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# Targeting IRFs by ubiquitination: regulating antiviral responses

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## Abstract

The IRF [IFN (interferon) regulatory factor] family of transcription factors control many cellular processes, including induction of key antiviral cytokines, type I IFNs, following viral infection. Recent studies have revealed several endogenous and viral proteins involved in ubiquitin-mediated regulation of IRF activity and thus having an impact on type I IFN signalling. Through the ubiquitin pathway, these proteins can manipulate the antiviral response either by initiating proteasomal degradation of the IRFs or, in contrast, by promoting activation of the IRFs.

## Introduction

Following viral infection, a crucial step in the antiviral response is the production of type I IFNs (interferons) [1], first described in 1957 by Isaacs and Lindemann as proteins that 'interfere' with viral replication [2]. Type I IFNs include 13–14 IFN- $\alpha$  subtypes, a single IFN- $\beta$  and other less characterized family members, such as IFN- $\epsilon$ , IFN- $\kappa$  and IFN- $\omega$  [3,4]. Secretion of type I IFNs by all nucleated cells establishes an antiviral state by inducing resistance to viral replication, increasing MHC class I expression and antigen presentation, and activating NK (natural killer) cells to kill virus-infected cells. In addition, IFNs signal through the IFNAR (type I IFN receptor) to activate ISGF3 (IFN-stimulated factor 3), which induces the transcription of IFN-inducible genes that contribute to the antiviral state, including pro-inflammatory cytokines and chemokines [3,4].

The PRRs (pathogen recognition receptors) responsible for the innate detection of viruses have been discovered in recent years and include the TLRs (Toll-like receptors) and the cytosolic PRRs [RIG-I (retinoic acid-inducible gene I) and MDA-5 (melanoma-differentiation-associated gene 5)] [5]. The TLRs recognizing viral nucleic acids (TLR3, TLR7, TLR8 and TLR9) have evolved in the endosomal compartment of cells, presumably as the endosome is commonly used for viral entry/exit and viral components collect in

the endosome following completion of viral replication [6]. Following activation of these TLRs, distinct intracellular signalling pathways are driven that regulate type I IFN production. The IRF (IFN regulatory factor) family of transcription factors are crucial in driving IFN responses [7]. IRF-3 is involved in the initial induction of IFN- $\beta$  downstream of TLR4 and TLR3 and the cytosolic PRR RIG-I, whereas IRF-5 and IRF-7 are critical for the induction of TLR7- and TLR9-mediated type I IFN production.

Pathways regulating activation of IRF-3 are well characterized. IRF-3 is expressed ubiquitously and is activated by serine phosphorylation in response to viral infection or TLR signalling. Evidence indicates that the kinases TBK1 {TANK [TRAF (tumour necrosis factor receptor-associated factor)-associated NF- $\kappa$ B (nuclear factor  $\kappa$ B) activator]-binding kinase 1} and IKK $\epsilon$  (inhibitor of NF- $\kappa$ B kinase- $\epsilon$ ) specifically phosphorylate serine residues in the C-terminal domain of IRF-3 and thereby activate it [8–10]. IRF-7 is similarly regulated by phosphorylation at C-terminal serine residues, and IKK $\epsilon$  and TBK1 are the kinases responsible [11].

TLR3 is expressed in a wide range of cells and recognizes dsRNA (double-stranded RNA), although it can also be activated *in vitro* with poly(I:C), an artificial dsRNA. It signals through the adaptor molecule TRIF {TIR [Toll/IL (interleukin)-1 receptor] domain-containing adaptor protein inducing IFN- $\beta$ } [12], which interacts with TRAF-3 to activate both TBK1 and IKK $\epsilon$  [13,14]. TLR4, which is located at the cell surface and recognizes Gram-negative endotoxin, also induces IFN- $\beta$  induction through this TRIF-mediated pathway, although an additional adaptor molecule, TRAM (TRIF-related adaptor molecule), is required as a link between TLR4 and TRIF [15,16].

TLR7 and TLR9 signal through the TRIF molecule MyD88 (myeloid differentiation factor 88), which forms a complex with TRAF-6 and IRF-7 in the cytoplasm [17]. Formation of this signalling complex has been shown to be dependent on the serine/threonine kinases IRAK-1 (IL-1-receptor associated kinase-1) and IRAK-4, with ubiquitination and phosphorylation of TRAF-6 (possibly

**Key words:** antiviral response, cell signalling, interferon regulatory factor (IRF), Toll-like receptor, type I interferon, ubiquitination.

