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Regulation and function of key RNA processing factors in innate immunity

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1. Abstract

Accurate regulation of innate immune signalling is essential to balance pathogen clearance and immunosurveillance with prevention of chronic inflammation and autoimmunity. While post-translational regulation of interferon- γ (IFN γ) signalling via JAK/STAT factors is well characterised, co- and post-transcriptional mechanisms that shape pathway output remain poorly understood. This thesis systematically investigates how RNA processing contributes to IFN γ signalling and identifies novel RNA regulatory nodes with broad implications for immune homeostasis.

Using a genome-wide CRISPR/Cas9 screen measuring endogenous interferon regulatory factor 1 (IRF1) protein abundance as a proxy for IFN γ signalling, I identified the highly conserved factor Enhancer of Rudimentary Homolog (ERH) and several associated factors – including serine/arginine-rich splicing factor 1 (SRSF1), the exon junction complex (EJC), and the export adaptor ALYREF – as essential regulators of IFN γ signalling. Mechanistic dissection revealed that ERH safeguards proper splicing of AU-rich introns, notably within the *JAK2* transcript, thereby maintaining JAK2 expression and downstream pathway responsiveness. ERH loss caused widespread intron retention and nuclear retention/degradation of affected transcripts, positioning ERH as a critical checkpoint linking splicing, nuclear surveillance, and export, as well as a key enabler of IFN γ -driven immune activation.

Additional work in this thesis characterised further regulatory layers of key innate immune factors involved in transcription and RNA decay. I contributed to the discovery that the E3 ubiquitin ligase receptor speckle type BTB/POZ protein (SPOP) directly ubiquitinates IRF1, controlling its proteasomal degradation and thereby its transcriptional activity. Furthermore, I helped uncover HUWE1 as a dominant regulator of the mRNA decay factor tristetraprolin (TTP), revealing that HUWE1 controls TTP protein stability by modulating previously underappreciated TTP phosphorylation events. This work expands upon previous models of how phosphorylation coordinates TTP turnover with inflammatory resolution.

Together, these studies highlight RNA processing and protein turnover as central regulatory layers of innate immunity. They establish ERH-mediated intron retention control as a pivotal determinant of IFN γ signalling and provide new mechanistic insights into how IRF1 and TTP abundance are precisely tuned to balance effective innate immune defence with the prevention of immunopathology.

2. Zusammenfassung

Die präzise Regulation der angeborenen Immunantwort ist entscheidend, um Pathogenelimination und Immunkontrolle mit der Vermeidung chronischer Entzündungen und Autoimmunität in Einklang zu bringen. Während die posttranslationale Regulation der Interferon- γ -(IFN γ)-Signalübertragung über JAK/STAT-Faktoren gut charakterisiert ist, sind co- und posttranskriptionale Mechanismen, die den Ausgang des Signalwegs beeinflussen, noch weitgehend unverstanden. Diese Dissertation untersucht systematisch, wie RNA-Prozessierung zur IFN γ -Signalübertragung beiträgt, und identifiziert neue regulatorische Knotenpunkte auf RNA-Ebene mit weitreichenden Implikationen für die Immunhomöostase.

Mittels eines genomweiten CRISPR/Cas9-Screens, der die endogene Proteinmenge des interferon regulatory factor 1 (IRF1) als Proxy für die IFN γ -Signalaktivität misst, identifizierte ich den hochkonservierten Faktor Enhancer of Rudimentary Homolog (ERH) sowie mehrere assoziierte Faktoren – darunter serine/arginine-rich splicing factor 1 (SRSF1), der exon junction complex (EJC) und der Export-Adaptor ALYREF – als essenzielle Regulatoren der IFN γ -Signalübertragung. Mechanistische Analysen zeigten, dass ERH das korrekte Spleißen von AU-reichen Introns, insbesondere im JAK2-Transkript, sicherstellt und so die JAK2-Expression und die nachgeschaltete Signalantwort aufrechterhält. Der Verlust von ERH führte zu weitverbreiteter Intron-Retention und nuklearer Retention bzw. Degradation der betroffenen Transkripte und positioniert ERH damit als kritischen Kontrollpunkt, der Spleißen, nukleare Qualitätskontrolle und Export verbindet – sowie als zentralen Ermöglicher der IFN γ -vermittelten Immunaktivierung.

Zusätzliche Arbeiten in dieser Dissertation charakterisierten weitere Regulierungsebenen zentraler Faktoren der angeborenen Immunität, die an Transkription und RNA-Abbau beteiligt sind. Ich trug zur Entdeckung bei, dass der E3-Ubiquitin-Ligase-Rezeptor speckle-type BTB/POZ protein (SPOP) IRF1 direkt ubiquitiniert, dessen proteasomalen Abbau steuert und so seine Transkriptionsaktivität reguliert. Darüber hinaus half ich, HUWE1 als dominanten Regulator des mRNA-Abbauproteins Tristetraprolin (TTP) zu identifizieren, wobei HUWE1 die TTP-Protein-Stabilität durch Modulation bislang wenig beachteter TTP-Phosphorylierungsereignisse kontrolliert. Diese Arbeit erweitert bisherige Modelle

darüber, wie Phosphorylierung den TTP-Umsatz mit der Auflösung von Entzündungen koordiniert.

