ligand that specifically activates RIG-I (Fig. 5B; [60]. As expected, *TREX1*-targeted THP-1 cells showed a decrease in ISG expression in *TMEM173* (STING)-targeted cells, but not in *MAVS*-targeted cells (Fig. 4.1B). However, and to our surprise, neither targeting of STING nor MAVS had any impact on the potent expression of *IFNB1, IFI27,* or *ISG15* in SAE2-targeted THP-1 cells (Fig 4.1C). Our data reveal that loss of sumoylation triggers an IFN response that is independent of the two known intracellular nucleic acid sensing pathways.

IFNAR is not required for the spontaneous IFN response in sumoylation-deficient cells

We investigated whether induction of type I IFNs in sumoylation-deficient cells was due to enhanced signaling through the type I interferon receptor instead of through a primary ligand-activated pathway. We made a lentiCRISPR construct targeting *IFNAR1*, generated a population of robustly targeted cells verified by RFLP (Fig. 4.2A), and then transduced these cells with non-targeting control, *UBA2* (SAE2), or *UBE2I* (UBC9) lentiCRISPR constructs. Functional disruption of *IFNAR1* was evaluated by treatment with recombinant human IFN-β and by transfection of calf thymus DNA (Fig. 4.2B). In response to IFN-β treatment, we observed robust *IFI27* and *ISG15* that was absent in *IFNAR1*-targeted cells. In response to calf-thymus DNA, we observed intact but decreased inducible expression of *IFNB1* in *IFNAR1*-targeted cells, consistent with the known enhancement of IFN production by IFNAR signaling. Importantly, the DNA-activated expression of *IFI27* and *ISG15* was severely impaired in *IFNAR1*-targeted cells (Fig. 4.2C), verifying functional disruption of IFNAR signaling.

TBK1/IKKε/IKKα/IKKβ are not required for the spontaneous IFN response in sumoylation-deficient cells

We next explored the possibility that sumoylation may regulate type I IFN responses through shared signaling components downstream of STING and MAVS or alternative IFN-inducing pathways (e.g. the TLRs). Downstream of both STING and MAVS, the related kinases TBK1 and IKKε phosphorylate and activate the transcription factors IRF3 and IRF7 [61,62]. The Toll-like receptors TLR3 and TLR4 signal through TRIF to activate TBK1-dependent IRF3 phosphorylation [10]. Finally, in plasmacytoid dendritic cells, TLR7/8/9 signal through the adaptor protein MyD88, which recruits the kinase IKKα to phosphorylate and activate IRF7 [63]. Thus, the kinases TBK1/IKKε and IKKα/IKKβ represent the essential signaling nexus required for all canonical IFN-inducing pathways. To test whether these kinases contribute to the IFN response in sumoylation-deficient cells, individually or together, we took advantage of the chemical inhibitors BX795 and TPCA-1, which inhibit TBK1/IKKε and IKKα/IKKβ respectively. Non-targeted and SAE2-targeted, differentiated THP-1 cells were treated for 48 hours with either BX795, TPCA-1, or both of these inhibitors in combination. To verify the efficacy of these inhibitors over the complete course of treatment, non-targeted cells were treated with lipopolysaccharide (LPS) for the final four hours prior to RNA harvest. LPS signals through TLR4 to activate both a type I IFN response dependent on TBK1/IKKε and an inflammatory response dependent on the activation of NF-κB by IKKα/IKKβ. We found that BX795 blocked the IFN response to LPS (Fig 4.3A), whereas TPCA-1 blocked both IFN and the proinflammatory TNF response (Fig 4.3 A and B). However, the IFN response in SAE2-targeted cells was unaffected by either inhibitor, alone or in combination (Fig 4.3A). These data suggest that none of the four TBK1-related kinases are essential for the IFN response caused by loss of sumoylation.

Neither IRF3 nor IRF7 are required for the spontaneous IFN response in sumoylation-deficient cells

We next investigated the role of the canonical IFN-inducing transcription factors IRF3 and IRF7. IRF3 is essential for STING-, MAVS- and TRIF-dependent IFN production in resting cells. However, IRF7 can functionally compensate for IRF3 in cells that have been treated with IFN because IRF7 is a potent ISG. Together, these two transcription factors are essential for all canonical IFN-inducing pathways [11,53]. To test whether these transcription factors are essential for the spontaneous IFN response in sumoylation-deficient THP-1 cells, we generated lentiCRISPR constructs targeting IRF3 and IRF7 in vectors with distinct selection markers. We transduced THP-1 cells with constructs targeting IRF3, IRF7, or both, together with corresponding non-targeting controls. To avoid the complication of incomplete targeting and the increased expression of IRF7 that would result from residual untargeted cells, we derived clonal lines of IRF3-, IRF7- and IRF3/7-targeted cells. Successful targeting was verified by western blot (Fig. 4.4A) and Sanger sequencing of the corresponding targeting site to ensure complete knockout (data not shown). To validate functional disruption of IRF3 and IRF7, we transfected each clonal line with either calf thymus DNA or RIG-I Ligand (Fig. 4.4B) or treated cells with

LPS (Fig. 4.3C). IRF3 KO cells failed to mount an IFN response to DNA, RNA, or LPS, but pretreatment of these cells with recombinant IFN-β restored the IFN response (Fig. 4.4B and C). This restoration after IFN pretreatment was entirely dependent on IRF7, because IRF3/7 double KO cells were completely unable to respond to any of the stimuli, even with IFN-β pre-treatment. Thus, our IRF3/7 double knockout human cells are functional knockouts for the STING-, MAVS-, and TRIF-IFN pathways. Importantly, these data also demonstrate that no other transcription factor in these cells can functionally compensate for the combined loss of IRF3 and IRF7. We did not test MyD88-dependent IFN signaling in these cells because the IFN response activated by TLR7/9 is specifically restricted to plasmacytoid dendritic cells, and THP-1 cells do not make IFN in response to TLR7/9 ligands (Data not shown). We transduced these targeted cells with a non-targeting or SAE2-targeting gRNA evaluated the IFN response. Remarkably, the expression of *IFNB1*, *IFI27*, and *ISG15* caused by loss of SAE2 was completely intact in clonal THP-1 cells lacking IRF3, IRF7, or both (Fig 4.4D). Thus, the potent IFN response caused by loss of sumoylation is independent of all known IFN-inducing pathways, revealing a novel mechanism of IFN regulation.