**Abbreviations used:** CBP, CREB (cAMP-response-element-binding protein)-binding protein; DBD, DNA-binding domain; dsRNA, double-stranded RNA; HEK-293 cells, human embryonic kidney cells; HEK-293T cells, HEK-293 cells expressing the large T-antigen of SV40 (simian virus 40); ICPO, infected cell protein 0; biCPO, BHV1 (bovine herpesvirus 1)-ICPO; IFN, interferon; IFNAR, type I IFN receptor; IL, interleukin; IRF, IFN regulatory factor; IAD, IRF association domain; ISRE, IFN-stimulated response element; MDA-5, melanoma-differentiation-associated gene 5; NF- $\kappa$ B, nuclear factor  $\kappa$ B; IKK $\epsilon$ , inhibitor of NF- $\kappa$ B kinase- $\epsilon$ ; NSP1, non-structural protein 1; PRD, positive regulatory domain; PRR, pathogen recognition receptor; RIG-I, retinoic acid-inducible gene I; RIP, receptor-interacting protein; SLE, systemic lupus erythematosus; TLR, Toll-like receptor; TRAF, tumour necrosis factor receptor-associated factor; TBK1, TANK (TRAF-associated NF- $\kappa$ B activator)-binding kinase 1; TRIF, TIR (Toll/IL-1 receptor) domain-containing adaptor protein inducing IFN- $\beta$ ; TRIM, tripartite motif.

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IKK $\epsilon$  and TBK1 occurs on Ser<sup>385</sup> and Ser<sup>386</sup> and on a serine/threonine cluster located between amino acids 396 and 405, as determined by the generation of point mutations at these residues [23,24]. Phosphorylation of IRF-3 disrupts an interaction between the helices H1 and H5 that flank the  $\beta$ -sandwich core of the IAD, thus liberating the DNA-binding activity [25]. Following this, IRF-3 becomes activated, dimerizes and translocates to the nucleus, where it associates with the co-activator CBP [CREB (cAMP-response-element-binding protein)-binding protein]/p300 [23,24]. IRF-3 induces IFN- $\beta$  transcription by binding to overlapping regions of PRD III (positive regulatory domain III) and PRD I on the enhancer region of the IFN- $\beta$  promoter. The importance of IRF-3 in the antiviral response has been confirmed since IRF-3<sup>-/-</sup> mice are more vulnerable to viral infection and have lower serum type I IFN levels [26]. Furthermore, cells lacking both IRF-3 and IRF-7 failed to induce type I IFN mRNA following viral infection, an effect that could be reversed by co-expression of both proteins [26].

### IRF-7

IRF-7 is an inducible member of the IRF family and is vital for IFN- $\alpha$  production following viral infection, as shown using IRF-7<sup>-/-</sup> mice [11]. IRF-7 expression can be induced directly after viral infection by TLR7 and TLR9 in pDCs (plasmacytoid dendritic cells), although IFN signalling through the IFNAR also promotes IRF-7 expression and is important for the secondary IFN response [27]. In addition to its inducibility, IRF-7 has a more transient expression than IRF-3, with a half-life of 30–60 min [26]. The structure of IRF-7 shares similarities to that of IRF-3 and contains multiple C-terminal regulatory regions that are the target of virus-induced phosphorylation by TBK1, and, to a lesser extent, IKK $\epsilon$  [28]. Specifically, Ser<sup>477</sup>–Ser<sup>479</sup> have been shown to be important for IRF-7 phosphorylation, whereas Ser<sup>471</sup>–Ser<sup>472</sup> are important for IRF-7 activation [29]. In addition, it has been shown that IRF-7 is acetylated *in vivo* at Lys<sup>92</sup> by the histone acetyltransferases P/CAF (p300/CBP-associated factor) and GCN5 (positive general control of transcription-5), a post-translational modification that inhibits its DNA-binding activity [30].