Zusammenfassend heben diese Studien RNA-Prozessierung und Proteinumsatz als zentrale Regulierungsebenen der angeborenen Immunität hervor. Sie etablieren die ERH-vermittelte Kontrolle der Intron-Retention als entscheidenden Bestimmungsfaktor der IFN γ -Signalübertragung und liefern neue mechanistische Einblicke, wie IRF1- und TTP-Mengen präzise abgestimmt werden, um eine effektive Immunabwehr zu gewährleisten und gleichzeitig Immunpathologien zu verhindern.

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4. Introduction

4.1 Interferon signalling

Interferons (IFNs) are central cytokines of the innate immune system that mediate the first line of defence against pathogens and support the adaptive immune response during recurring encounters. Discovered in 1957 as “interfering” factors secreted from influenza-infected chick embryo cells^{1,2}, IFNs were initially appreciated for their antiviral effects. Decades of research thereafter revealed that their functions extend far beyond this, establishing IFNs as pleiotropic regulators of immunity. They are critical for anti-microbial defence across pathogen types^{3–8}, but also play complex roles in tumour surveillance and escape^{9–13}, and in the development of autoimmune diseases^{14–19}. Their broad impact highlights how IFN activity is essential for protective immunity but, if dysregulated, can also contribute to immunopathology.

Mechanistically, IFNs act by binding distinct cell-surface receptors in a variety of cell types, which activates associated Janus kinases (JAKs). These in turn phosphorylate signal transducer and activator of transcription (STAT) proteins, which translocate to the nucleus and function as transcription factors (TFs) to induce expression of a plethora of IFN-stimulated genes (ISGs). Through this cascade, extracellular cues are translated into diverse cellular outputs, exemplifying how tightly regulated signalling pathways can coordinate broad immune functions.

The winding path of identifying the different IFN signalling pathways²⁰ relied on several instrumental discoveries. First, the identification of IFN-specific gene promotor elements on ISGs^{21–26}, termed interferon-stimulated response element (ISRE)^{27,28} and gamma IFN activation site (GAS)²¹, indicated that different IFNs activate distinct signalling pathways and transcription factor complexes^{29,30}. The subsequent discovery of the JAK family of kinases^{31–38}, in which distinct JAK members were required for different IFN pathways^{39–41}, confirmed that discrete IFN-kinase-STAT modules exist.

Today, IFNs are classified into three groups based on their reliance on distinct receptor complexes and signalling pathways: Type I (including IFN α and IFN β , the most ubiquitously expressed and best-studied members in human), type II (IFN γ), and type III (IFN λ family).

4.1.1 Type II Interferon signal transduction

IFN γ , the only type II IFN, is primarily produced by immune cells such as innate lymphoid cells (ILCs), including natural killer (NK) cells^{42,43} and ILC1s⁴⁴, as well as select adaptive immune cells, primarily CD4⁺ T helper 1 (Th1) cells⁴⁵ and CD8⁺ cytotoxic T lymphocytes (CTLs)⁴⁶. Initiation of IFN γ production can be triggered in multiple ways. In innate immune cells, IFN γ is produced rapidly upon contact with cytokines like interleukin-12 (IL-12) and IL-18^{47,48}, released primarily by professional antigen-presenting cells (APCs)^{49,50}, or by activation of pattern recognition receptors (PRRs) and broadly reactive antigen receptors that detect pathogen- and damage-associated molecular patterns (PAMPs and DAMPs) during infection or tissue damage⁵¹. In this way, IFN γ is rapidly produced at the onset of infection and aids the innate immune response before antigen-specific adaptive immunity is established. In contrast, sustained IFN γ production by adaptive immune cells typically requires T cell receptor (TCR)-mediated recognition and presentation of antigens by APCs through MHC class I and MHC class II molecules⁵².

Once secreted, IFN γ homodimers bind the ubiquitously expressed IFN γ receptor on target cells⁵³. The receptor is composed of two IFNGR1/IFNGR2 heterodimers^{54–56}, which, upon ligand binding, trigger activation of the receptor-associated kinases JAK1 and JAK2 by auto- and trans-phosphorylation events^{57–65}. These kinases phosphorylate STAT1 homodimers, which subsequently undergo conformational changes, translocate to the nucleus, and bind GAS promoter sequences, thereby inducing ISG expression^{21,66–69} (Fig. 1, adapted from Soderholm et al.⁷⁰).