Discussion

We unexpectedly found that neither the cGAS-STING pathway nor any other known IFN-inducing pathway is responsible for the potent IFN response caused by loss of sumoylation. Moreover, the transcription factors IRF3 and IRF7, which are essential for all known type I IFN responses, are completely dispensable for this ectopic IFN response. How, if not through IRF3 or IRF7, does sumoylation regulate type I IFN production? Two major possibilities remain. One possibility is that alternative signaling pathways or transcription factors are regulated by sumoylation, and in sumoylation-deficient cells they are able to trigger an IFN response. For example, IRF1 was originally identified as a transcription factor that drives expression of IFNβ, though later experiments demonstrated that IFN production by cytosolic nucleic acid sensing pathways and TLRs are unaffected by the loss of IRF1 [53]. However, isolated examples persist in the literature of non-canonical IFN responses driven by IRF1 activation[64-66]. Alternatively, IRF5 has been shown to be important for the IFN response to some RNA viruses[67], and SNPs in IRF5 have been associated with increased susceptibility to SLE [68,69], an autoimmune disease associated with a chronic type I IFN signature. Whether these or other related factors are activated by loss of sumoylation will require further work, but we emphasize that these factors are not able to compensate for combined loss of IRF3 and IRF7 in all known canonical IFN-inducing pathways (Fig. 7). Thus, their contributions to IFN production must be through a currently uncharacterized mechanism. A second possibility is that sumoylation modifies the IFNβ locus itself, potentially altering the chromatin structure, and that increased accessibility or activation of genomic elements such as enhancers allows for the activation of *IFNB* transcription by non-canonical transcription factors. The identification of relevant targets of SUMO2/3 monosumoylation may reveal new components of the IFN response that will help distinguish between these two possibilities.

In summary, we have found that SUMO2 and SUMO3 are specific and essential negative regulators of a non-canonical mechanism of type I IFN induction that cannot be placed within the known IFN-inducing pathways. We propose that further definition of this pathway will provide insights into the protective and pathological functions of type I IFNs.

Figures

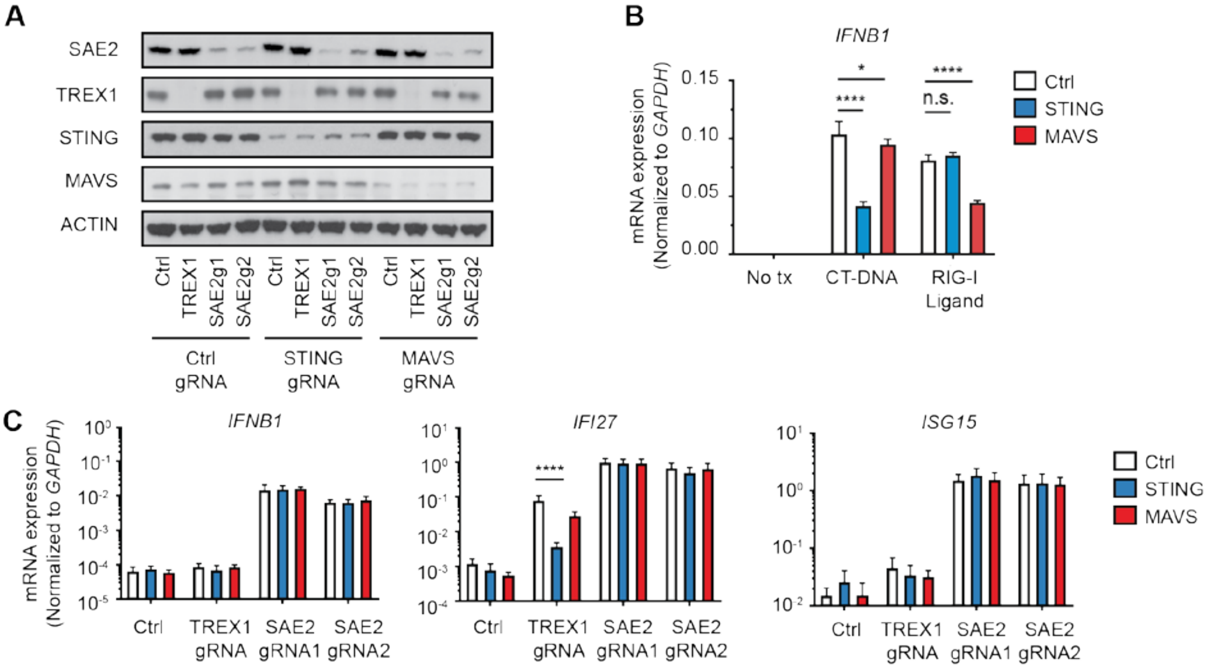


Figure 4.1: The spontaneous IFN response in sumoylation-deficient cells is not dependent on either STING or MAVS. THP-1 monocytes were transduced with lentiCRISPR lentiviral constructs encoding Cas9 and the indicated guide RNAs (A) Western blot evaluation of SAE2, Trex1, STING, MAVS, and ACTIN protein expression (B) PMA-differentiated THP-1 cells targeted with the indicated guide RNAs were transfected with either 1 ug of CT-DNA or 1 ug of RIG-I ligand. 4 hours after transfection, RNA was harvested and expression of *IFNB1*, *IFI27*, and *ISG15* was evaluated quantitative RT-PCR. Statistical analysis was performed using a two-way ANOVA and comparing STING and MAVS-targeted cells to the non-targeted control, correcting for multiple comparisons using the Holm-Sidak method. N =3 where N is the number of transfected cell lines. The data is representative of two experiments. (C) Evaluation of IFNB1, IFI27, and ISG15 mRNA expression by quantitative RT-PCR in PMA-differentiated THP-1 cells. Statistical analysis was performed using a two-way ANOVA and comparing STING and MAVS-targeted cells to the non-targeted control, correcting for multiple comparisons using the Holm-Sidak method. N = 3 where N is the number of unique polyclonal cell lines generated. * P ≤ 0.05, **** P ≤ 0.0001