### Ubiquitin-mediated regulation of IRFs

Cellular mechanisms that limit or down-regulate type I IFN production downstream of pathogen recognition are critical to protection against overproduction of these cytokines, which can contribute to the adverse pathogenic effects characteristic of many autoimmune disorders. For example, SLE (systemic lupus erythematosus) patients present with high levels of serum type I IFN, which correlates with both disease severity and pathogenesis [31]. The molecular mechanisms that contribute to the overproduction of type I IFNs in SLE patients is under intense investigation. Studies in SLE-prone mice have linked both the TLR7- and TLR9-dependent pathways with this effect, with autoimmune complexes being shown to activate both TLR7 and TLR9

in driving autoantibody and type I IFN production [32,33]. Intriguingly, polymorphisms in the transcription factor IRF-5, important in regulating IFN production downstream of TLR7 and TLR9, have been linked with SLE, although the functional relevance of these polymorphisms is as yet unknown. Thus understanding how IRF activity is both positively and negatively regulated is critical for understanding how defects in these pathways contribute to both antiviral defence and autoimmunity.

It has become apparent in recent years that an effective mechanism to limit type I IFN production is through ubiquitin-mediated degradation of the IRF family members, specifically IRF-3 and IRF-7. Ubiquitin-mediated proteasomal degradation is an efficient cellular process that contributes to many biological events including cell cycle control, signal transduction, DNA repair and apoptosis [34,35]. In this process, ubiquitin associates with, and is activated by, an E1 ubiquitin-activating enzyme. It is then transferred via an E2 ubiquitin-conjugating enzyme to specific lysine residues of target proteins, an action that is mediated by one of a large heterogeneous series of E3 ubiquitin ligases. The E3 ligase is vital and provides the specificity in the ubiquitin process as it recruits both the E2-ubiquitin complex and the substrate protein [36]. Addition of further ubiquitin molecules through lysine-glycine bonding results in the formation of a polyubiquitin chain that targets the substrate protein for proteasomal degradation by the 26S protein complex. The specific lysine targeted by the E3 ligase to generate polyubiquitin chains on the target protein is critical, as Lys<sup>48</sup>-linked ubiquitination generally leads to proteasomal degradation, whereas Lys<sup>63</sup>-linked ubiquitination appears to promote activation of the substrate protein.

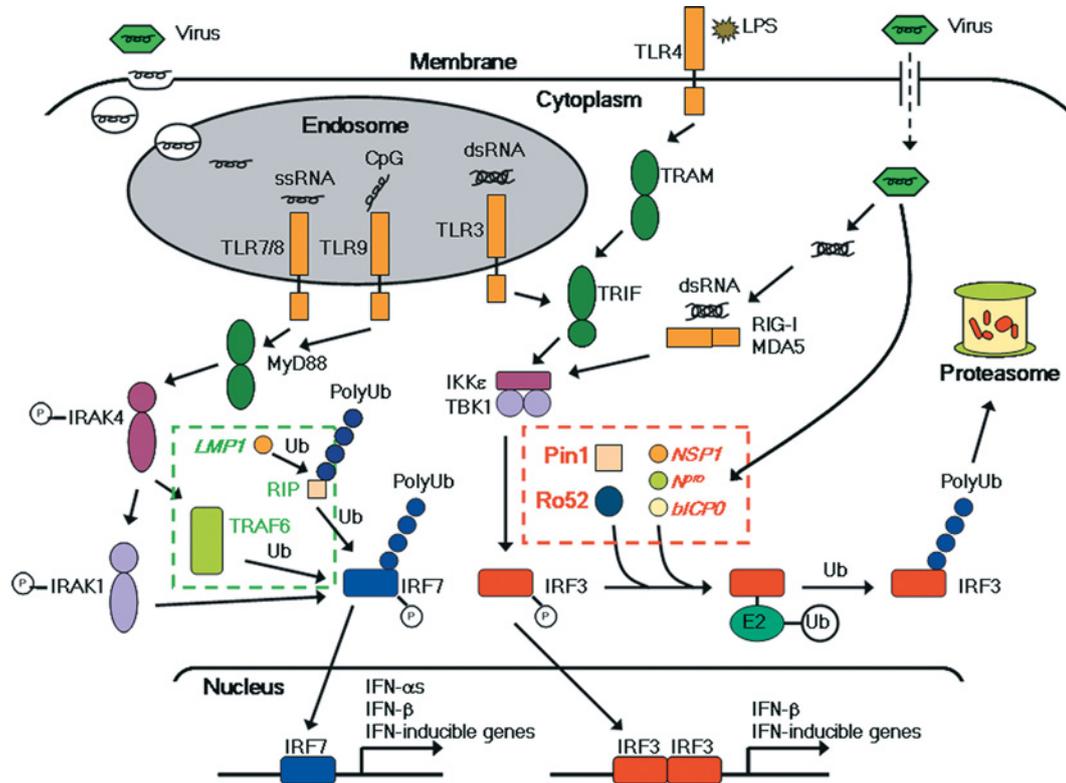
### Negative ubiquitin-mediated regulation