Conceptually, this pathway illustrates the non-redundant architecture of IFN γ signalling. Each component in the JAK/STAT cascade plays an indispensable role, with their loss abrogating downstream transcriptional responses⁷¹. This design highlights how the immune system relies on tightly

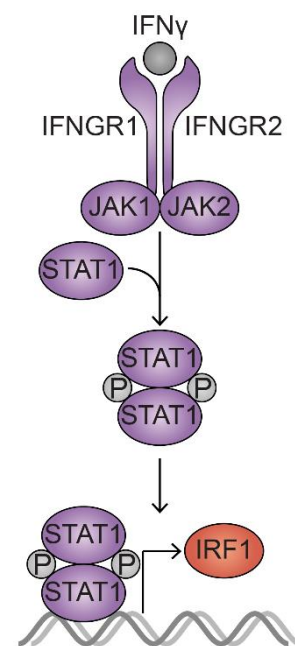


Figure 1 The JAK/STAT pathway. Schematic representation of the IFN γ -activated JAK/STAT pathway, which induces expression of various immune factors, including IRF1 (adapted from Fig. 1A⁷⁰).

coupled, non-substitutable signalling nodes to ensure robust execution of IFN γ -mediated programmes. At the same time, this reliance creates vulnerabilities, as perturbation of core factors can either disable or exaggerate pathway activity, with broad consequences for IFN γ outputs.

4.1.2 Outcomes of IFN γ signalling

The transcriptional programmes induced by IFN γ are broad, with hundreds of induced ISGs that are activated in a cell- and context-dependent manner. These genes encode immediate antimicrobial effectors, as well as secondary TFs that reinforce or extend the response in a feedforward loop⁷². In this way, IFN γ signalling exemplifies how a single cytokine can generate controlled, multilayered, and self-amplifying outputs.

Direct antipathogenic IFN γ effectors include oxidoreductases that increase microbicidal reactive oxygen and nitrogen species (ROS and RNS)^{73–75}, GTPases and cytosolic ubiquitin-binding receptors that promote bacterial engulfment^{76–84}, as well as efflux pumps and catabolic enzymes that restrict access of key nutrients for microbial growth^{85–94}. In addition, IFN γ induces antiviral factors acting at virtually every step of the viral life cycle, from entry^{95–102} and replication^{103–106} to assembly and release^{107–114}.

Beyond these immediate effectors, IFN γ also induces TFs that extend and broaden ISG expression. A central mediator in both type I and type II IFN responses is IRF1^{115–118}. Unlike constitutively expressed IRFs such as IRF9, which drives expression of initial response factors in the type I IFN response¹¹⁹, IRF1 is normally present at low levels and requires STAT1-dependent induction¹¹⁵. Once expressed, IRF1 cooperates with STAT1, IRF8, and other partners to upregulate antigen-presenting molecules^{120,121} and chemokines that recruit adaptive immune cells^{122–124}, thereby bridging innate and adaptive immunity. Furthermore, IRF1 plays a pivotal role in pro-inflammatory (M1) macrophage polarisation and in Th1 differentiation^{125,126}, while suppressing anti-inflammatory programmes such as M2 macrophages^{127–131,125}. This feedforward reinforcement, mediated by factors like IRF1, ensures that IFN γ not only triggers immediate defences but also reshapes the immune landscape to sustain inflammation and adaptive responses.

These IFN γ outputs are executed through a variety of effector immune cells^{132,133}. Macrophages are major IFN γ effectors that exemplify the concept of

“priming”: once stimulated with IFN γ they become hyper-responsive to other inflammatory cues, including cytokines like tumour necrosis factor (TNF)^{118,134} and microbial ligands like lipopolysaccharide (LPS)^{135–138}. This priming is partly mediated by the induction of latent enhancers¹³⁹ and epigenetic reprogramming, which open chromatin at pro-inflammatory loci¹⁴⁰ while depositing repressive marks at anti-inflammatory genes^{141,142}. IFN γ also shapes adaptive responses by skewing naive CD4⁺ T cells away from Th2 and Th17 differentiation, lineages important for macrophage-independent responses^{143–147}, while instead promoting pro-inflammatory Th1 differentiation¹⁴⁸. In parallel, it drives monocytes toward dendritic cells¹³², expanding the pool of professional APCs. Importantly, IFN γ also induces a subset of regulatory T cells (Tregs) that critically restrain over-activation of the inflammatory response as well as the return to tissue homeostasis^{149–151}. Their key role is exemplified by experimental autoimmune encephalomyelitis (EAE), in which loss of IFN γ -induced Tregs exacerbates disease severity^{152,153}.

Together, these cellular programmes illustrate how IFN γ generates potent pro-inflammatory environments. However, they also highlight the need for proper control: the same feedforward loops and epigenetic reprogramming that ensure robust pathogen and tumour clearance can, if unchecked, fuel excessive or chronic inflammation. This delicate balance relies on precise regulatory mechanisms operating at multiple levels of the pathway. Understanding how such mechanisms fine-tune IFN γ outputs remains a central open question, with direct relevance for diseases such as autoimmunity and cancer.

4.1.3 Molecular regulatory mechanisms of IFN γ signalling

At the core of IFN γ signalling is the JAK/STAT cascade, in which components act as tightly coupled, non-redundant nodes. Regulation of these central players has therefore been a major focus of past research, particularly at the protein and post-translational level. The canonical example is STAT1 activation: activated JAK kinases^{57–65} phosphorylate Tyr701 on STAT1⁶⁸, inducing its nuclear translocation. Additional serine phosphorylation, notably on Ser727, further enhances STAT1 activity at specific genes^{154–156}. These non-canonical inputs, mediated by kinases such as cyclin-dependent kinase 8 (CDK8, a component of the transcriptional coactivator complex Mediator) or stress-induced p38/mitogen-activated protein kinase

(p38/MAPK) signalling^{156–159}, illustrate how external pathways can fine-tune IFN γ responses rather than functioning as a simple on/off switch.