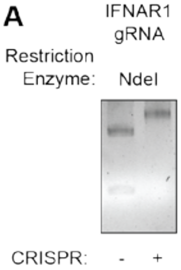
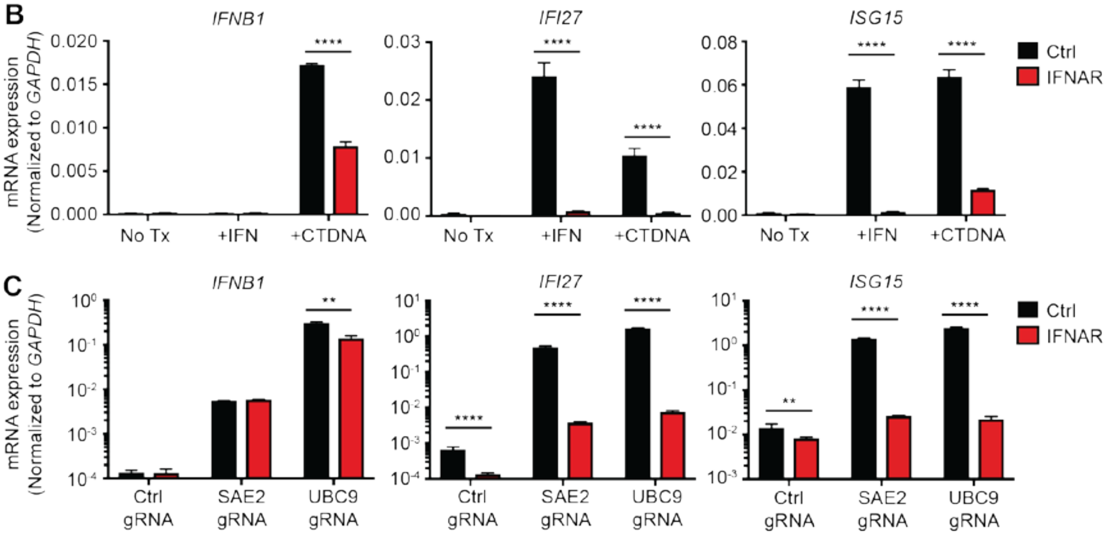


Figure 4.2: The spontaneous IFN response in sumoylation-deficient cells is not dependent on IFNAR. THP-1 monocytes were transduced with lentiCRISPR lentiviruses encoding Cas9 the indicated guide RNAs. Transduced THP-1s were selected with hygromycin B for at least 6 days. (A) Evaluation of *IFNAR1* lentiCRISPR-targeting by restriction fragment length polymorphism assay (B) Evaluation of *IFNB1*, *IFI27*, and *ISG15* mRNA expression in PMA-differentiated THP-1s treated with either 50 U/mL recombinant interferon-β or transfected with 1 ug CT-DNA for 6 hours. (C) Control and *IFNAR1*-targeted THP-1 cells were transduced with lentiCRISPR lentiviruses encoding Cas9 and the indicated guide RNA. Transduced cells were selected with puromycin for at least 3 days. Evaluation of *IFNB1*, *IFI27*, and *ISG15* mRNA expression in PMA-differentiated THP-1s by quantitative RT-PCR. Statistical analysis was performed using a two-way ANOVA and comparing *IFNAR1*-targeted cells to the control line, correcting for multiple comparisons using the Holm-Sideak method. N= 3 where N is the number of unique polyclonal cell lines. * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001, **** P ≤ 0.0001



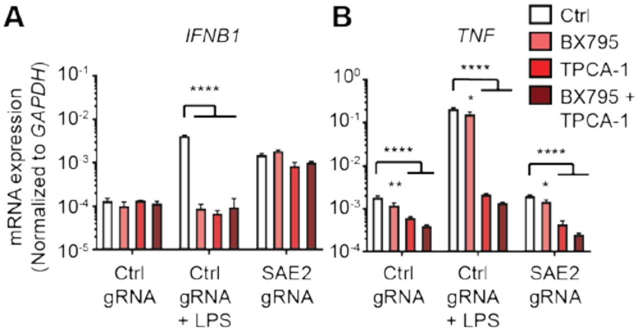


Figure 4.3: Neither TBK1/IKKε nor IKKα/IKKβ are required for the spontaneous IFN response in sumoylation-deficient cells. THP-1 monocytes were transduced with lentiCRISPR lentiviruses encoding Cas9 and the indicated guide RNAs. PMA-differentiated THP-1s were treated with DMSO, 1 uM BX795, 20 uM TPCA-1, or 1 uM BX795 and 20 uM TPCA-1. 4 hours prior to harvest, 1 ug LPS was added to non-targeted THP-1 cells. (A and B) Evaluation of *IFNB1* (A) and *TNF* (B) mRNA expression by quantitative RT-PCR. Statistical analysis was performed using a two-way ANOVA and comparing BX795 and TPCA-1 treated cells to the DMSO treated control, correcting for multiple comparisons using the Holm-Sidak method. N = 3 where N is the number of treated cell lines. Data is representative of 4 separate experiments. * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.0001