Ubiquitin-mediated degradation of IRF-3 has been described in several studies and falls into two main categories summarized in Figure 2. First, several endogenous proteins have been described that, after viral infection, directly or indirectly contribute to the polyubiquitination and subsequent proteasomal degradation of IRF-3 in order to protect the host from overproduction of type I IFNs. Secondly, it has been shown that viral proteins can act as E3 ubiquitin ligases and similarly target IRF-3 for proteasomal degradation, thus limiting the antiviral response and creating a more promising cellular environment for viral replication.

The existence of endogenous or viral proteins that target IRF-3 for degradation was suggested from early work in which the proteasome inhibitor MG-132 (carbobenzoxy-L-leucyl-L-leucyl-leucinal) inhibited IRF-3 degradation in response to viral infection [23]. Recently, IRF-3 activation was shown to be negatively regulated by the peptide-prolyl isomerase Pin1. Specifically, Pin1 was shown to interact with phosphorylated IRF-3 at Ser<sup>339</sup> following polyI:C stimulation of HEK-293T cells [HEK-293 cells (human embryonic kidney cells) expressing the large T-antigen of SV40 (simian virus 40)] [37]. This interaction was necessary

**Figure 2 | Ubiquitin-mediated negative regulation of IRF-3 signalling**

Viral nucleic acid is recognized by endosomal TLRs and intracellular PRRs (RIG-I and MDA-5). Activation of TLR3 and RIG-I results in phosphorylation of IRF-3 by IKK $\epsilon$  and TBK1. Following this, several endogenous or viral proteins (indicated in a broken red box) interact with IRF-3 and induce polyubiquitination of the transcription factor. Polyubiquitinated IRF-3 is targeted for degradation in the 26S proteasome, thus limiting type I IFN production. The endogenous proteins Pin1 and Ro52 are indicated in red, and the viral proteins NSP1, NP10 and bICP0 are indicated in red and italics. IRF-7 can be ubiquitinated by TRAF-6 or RIP (indicated in dashed green box), leading to activation of the transcription factor. Protein phosphorylation is indicated by an encircled 'P'.



for the polyubiquitination and proteasomal degradation of the IRF-3 and the subsequent negative regulation of IFN- $\beta$  production [37]. Accordingly, Pin1<sup>-/-</sup> mice had elevated serum levels of IFN- $\beta$  in comparison with wild-type mice following polyI:C injection [37]. However, Pin1 does not directly catalyse the ubiquitination of IRF-3. A separate study into the endogenous mechanisms to down-regulate type I IFN signalling downstream of pathogen detection demonstrated the involvement of a cullin-based ubiquitin ligase in Sendai virus-induced IRF-3 degradation [38], suggesting that endogenous enzymes may contribute to the negative regulation of IRF-3. In this context, recent evidence in our laboratory suggests that the E3 ubiquitin ligase Ro52 [TRIM (tripartite motif) 21] is directly responsible for ubiquitinating and targeting IRF-3 for proteasomal degradation. Ro52 is a member of the TRIM/RBCC [RING (really interesting new gene) finger B-box coiled-coil] family of single-protein E3 ligases, members of which have been shown to play important roles in antiviral defence (reviewed in [39,40]). Ubiquitination and degradation of IRF-3 were substantially increased in

HEK-293T cells co-expressing Ro52, an effect that could be inhibited with the proteasome inhibitor MG-132 (R. Higgs, J. Ní Gabhann, N. Ben Larbi, E.P. Breen, K.A. Fitzgerald and C.A. Jefferies, unpublished work). Moreover, knockdown of Ro52 expression with shRNA (small-hairpin RNA) resulted in enhanced TLR3-driven IFN- $\beta$  production and Sendai virus-stimulated RANTES (regulated upon activation, normal T-cell expressed and secreted) production (R. Higgs, J. Ní Gabhann, N. Ben Larbi, E.P. Breen, K.A. Fitzgerald and C.A. Jefferies, unpublished work). In a separate study, Ro52 was shown to ubiquitinate the transcription factor IRF-8, which positively regulates IL-12p40 production in murine macrophages [41]; thus Ro52 may have a global role in the regulation of IRF activity.