Such activation is counterbalanced by equally critical inhibitory mechanisms. First, protein tyrosine phosphatases (PTPs) inhibit the pathway at multiple levels^{160,161}: T cell PTP (TC45) dephosphorylates nuclear STAT1¹⁶², while PTP1B and CD45 target receptor-associated JAK kinases^{163,164}. Secondly, suppressor of cytokine signalling (SOCS) proteins are induced as part of a negative feedback loop^{165–167}. SOCS1 and SOCS3 bind to JAK1 and JAK2 to directly inhibit their activity^{168–172}, and further recruit E3 ubiquitin ligase complexes that target the kinases for degradation^{173–175}, thereby reducing their protein abundance. Similarly, the protein inhibitor of activated STAT (PIAS) family exert control on STATs^{176,177}, with PIAS1 binding nuclear STAT1 and blocking its DNA-binding^{177,178}. Its protein abundance is also similarly regulated by the action of ubiquitin E3 ligases^{179,180}. Collectively, these protein and post-translational regulators shape both the intensity and the temporal resolution of IFN γ signalling, preventing rampant amplification.

Beyond direct control of JAKs and STATs, IFN γ signalling is also shaped by transcriptional cofactors and chromatin dynamics. A central mediator is IRF1, which cooperates with STAT1 and IRF8 to expand the repertoire of ISGs by binding GAS or IRF motifs in promoter regions¹⁸¹. Efficient induction requires remodelling of local chromatin, with STAT1 recruiting histone acetyltransferases such as CREB-binding protein (CBP)/p300 that establish activating histone modifications¹⁸², promoting DNA accessibility and faster transcriptional elongation^{181,183}. While such mechanisms were appreciated two decades ago, only the development of high-throughput methods such as chromatin immunoprecipitation (ChIP) sequencing made genome-wide characterisation possible, revealing how IFN γ rapidly remodels DNA accessibility at ISG loci¹⁸⁴. More recently, cross-talk with stress pathways like p38/MAPK has been shown to further enhance ISG expression by similar deposition of activating histone marks and recruitment of additional coactivators¹⁸⁵. Altogether, these insights highlight that regulation of IFN γ responses extends well beyond post-translational control of JAK/STAT proteins and encompass mechanisms such as chromatin remodelling.

By contrast, co- and post-transcriptional RNA regulation of IFN γ signalling remains far less explored, despite being well established as critical control points in other innate immune pathways^{186–188}. Toll-like receptor (TLR) signalling exemplifies this well, in which every *TLR* gene encodes multiple alternatively spliced isoforms with

distinct features that modulate pathway activity^{189–193}. For example, one isoform of *TLR4* produces a truncated protein that dampens TNF expression in response to LPS, thereby limiting excessive inflammation^{190,191}. Such regulation is mediated by splicing factors like the SF3a complex, which preferentially target innate immune transcripts encoding signalling factors¹⁹⁴. Alternative splicing also shapes macrophage activation and development^{195,196}, with heterogeneous nuclear RiboNucleoProtein M (hnRNP M) playing a key role in regulating macrophage ISG expression¹⁹⁷. Rapid RNA decay provides yet another checkpoint, exemplified by tristetraprolin (TTP) that binds AU-rich elements (AREs) in induced mRNAs and drives their turnover^{198–200}, thereby ensuring proper resolution of inflammation²⁰¹. Taken together, these examples underscore the central role of RNA-level control in innate immunity; a layer of regulation that remains largely unexplored for IFN γ signalling.

Systematic exploration of RNA processing in IFN γ signalling has likely been hampered by the cell essential nature of many RNA regulators. This essentially stems from their broad control of numerous transcripts, including those required for fundamental cellular functions^{202,203}. As a result, genetic screening approaches often miss them, because cells with disrupted RNA processing often die before they can be analysed. For example, while a gene trap-mediated genetic screen of IFN γ signalling successfully identified all canonical JAK/STAT factors, which are not essential for viability, it likely overlooked RNA-processing components due to insufficient recovery of viable mutants⁷¹. Consequently, although microRNAs have been shown to modulate *STAT* mRNA abundance and thus STAT protein levels^{204–206}, the role of RNA-binding proteins and splicing/export factors remains unexplored for IFN γ . This represents a critical gap in the field: while protein-level regulation of IFN γ signalling is well established, RNA-based regulation may be an equally important but underappreciated layer of control.

The need to better understand how IFN γ signalling is regulated becomes especially clear in disease contexts. In particular, autoimmunity and cancer provide striking examples in which unchecked IFN γ activity directly contributes to pathology.