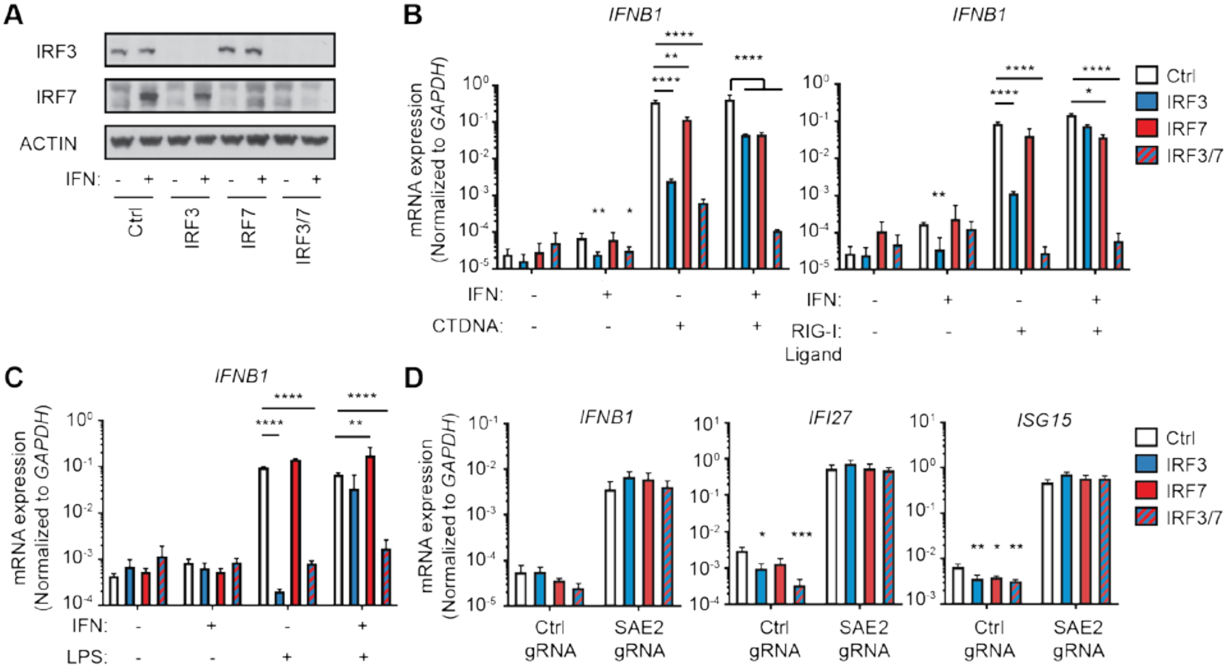


Figure 4.4: The spontaneous IFN response in sumoylation-deficient cells is not dependent on IRF3 or IRF7. THP-1 monocytes were transduced with lentiCRISPR lentiviruses encoding Cas9 the indicated guide RNAs. Transduced THP-1s were selected with both blasticidin and hygromycin B for 6 days and then single cell cloned. (A) Western blot evaluation of IRF3, IRF7, and ACTIN protein in clonal THP-1 lines. (B and C) Evaluation of *IFNB1* mRNA expression in PMA-differentiated THP-1s treated with 50 U/mL recombinant IFNβ overnight and then transfected with either (B) 1 ug CT-DNA or 1 ug RIG-I ligand for 4 hours (C) or treated with 100 ng/mL LPS for 4 hours. Statistical analysis was performed using a two-way ANOVA and comparing each clonal line to the control, correcting for multiple comparisons using the Holm-Sidak method. N= 3 where N is the number of treated cell lines. Representative of 1 experiment. (D) Clonal THP-1 cells were transduced with lentiCRISPR lentiviruses encoding Cas9 and the indicated guide RNA. Evaluation of *IFNB1*, *IFI27*, and *ISG15* mRNA expression in PMA-differentiated THP-1s by quantitative RT-PCR. Statistical analysis was performed using a two-way ANOVA and comparing IRF3, IRF7, and IRF3/7-targeted cells to the control line, correcting for multiple comparisons using the Holm-Sideak method. N= 3 where N is the number of unique polyclonal cell lines. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, *** $P \leq 0.0001$

Chapter 5: Materials and Methods

Cell Lines and tissue culture

293T cells were grown in DMEM supplemented with 10% fetal calf serum, L-glutamine, penicillin/streptomycin, sodium pyruvate, and HEPES. THP-1 cells were cultured in RPMI supplemented as above. Where indicated, THP-1 cells were differentiated by culturing in 100 nM PMA for 24 hours and then culturing for an additional 24 hours in fresh medium (without PMA) prior to treatment.

Lentiviral Transduction

VSV-G pseudotyped, self-inactivating lentivirus was prepared by transfecting a nearly confluent 10 cm plate of 293T cells with 1.5 ug pVSV-G, 3 ug psPAX-2, and 6 ug of the pRRL lentiCRISPR vector for 24 hours and then aspirating and replacing the media with fresh media. Between 2 and 4 x 10^6 THP-1 cells were transduced with 0.22 uM sterile filtered lentiviral supernatant. 24 hours after transduction the lentiviral supernatants were replaced with fresh media, and 48 hours after transduction the cells were placed under selection with either 5 ug/mL puromycin (Thermo Fisher), 10 ug/mL blasticidin (Thermo Fisher) or 80 ug/mL hygromycin B (Thermo Fisher) for at least 6 days.