Another emerging area of study is the down-regulation of IRF-3 activation by viral proteins. For example, following rotaviral infection of Caco-2 cells, the rotavirus gene 5 product, NSP1 (non-structural protein 1), associates with IRF-3 and mediates degradation of the transcription factor [42]. In contrast, less IRF-3 degradation was observed in

Caco-2 cells infected with a rotaviral strain expressing a C-terminal truncated version of NSP1, confirming the immunosuppressive role of this viral protein [42]. Further work showed that NSP1-mediated IRF-3 degradation is dependent on the integrity of the N-terminal zinc-binding domain of NSP1, supporting the hypothesis that NSP1 is an E3 ubiquitin ligase [43]. The pestiviruses CSFV (classical swine fever virus) and BVDV (bovine viral diarrhoea virus) interact with and induce degradation of IRF-3, but not IRF-7, through the protein N<sup>pro</sup> [44,45]. In addition, bICP0 [BHV1 (bovine herpesvirus 1)-ICP0 (infected cell protein 0)] has been shown to act as an E3 ligase and promote IRF-3 degradation in a proteasome-dependent manner, thus inhibiting the IFN- $\beta$  promoter [46]. Accordingly, HSV-1 (herpes simplex virus 1) ICP0 protein has also been shown to inhibit IRF-3 activity in a proteasome-dependent manner [47]. Furthermore, a recent study has shown that the HIV-1 accessory proteins VPR (viral protein R) and VIF (viral infectivity factor) can modulate the antiviral response by targeting IRF-3 for degradation [48].

Ubiquitin-mediated degradation of other IRF family members has also been reported. The C-termini of both IRF-1 and IRF-8 were shown to be critical for ubiquitination and proteasomal degradation of these transcription factors [49,50]. IRF-8 ubiquitination was mediated by the E3 ligase Cbl and shown to inhibit IL-12p40 production [50], in contrast with Ro52-mediated ubiquitination of IRF-8, which appears to regulate functional activity of this transcription factor [41]. Taken together, these studies demonstrate a pivotal role for ubiquitin-mediated degradation of IRF family members, particularly IRF-3, through endogenous or viral proteins to negatively regulate IFN signalling.

## Positive ubiquitin-mediated regulation

Protein degradation is not the only outcome of ubiquitination, and studies have indicated that IRF activity can also be enhanced following Lys<sup>63</sup>-linked ubiquitination (Figure 2). For example, IRF-7 activity is increased following Lys<sup>63</sup>-linked ubiquitination by TRAF-6, which is known to act as an E3 ubiquitin ligase in TLR signalling [17]. This IRF-7 ubiquitination occurs in association with the E2 enzyme *UBC13*, and reduced IFN- $\alpha$  and IFN- $\alpha$ 6 promoter activity was observed in cells treated with *UBC13* siRNA (small interfering RNA) [17]. In addition, IRF-7 is ubiquitinated and shows enhanced activity following Epstein–Barr virus infection [17,51]. Specifically, the viral oncoprotein LMP1 (latent membrane protein 1) stimulates RIP (receptor-interacting protein)-dependent Lys<sup>63</sup>-linked ubiquitination of IRF-7, which leads to an increase in ISRE promoter activity in luciferase reporter gene assays in HEK-293 cells [51]. Also, as described above, the TRIM family member Ro52 acts as an E3 ligase to ubiquitinate the transcription factor IRF-8, which positively regulates IL-12p40 production in murine macrophages [41]. Thus activation of IRFs via the ubiquitin pathway following viral infection appears to be equally as important as proteasomal degradation.

## Conclusion

Following viral infection, ubiquitination of the IRF family of transcription factors directly regulates type I IFN signalling by both activating and inhibiting IRF activity. Thus viruses have evolved mechanisms to manipulate the ubiquitin–proteasome pathway to aid viral survival. In addition, proteins such as SUMOs (small ubiquitin-related modifiers) and DUBs (deubiquitinating enzymes) contribute to the regulation of this complex system. Further understanding of the role of ubiquitin in type I IFN pathways may provide cellular targets for both antiviral treatment and autoimmune therapy.

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