4.1.4 IFN γ in autoimmune diseases

The potent pro-inflammatory activity of IFN γ , essential for host defence, also makes it a potential driver of autoimmune disease. Sustained activation of ISGs, also

detectable as an “IFN γ signature”, is a hallmark of several autoimmune disorders, including systemic lupus erythematosus (SLE)^{15,17,207}, rheumatoid arthritis (RA)²⁰⁸, multiple sclerosis (MS)^{209–213}, Sjögren’s syndrome (SjS)^{214,215}, and autoimmune mice models^{216–218}. Beyond transcriptional signatures, IFN γ -regulated immune cells directly contribute to pathology, with macrophage infiltration commonly being observed in autoimmune lesions^{219,220}, and RA patients displaying primed monocytes²²¹. These features highlight the potential of IFN γ in perpetuating rampant inflammation and tissue damage.

Given this prominent role, IFN γ and its downstream kinases have long been pursued as therapeutic targets. Indeed, multiple JAK inhibitors are already in clinical use for treatment of RA²²², including the JAK1/JAK2 inhibitor “baricitinib”, approved in 2017^{223,224}. However, as baricitinib also targets type I IFN signalling via JAK1, its beneficial effects cannot be attributed solely to IFN γ inhibition²²⁵. Other approaches aimed more directly towards this signalling, including monoclonal neutralising antibodies against IFN γ in SLE, advanced to phase I clinical trials and demonstrated acceptable safety profiles. However, although they showed promising molecular effects, such as reduced IFN γ signatures, little to no clinical benefit was observed^{226–228}. Similarly, JAK1/JAK2 inhibition with baricitinib did not convincingly improve primary or secondary health outcomes in two large phase III clinical trials for SLE^{229,230}. Likely due to this limited success, several IFN γ -targeting strategies have been discontinued for further clinical development in several autoimmune diseases^{228,230–232}.

One explanation for these disappointing outcomes is the potential paradoxical nature of IFN γ in autoimmunity. MS illustrates this complexity well: IFN γ contributes to pathology, yet in the related demyelinating disease model EAE, it also exerts protective effects through multiple self-regulatory mechanisms^{233–236}. For example, IFN γ can suppress pathogenic self-reactive Th17 cells via IL-27 induction²³⁷, promote immune suppression through nitric oxide synthase (NOS) production^{238,239}, and expand Treg populations that dampen inflammation^{240,241}. The helpful or harmful effect of IFN γ therefore depends on contextual variables such as disease timing and the affected tissue²³⁴.

Altogether, these observations highlight both the therapeutic potential and the risks of targeting IFN γ . Its activity is not inherently pathogenic but instead demands precise regulation. The molecular mechanisms that fine-tune its outputs remain incompletely understood, particularly beyond the well-characterised protein-level

controls. Deciphering these additional layers of regulation may help explain why broad IFN γ blockade has failed clinically and could point toward more refined, context-specific strategies better suited for a precision medicine approach.

4.1.5 IFN γ in cancer

Similarly to its role in autoimmune diseases, properly regulated IFN γ signalling is critical for anti-tumour immunity. On one hand, IFN γ exerts multiple anti-tumour effects and is a key mediator of tumour surveillance. On the other hand, chronic or dysregulated signalling can paradoxically promote tumour progression and metastatic spread (Fig. 2). This duality has major implications for cancer therapies, particularly for the breakthrough treatment that is immune checkpoint blockade (ICB), where IFN γ can act as both a mediator of therapeutic success and a driver of resistance.

The first indications that IFN γ signalling protects against tumour development came from loss-of-function studies. Cancer cell lines lacking the receptor component IFNGR1 displayed enhanced tumourigenicity¹⁰, and mice deficient in JAK/STAT signalling factors were more susceptible to induced cancers, an effect reversible by reintroduction of the missing factors²⁴². More recently, acquired resistance to IFN γ signalling through JAK/STAT deficiency has been shown to aid tumour immune evasion in cancers such as melanoma and chronic myeloid leukaemia²⁴³. Today, it is well established that IFN γ is a key mediator of tumour immunosurveillance.

Mechanistically, IFN γ acts on virtually all cellular compartments of the tumour environment, including cancer cells, immune cells, and the local stromal tissue. In tumour cells, it inhibits proliferation by upregulating expression of CDK inhibitor 1 (p21), and in cooperation with TNF drives permanent senescence through activation of the CDK inhibitor p16^{244,245}. IFN γ also induces tumour apoptosis by promoting expression of caspases^{246–251}, FAS and FAS ligand²⁵², and TNF-related apoptosis-inducing ligand (TRAIL)^{253,254}. Beyond direct effects on cancer cells, IFN γ reshapes the immune microenvironment. Macrophages acquire a tumouricidal phenotype^{255,256}, suppressing anti-inflammatory and tumour growth factors²⁵⁷, and their presence often correlates with better health outcomes^{258–262}. In parallel, IFN γ -induced chemokines such as CXCL9/10/11 recruit T cells, NK cells, and NKT cells^{263–265}, while enhancing the tumouricidal activity of key CD8⁺ T cells²⁶⁶. Finally, IFN γ also acts on non-immune stromal components by reducing vascular growth factor production in fibroblasts and

impairing endothelial cell proliferation and survival, which effectively inhibits angiogenesis and induces tumour ischemia^{267–271}.