LentiCRISPR/Cas9 Gene Targeting

For CRISPR/Cas9 gene targeting, we generated pRRL lentiviral vectors in which a U6 promoter drives expression of a guide RNA, and an MND promoter drives expression of Cas9, a T2A peptide, and either a puromycin, blasticidin, or hygromycin resistance cassette. Guide RNAs were designed in Benchling. The sequences of the guide RNA (gRNA) target sites are as follows, with the protospacer adjacent motif (PAM) sequence underlined: non-targeting control gRNA: 5’-(G)ACGGAGGCTAAGCGTCGCAA [70], where the (G) denotes a nucleotide added to enable robust transcription off the U6 promoter; *TREX1* gRNA: GAGCATCCACCCACCGCAGCAGG; *SAE1* gRNA1: (G)CTCAGTTCTTGATTCGTACTGGG; *SAE1* gRNA2: (G)CAACCCCATGGTGGATGTGAAGG; *UBA2* (SAE2) gRNA1: (G)TGGTCTTTCAGGCCTAACTGGGG; *UBA2* (SAE2) gRNA2: GGATGGAGCTGAGCTCATATGGG; *UBE2I* (UBC9) gRNA1: (G)CATGAGGTTCATCGTGCCATCGG ; UBE2I (UBC9) gRNA2: (G)AACTGGGAGTGCGCCATTCCAGG; PIAS1 gRNA: (G)CATAGGACTTGAATGTACGTTGG; *PIAS2*: (G)AATGCAGCGCCCTCATCAGGAGG; *PIAS3* gRNA: GGGCACAGCTGGACTTCAGGAGG; *PIAS4* gRNA: (G)TGATCTTCTTGAACAGCTCAAGG; *SUMO1* gRNA: GTGTTCCAATGAATTCACTCAGG; *SUMO2* gRNA: GAAAGCCTATTGTGAACGACAGG; *SUMO3* gRNA: GGCCGGGCAGGACGGCTCCGTGG; *RNF4* gRNA: GCCAGGGATGAGGGCGCTACAGG; *RNF111* gRNA: (G)TAAAAAATAATTCCTCACAGAGG; *TMEM173* (STING) gRNA: GGTGCCTGATAACCTGAGTATGG; *MAVS* gRNA: GTCCTGCTCCTGATGCCCGCTGG; IFNAR1 gRNA: GCTCGTCGCCGTGGCGCCATGGG; *IRF3* gRNA: (G)TTGGAAGCACGGCCTACGGCAGG: *IRF7* gRNA: (G)CCACCTCCCCTGCTGCTAGGCGG.

Where indicated, THP-1 cells were transduced with pSYG lentiviral vectors encoding an HA epitope tag, the protein of interest, an internal ribosome entry site, and a blasticidin resistance cassette using the protocol described previously. After selection, these cells were transduced with lentiCRISPR vectors using the lentiviral transduction protocol described previously.

Western Blot

Where indicated, targeting of genes by lentiCRISPR was confirmed at least 10 days after transduction by immunoblot analysis of whole cell extracts according to standard techniques. Cells were harvested in lysis buffer (20mM HEPES pH 7.4, 150mM NaCl, 10% glycerol, 1%

Triton-X 100, 1mM EDTA, 1mM DTT) supplemented with Complete protease inhibitor cocktail (Thermo Fisher), incubated on ice for 15 minutes, and cleared of insoluble material by centrifugation. Cleared lysates were separated using a 4-12% Bis-Tris SDS-PAGE gel (Life Technologies) and transferred to Immobilon-P PVDF membrane (Millipore). Membranes were probed with the following primary antibodies: anti-SAE1 (ab185949; Abcam), anti-SAE2 (CST8688; Cell Signaling Technology), anti-UBC9 (CST4786; Cell Signaling Technology), anti-human TREX1 (CST12215, Cell Signaling Technology), anti-murine TREX1 (T28920; Transduction Labs), anti-ACTIN (AC-74, Sigma); anti-HA (CST2367; Cell Signaling Technology), anti-GFP (ab6556; Abcam), anti-SUMO1 (CST4930; Cell Signaling Technology), anti-SUMO2/3 (CST4971; Cell Signaling Technology), anti-STING (CST13647; Cell Signaling Technology), anti-MAVS (A300-782; Bethyl Laboratories), anti-IRF3 (CST4320; Cell Signaling Technology), anti-IRF7 (CST4920, Cell Signaling Transduction).

Restriction Fragment Length Polymorphism Assay

Targeting of genes by lentiCRISPR was confirmed by a restriction fragment length polymorphism assay where indicated. PCR products were amplified with Phusion High-Fidelity DNA polymerase (Thermo Fisher) or EmeraldAmp (Clontech) Primers used for RFLP are as follows: *UBA2* (SAE2) Fwd: 5'- TGGTCGGGGATTGAACTTGT-3', *UBA2* (SAE2) Rev: 5'-AGAGCTGAAAATATCCAAGCCA-3', *PIAS1* Fwd: 5'-CTTAAGTAAACCATAAGAAGGGGGT-3', *PIAS1* Rev: 5'-TATCCGGATGGACTGGGTGAA-3', *PIAS2* Fwd: 5'-CATGCCAATTGATAGAATGCTGGA-3', *PIAS2* Rev: 5'-CTGAAGTGGAAGGCAACGAG-3', *PIAS3* Fwd: 5'-TGCTGGAGAGAAAGGCACTG-3', *PIAS3* Rev: 5'-GGGGAATGGGAGCTAGAGGA-3', *PIAS4* Fwd: 5'-GAGTGGGTGTCTTTCACGGC-3', *PIAS4* Rev: 5'-CAACCGTCCCAGTCCGTTT-3', *RNF4* Fwd: 5'-GAGGGTCTTAAGAGGCGTCAGG-3', *RNF4* Rev: 5'- TATCCAGATGCTGGGTAGAGGCT-3', *RNF111* Fwd: 5'-GGGGCAGCCAAAAGTTTTCC-3', RNF11 Rev: 5'-TGCTGAGGTCATTCTCACTGG-3', IFNAR1

Fwd: 5’-GCCATAGGCCGGAAAGAGTG-3’, IFNAR1 Rev: 5’-GGTCGGGTTTAATCTTTGGCG-3’.. PCR products were gel purified and then digested with the following restriction enzymes (New England Biolabs): NdeI, HpyCH4IV, BslI, BfaI, AcuI, SfcI, and NcoI.

Cell Treatments

For transfections, calf-thymus DNA (Thermo Fisher) or RIG-I ligand (prepared as described in Saito et al, 2008) were complexed with lipofectamine 2000 (Thermo Fisher) at a ratio of 1 ug nucleic acid to 1 uL lipid. For LPS stimulations, LPS (Sigma) was added directly to PMA differentiated THP-1 cells. For treatment with chemical inhibitors, BX795 (Selleckchem) and TPCA-1 (Sigma) were diluted to 1 uM or 20 uM respectively in fresh media and added to cells for 48 hours. Cells were either pretreated with IFN-β (R&D Systems) overnight or stimulated with IFN-β for 6 hours where indicated.