Together, these studies highlight how IFN γ can act on multiple cell types in the tumour microenvironment to mount a coordinated anti-cancer response. Yet, the very same mechanisms that provide protection can, in different contexts, shift toward tumour-promoting outcomes. Notably, many of these protective mechanisms were identified in reductionist experimental systems with tightly regulated IFN γ signalling. Tumour environments are complex and heterogeneous, and cancers that evolve under chronic or dysregulated IFN γ signalling often exploit these same pathways for growth and survival.

IFN γ can foster tumour-promoting conditions in several ways. A major mechanism is through its own immunosuppressive feedback loops, in which it induces inhibitory checkpoint receptor ligands such as programmed death-ligand 1 (PD-L1)^{272–279} and anti-inflammatory enzymes like indoleamine-2,3-dioxygenase (IDO)^{278–283}, dampening immune activity and promoting resistance to immunotherapy^{284,285}. These inhibitory programmes likely evolved to restrain excessive inflammation but are abused by cancers to enable immune evasion. Furthermore, chronic IFN γ signalling, due to loss of regulatory breaks²⁸⁶ or mutations that drive constitutive JAK/STAT activation^{287–289}, can itself drive tumourigenesis. For example, IFN γ can enhance metastatic potential^{290–293} by inducing migratory stimulating chemokines²⁹⁴ and promoting dissociation of blood vessel pericytes²⁹⁵. Even its anti-tumourigenic functions can backfire, as IFN γ -induced apoptosis in low tumour burden states may deplete essential immune populations, including effector T cells²⁹⁶. Notably, the outcome often depends on dose, timing, and cellular context, with prolonged low IFN γ levels likely promoting metastasis, whereas acute, high-intensity signalling tends to be tumouricidal²⁴⁸. This context dependence helps explain why IFN γ can be either protective or harmful in different cancer settings.

The importance of IFN γ 's dual role is highlighted in cancer immunotherapy, particularly in the context of ICB. This treatment blocks inhibitory receptors such as CTLA4 and PD1 on effector T cells, relieving immune cells of negative signals that would otherwise prevent killing of cancer cells^{284,297,298}. Therapeutic success is seemingly dependent on IFN γ activity. In responsive patients, ICB normally promotes IFN γ production within the tumour environment^{299–302}, and an IFN γ signature is a predictive marker for ICB efficacy^{303–307}. By contrast, non-responsive tumours often

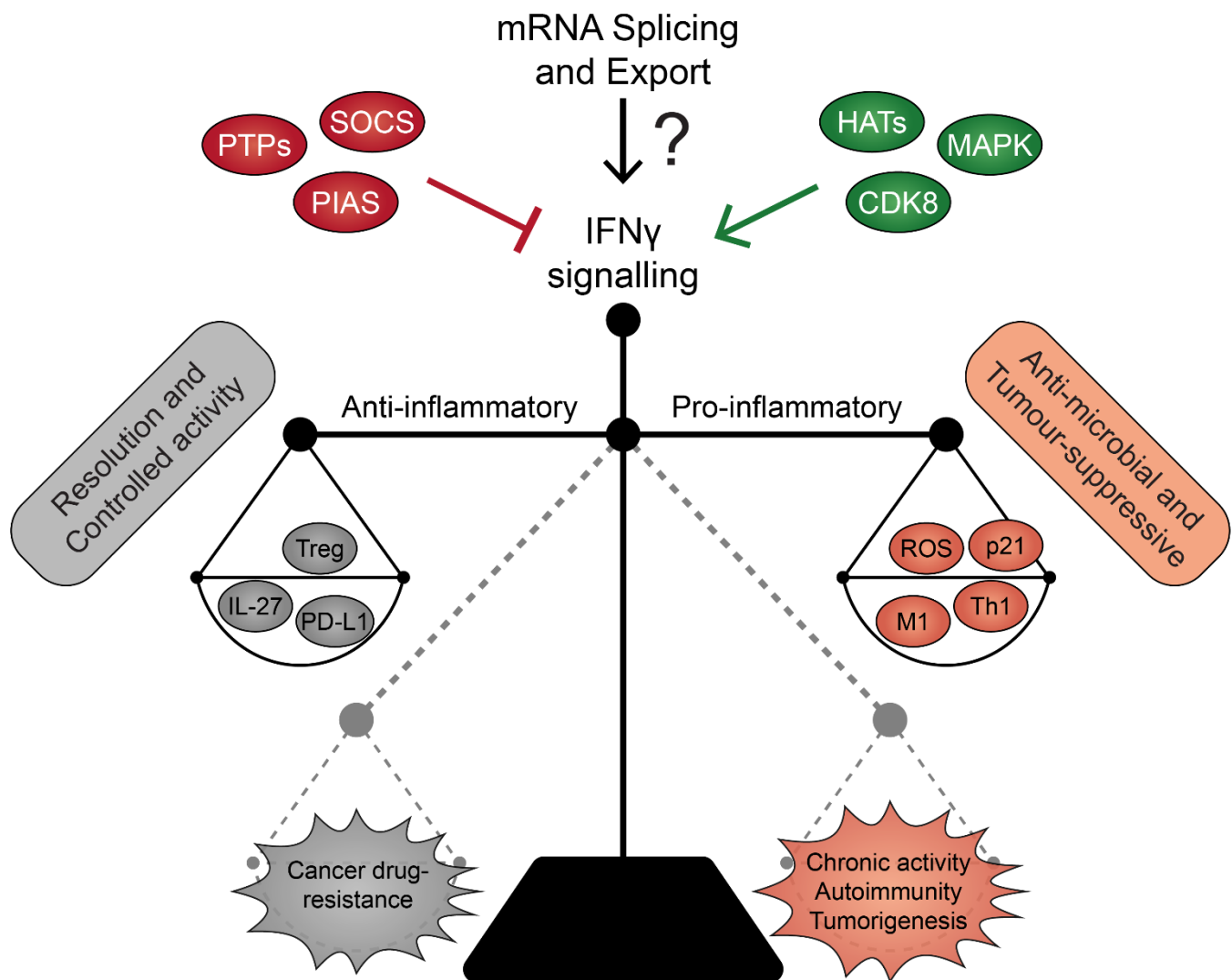


Figure 2 IFN γ signalling is tightly regulated to ensure balanced innate immune activity. IFN γ signalling is well recognized to be regulated post-translationally by multiple inhibitory and facilitating factors. In contrast, the contribution of RNA processing to this regulation has remained largely unexplored. The balance of IFN γ pathway activity has major consequences for immune outcomes: appropriate pro- and anti-inflammatory functions are critical for anti-microbial defence, tumour suppression, and resolution of inflammation, whereas dysregulated or chronic activity can drive autoimmunity, tumorigenesis, and resistance to cancer therapies.

harbours defective mutations in JAK/STAT factors or downstream mediators like IRF1^{308–310}. Consistently, genome-wide CRISPR screens have identified defects in IFN γ signalling as major contributors to immunotherapy resistance and loss of cytotoxic T cell function^{307,311}. In light of these findings, early-phase clinical trials combining IFN γ and immunotherapy have shown promising results^{312,313}, although larger studies are needed to establish efficacy.

At the same time, IFN γ can also underlie resistance to ICB. By inducing factors like PD-L1 and IDO, IFN γ signalling blunts antitumour immunity and fosters tumour escape^{284,297,298}. This paradox may explain why some patients fail to respond to ICB despite exhibiting robust IFN γ activity. Importantly, inhibition of these IFN γ -induced factors has shown significantly improved pre-clinical and clinical results for antitumour activity and ICB response^{314–317}, underscoring that the therapeutic impact of IFN γ depends not only on its induction, but also on how its downstream suppressive programmes are managed.

Altogether, IFN γ emerges as both a driver of therapeutic success and a potential contributor to resistance. Its combinatorial usage with other conventional treatments, such as chemotherapy or radiation, has shown great potential^{318–321}. The challenge ahead is to harness its tumouricidal functions while avoiding its potential tumour-promoting effects. Meeting this challenge will require a deeper understanding of the molecular mechanisms that fine-tune IFN γ responses. While protein-level regulators have been studied extensively, less explored layers such as RNA processing may be critical checkpoints that shape IFN γ outcomes. Identifying these RNA regulatory mechanisms could help guide more precise therapeutic strategies.

4.2 Mechanisms of RNA regulation

RNA regulation underpins virtually every step of gene expression, from transcript maturation to decay, and is critical for shaping immune responses. Yet its systematic dissection in specific pathways, such as IFN γ signalling, has long been hindered by the fact that many RNA processing factors are essential for cell viability. A central challenge is therefore to disrupt their function in a time-controlled manner, so that regulatory effects can be measured before cells lose viability.

RNA interference (RNAi) was one of the first broadly adopted approaches. By introducing short interfering RNAs (siRNAs) or related precursors^{322–324}, transcripts can be selectively degraded through the cell's endogenous machinery³²⁵. RNAi can enable efficient knockdowns within days, and has been widely utilised in screens to identify pathway regulators^{326–329}. However, it comes with notable drawbacks, including off-target effects³³⁰ (possible with as little as seven complementary nucleotides³³¹) and incomplete loss-of-function, particularly for stable proteins³³². While improved RNAi strategies have mitigated some of these issues^{326,332}, the method remains limited for systematic dissection of essential factors.

CRISPR/Cas9 gene editing has since emerged as a powerful alternative³³³. By programming Cas9 with single guide RNAs (sgRNAs), frameshift mutations can be introduced via the cell's own error-prone non-homologous end joining (NHEJ) DNA repair pathway, thereby generating complete gene knockouts (KOs) rather than knockdowns³³⁴. Although off-target effects were initially a concern³³⁵, improved tools and algorithms now allow selection of optimal sgRNAs with both high efficiency (enhanced on-targeting) and specificity (limited off-targeting)³³⁶. Importantly, inducible Cas9 systems, for example under a doxycycline (dox) promoter, allow time-controlled KOs, enabling the study of genes that are otherwise essential for long-term cell survival. These advances have established CRISPR/Cas9 as the often preferred method for unbiased genetic screens, enabling identification of regulatory pathway factors irrespective of their impact on viability^{337,338}. In this thesis, this approach provided the foundation for uncovering key RNA regulators of IFN γ signalling.