Quantitative RT-PCR

For quantitative RT-PCR, cells were harvested in RNA-STAT60 (Amsbio) or Trizol (Thermo Fisher). RNA was reverse transcribed into cDNA with Superscript III (Thermo Fisher) or EcoDry Premix (Clontech). Quantitative RT-PCR was performed with EVA Green reagents (Bio-Rad Laboratories) on a Bio-Rad CFX96 Real-Time system. The primers used were as follows: *GAPDH* Fwd: 5’-AACAGCCTCAAGATCATCAGC-3’, *GAPDH* Rev: 5’-CACCACCTTCTTGATGTCATC-3’, *IFNB1* Fwd: 5’-ACGCCGCATTGACCATCTATG-3’, *IFNB1* Rev: 5’-CGGAGGTAACCTGTAAGTCTGT-3’, *IFI27* Fwd: 5’-TGCTCTCACCTCATCAGCAGT-3’, *IFI27* Rev: 5’-CACAACTCCTCCAATCACAACT-3’, *ISG15* Fwd: 5’-TCCTGGTGAGGAATAACAAGGG-3’*, ISG15* Rev: 5’-GTCAGCCAGAACAGGTCGTC-3’, *TNF* : 5’-GAGGCCAAGCCCTGGTATG-3’*, TNF* Rev: 5’-CGGGCCGATTGATCTCAGC-3’.

References

1 Arber, W. & Linn, S. DNA modification and restriction. *Annu Rev Biochem* **38**, 467-500, doi:10.1146/annurev.bi.38.070169.002343 (1969).

2 Jinek, M. *et al.* A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* **337**, 816-821, doi:10.1126/science.1225829 (2012).

3 Fire, A. *et al.* Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature* **391**, 806-811, doi:10.1038/35888 (1998).

4 Janeway, C. A., Jr. Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harb Symp Quant Biol* **54 Pt 1**, 1-13 (1989).

5 Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J. M. & Hoffmann, J. A. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. *Cell* **86**, 973-983 (1996).

6 Poltorak, A. *et al.* Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* **282**, 2085-2088 (1998).

7 Poltorak, A. *et al.* Genetic and physical mapping of the Lps locus: identification of the toll-4 receptor as a candidate gene in the critical region. *Blood Cells Mol Dis* **24**, 340-355 (1998).

8 Medzhitov, R., Preston-Hurlburt, P. & Janeway, C. A., Jr. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature* **388**, 394-397, doi:10.1038/41131 (1997).

9 Ewald, S. E. *et al.* The ectodomain of Toll-like receptor 9 is cleaved to generate a functional receptor. *Nature* **456**, 658-662, doi:10.1038/nature07405 (2008).

10 Yamamoto, M. *et al.* Role of Adaptor TRIF in the MyD88-Independent Toll-Like Receptor Signaling Pathway. *Science* (2003).

11 Honda, K. *et al.* IRF-7 is the master regulator of type-I interferon-dependent immune responses. *Nature* **434**, 772-777 (2005).

12 Sun, L., Wu, J., Du, F., Chen, X. & Chen, Z. J. Cyclic GMP-AMP synthase is a cytosolic DNA sensor that activates the type I interferon pathway. *Science* **339**, 786-791, doi:10.1126/science.1232458 (2013).

13 Yoneyama, M. *et al.* The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nat Immunol* **5**, 730-737, doi:10.1038/ni1087 (2004).

14 Stetson, D. B., Ko, J. S., Heidmann, T. & Medzhitov, R. Trex1 prevents cell-intrinsic initiation of autoimmunity. *Cell* **134**, 587-598, doi:10.1016/j.cell.2008.06.032 (2008).

15 Gao, D. *et al.* Activation of cyclic GMP-AMP synthase by self-DNA causes autoimmune diseases. *Proc Natl Acad Sci U S A* **112**, E5699-5705, doi:10.1073/pnas.1516465112 (2015).

16 Gray, E. E., Treuting, P. M., Woodward, J. J. & Stetson, D. B. Cutting Edge: cGAS Is Required for Lethal Autoimmune Disease in the Trex1-Deficient Mouse Model of Aicardi-Goutieres Syndrome. *J Immunol* **195**, 1939-1943, doi:10.4049/jimmunol.1500969 (2015).

17 Crow, Y. J. *et al.* Mutations in the gene encoding the 3'-5' DNA exonuclease TREX1 cause Aicardi-Goutieres syndrome at the AGS1 locus. *Nat Genet* **38**, 917-920, doi:10.1038/ng1845 (2006).

18 Crow, Y. J. & Manel, N. Aicardi-Goutieres syndrome and the type I interferonopathies. *Nat Rev Immunol* **15**, 429-440, doi:10.1038/nri3850 (2015).

19 Crowl, J. T., Gray, E. E., Pestal, K., Volkman, H. E. & Stetson, D. B. Intracellular Nucleic Acid Detection in Autoimmunity. *Annu Rev Immunol*, doi:10.1146/annurev-immunol-051116-052331 (2017).

20 Kim, H., Sanchez, G. A. & Goldbach-Mansky, R. Insights from Mendelian Interferonopathies: Comparison of CANDLE, SAVI with AGS, Monogenic Lupus. *J Mol Med (Berl)* **94**, 1111-1127, doi:10.1007/s00109-016-1465-5 (2016).

21 Lee-Kirsch, M. A. *et al.* Mutations in the gene encoding the 3'-5' DNA exonuclease TREX1 are associated with systemic lupus erythematosus. *Nat Genet* **39**, 1065-1067, doi:10.1038/ng2091 (2007).

22 Lee-Kirsch, M. A. *et al.* A mutation in TREX1 that impairs susceptibility to granzyme A-mediated cell death underlies familial chilblain lupus. *J Mol Med (Berl)* **85**, 531-537, doi:10.1007/s00109-007-0199-9 (2007).

23 Rice, G. *et al.* Heterozygous mutations in TREX1 cause familial chilblain lupus and dominant Aicardi-Goutieres syndrome. *Am J Hum Genet* **80**, 811-815, doi:10.1086/513443 (2007).

24 Morita, M. *et al.* Gene-targeted mice lacking the Trex1 (DNase III) 3'-->5' DNA exonuclease develop inflammatory myocarditis. *Mol Cell Biol* **24**, 6719-6727, doi:10.1128/MCB.24.15.6719-6727.2004 (2004).

25 Gall, A. *et al.* Autoimmunity initiates in nonhematopoietic cells and progresses via lymphocytes in an interferon-dependent autoimmune disease. *Immunity* **36**, 120-131, doi:10.1016/j.immuni.2011.11.018 (2012).

26 Wolf, C. *et al.* RPA and Rad51 constitute a cell intrinsic mechanism to protect the cytosol from self DNA. *Nat Commun* **7**, 11752, doi:10.1038/ncomms11752 (2016).

27 Mazur, D. J. & Perrino, F. W. Excision of 3' termini by the Trex1 and TREX2 3'-->5' exonucleases. Characterization of the recombinant proteins. *J Biol Chem* **276**, 17022-17029, doi:10.1074/jbc.M100623200 (2001).

28 Brunette, R. *Nucleic acid detection in anti-viral responses and autoimmunity* PhD in Immunology thesis, University of Washington, (2012).

29 O'Donnell, M. & Kuriyan, J. Clamp loaders and replication initiation. *Curr Opin Struct Biol* **16**, 35-41, doi:10.1016/j.sbi.2005.12.004 (2006).

30 Shibahara, K. & Stillman, B. Replication-dependent marking of DNA by PCNA facilitates CAF-1-coupled inheritance of chromatin. *Cell* **96**, 575-585 (1999).

31 Cubenas-Potts, C. & Matunis, M. J. SUMO: a multifaceted modifier of chromatin structure and function. *Dev Cell* **24**, 1-12, doi:10.1016/j.devcel.2012.11.020 (2013).

32 Peng, C. *et al.* The transcriptional regulation role of BRD7 by binding to acetylated histone through bromodomain. *J Cell Biochem* **97**, 882-892, doi:10.1002/jcb.20645 (2006).

33 Liu, B. *et al.* PIAS1 selectively inhibits interferon-inducible genes and is important in innate immunity. *Nat Immunol* **5**, 891-898, doi:10.1038/ni1104 (2004).

34 Li, R., Pan, Y., Shi, D. D., Zhang, Y. & Zhang, J. PIAS1 negatively modulates virus triggered type I IFN signaling by blocking the DNA binding activity of IRF3. *Antiviral Res* **100**, 546-554, doi:10.1016/j.antiviral.2013.09.001 (2013).

35 Zhao, R. *et al.* BRD7 plays an anti-inflammatory role during early acute inflammation by inhibiting activation of the NF-small ka, CyrillicB signaling pathway. *Cell Mol Immunol* **14**, 830-841, doi:10.1038/cmi.2016.31 (2017).

36 Yang, Y. G., Lindahl, T. & Barnes, D. E. Trex1 exonuclease degrades ssDNA to prevent chronic checkpoint activation and autoimmune disease. *Cell* **131**, 873-886, doi:10.1016/j.cell.2007.10.017 (2007).

37 Flotho, A. & Melchior, F. Sumoylation: a regulatory protein modification in health and disease. *Annu Rev Biochem* **82**, 357-385, doi:10.1146/annurev-biochem-061909-093311 (2013).

38 Desterro, J. M., Rodriguez, M. S., Kemp, G. D. & Hay, R. T. Identification of the enzyme required for activation of the small ubiquitin-like protein SUMO-1. *J Biol Chem* **274**, 10618-10624 (1999).

39 Wang, L. *et al.* SUMO2 is essential while SUMO3 is dispensable for mouse embryonic development. *EMBO Rep* **15**, 878-885, doi:10.15252/embr.201438534 (2014).

40 Tatham, M. H. *et al.* Unique binding interactions among Ubc9, SUMO and RanBP2 reveal a mechanism for SUMO paralog selection. *Nat Struct Mol Biol* **12**, 67-74, doi:10.1038/nsmb878 (2005).

41 Tatham, M. H. *et al.* Polymeric chains of SUMO-2 and SUMO-3 are conjugated to protein substrates by SAE1/SAE2 and Ubc9. *J Biol Chem* **276**, 35368-35374, doi:10.1074/jbc.M104214200 (2001).

42 Tatham, M. H. *et al.* RNF4 is a poly-SUMO-specific E3 ubiquitin ligase required for arsenic-induced PML degradation. *Nat Cell Biol* **10**, 538-546, doi:10.1038/ncb1716 (2008).

43 Hendriks, I. A. & Vertegaal, A. C. A comprehensive compilation of SUMO proteomics. *Nat Rev Mol Cell Biol* **17**, 581-595, doi:10.1038/nrm.2016.81 (2016).

44 Decque, A. *et al.* Sumoylation coordinates the repression of inflammatory and anti-viral gene-expression programs during innate sensing. *Nat Immunol* **17**, 140-149, doi:10.1038/ni.3342 (2016).

45 Liu, B. *et al.* Inhibition of Stat1-mediated gene activation by PIAS1. *Proc Natl Acad Sci U S A* **95**, 10626-10631 (1998).

46 Brehm, A. *et al.* Additive loss-of-function proteasome subunit mutations in CANDLE/PRAAS patients promote type I IFN production. *J Clin Invest* **125**, 4196-4211, doi:10.1172/JCI81260 (2015).

47 Poulsen, S. L. *et al.* RNF111/Arkadia is a SUMO-targeted ubiquitin ligase that facilitates the DNA damage response. *J Cell Biol* **201**, 797-807, doi:10.1083/jcb.201212075 (2013).

48 Fu, J., Xiong, Y., Xu, Y., Cheng, G. & Tang, H. MDA5 is SUMOylated by PIAS2beta in the upregulation of type I interferon signaling. *Mol Immunol* **48**, 415-422, doi:10.1016/j.molimm.2010.09.003 (2011).

49 Wu, J. *et al.* Cyclic GMP-AMP is an endogenous second messenger in innate immune signaling by cytosolic DNA. *Science* **339**, 826-830, doi:10.1126/science.1229963 (2013).

50 Ishikawa, H. & Barber, G. N. STING is an endoplasmic reticulum adaptor that facilitates innate immune signalling. *Nature* **455**, 674-678, doi:nature07317 [pii]

10.1038/nature07317 (2008).

51 Goubau, D., Deddouche, S. & Reis, E. S. C. Cytosolic sensing of viruses. *Immunity* **38**, 855-869, doi:10.1016/j.immuni.2013.05.007 (2013).

52 Barbalat, R., Ewald, S. E., Mouchess, M. L. & Barton, G. M. Nucleic acid recognition by the innate immune system. *Annu Rev Immunol* **29**, 185-214, doi:10.1146/annurev-immunol-031210-101340 (2011).

53 Honda, K., Takaoka, A. & Taniguchi, T. Type I interferon [corrected] gene induction by the interferon regulatory factor family of transcription factors. *Immunity* **25**, 349-360 (2006).

54 Liu, S. *et al.* Phosphorylation of innate immune adaptor proteins MAVS, STING, and TRIF induces IRF3 activation. *Science* **347**, aaa2630, doi:10.1126/science.aaa2630 (2015).

55 Hu, M. M. *et al.* Sumoylation Promotes the Stability of the DNA Sensor cGAS and the Adaptor STING to Regulate the Kinetics of Response to DNA Virus. *Immunity* **45**, 555-569, doi:10.1016/j.immuni.2016.08.014 (2016).

56 Cui, Y. *et al.* SENP7 Potentiates cGAS Activation by Relieving SUMO-Mediated Inhibition of Cytosolic DNA Sensing. *PLoS Pathog* **13**, e1006156, doi:10.1371/journal.ppat.1006156 (2017).

57 Hu, M. M., Liao, C. Y., Yang, Q., Xie, X. Q. & Shu, H. B. Innate immunity to RNA virus is regulated by temporal and reversible sumoylation of RIG-I and MDA5. *J Exp Med* **214**, 973-989, doi:10.1084/jem.20161015 (2017).

58 Mi, Z., Fu, J., Xiong, Y. & Tang, H. SUMOylation of RIG-I positively regulates the type I interferon signaling. *Protein Cell* **1**, 275-283, doi:10.1007/s13238-010-0030-1 (2010).

59 Kubota, T. *et al.* Virus infection triggers SUMOylation of IRF3 and IRF7, leading to the negative regulation of type I interferon gene expression. *J Biol Chem* **283**, 25660-25670, doi:10.1074/jbc.M804479200 (2008).

60 Saito, T., Owen, D. M., Jiang, F., Marcotrigiano, J. & Gale, M., Jr. Innate immunity induced by composition-dependent RIG-I recognition of hepatitis C virus RNA. *Nature* **454**, 523-527, doi:nature07106 [pii]

10.1038/nature07106 (2008).

61 Fitzgerald, K. A. *et al.* IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway. *Nat Immunol* **4**, 491-496, doi:10.1038/ni921 (2003).

62 Sharma, S. *et al.* Triggering the interferon antiviral response through an IKK-related pathway. *Science* **300**, 1148-1151, doi:10.1126/science.1081315 (2003).

63 Hoshino, K. *et al.* IkappaB kinase-alpha is critical for interferon-alpha production induced by Toll-like receptors 7 and 9. *Nature* **440**, 949-953, doi:10.1038/nature04641 (2006).

64 Yarilina, A., Park-Min, K. H., Antoniv, T., Hu, X. & Ivashkiv, L. B. TNF activates an IRF1-dependent autocrine loop leading to sustained expression of chemokines and STAT1-dependent type I interferon-response genes. *Nat Immunol* **9**, 378-387, doi:10.1038/ni1576 (2008).

65 Schmitz, F. *et al.* Interferon-regulatory-factor 1 controls Toll-like receptor 9-mediated IFN-beta production in myeloid dendritic cells. *Eur J Immunol* **37**, 315-327, doi:10.1002/eji.200636767 (2007).

66 Negishi, H. *et al.* Evidence for licensing of IFN-gamma-induced IFN regulatory factor 1 transcription factor by MyD88 in Toll-like receptor-dependent gene induction program. *Proc Natl Acad Sci U S A* **103**, 15136-15141, doi:10.1073/pnas.0607181103 (2006).

67 Lazear, H. M. *et al.* IRF-3, IRF-5, and IRF-7 coordinately regulate the type I IFN response in myeloid dendritic cells downstream of MAVS signaling. *PLoS Pathog* **9**, e1003118, doi:10.1371/journal.ppat.1003118 (2013).

68 Graham, R. R. *et al.* A common haplotype of interferon regulatory factor 5 (IRF5) regulates splicing and expression and is associated with increased risk of systemic lupus erythematosus. *Nat Genet* **38**, 550-555 (2006).

69 Sigurdsson, S. *et al.* Polymorphisms in the tyrosine kinase 2 and interferon regulatory factor 5 genes are associated with systemic lupus erythematosus. *Am J Hum Genet* **76**, 528-537, doi:10.1086/428480 (2005).

70 Sanjana, N. E., Shalem, O. & Zhang, F. Improved vectors and genome-wide libraries for CRISPR screening. *Nat Methods* **11**, 783-784, doi:10.1038/nmeth.3047 (2014).