In the following sections I will follow the RNA maturation process from initial synthesis to degradation, highlighting regulatory factors that we initially investigated or subsequently identified as critical for innate immunity, with a focus on IFN γ signalling (Fig. 3).

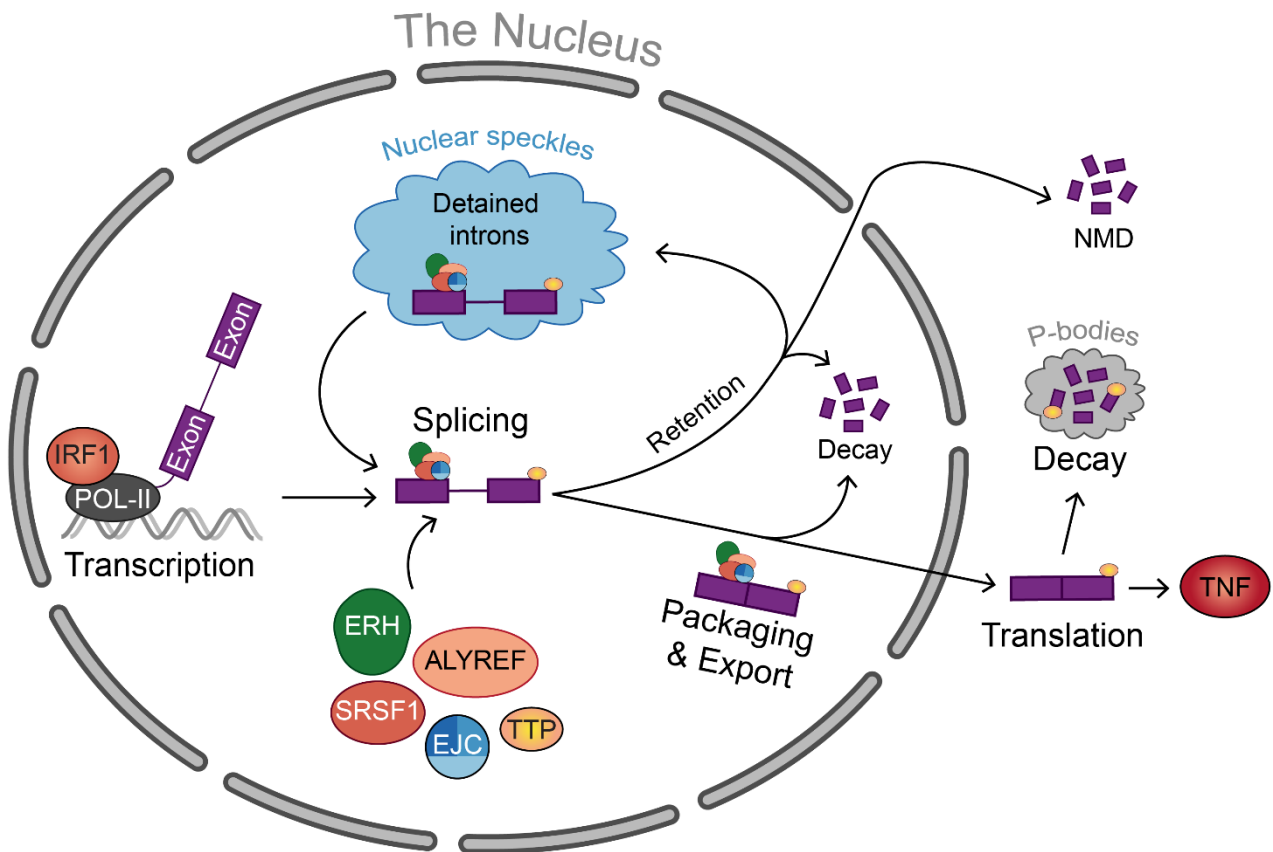


Figure 3 RNA processing as a critical regulatory layer of innate immunity. Schematic overview of mRNA maturation and decay pathways, highlighting factors investigated in this thesis. Transcription produces nascent mRNAs under the control of transcription factors such as IRF1. Co- and post-transcriptional steps involve multiple regulators, with ERH, ALYREF, the EJC, and SRSF1 identified here as essential for IFN γ signalling. Alternative splicing can generate transcripts with retained introns, which may be detained in nuclear speckles, degraded in the nucleus, or exported and eliminated by nonsense-mediated decay (NMD). Properly spliced mRNAs are packaged, exported, and translated, with TTP promoting degradation of cytokine mRNAs during resolution of inflammation.

4.2.1 Transcription – the birth of RNAs

Although this thesis primarily focuses on co- and post-transcriptional RNA regulation, some of the investigated factors have been proposed to function in transcription itself. Such associations, however, may actually reflect their roles in RNA processing rather than direct DNA regulation. To place this in context, I briefly review how genes are transcribed and highlight where uncertainties remain.

Transcription of messenger RNAs (mRNAs) is predominantly carried out by RNA polymerase II (Pol II) in three interconnected stages: initiation, elongation, and termination^{339–341}. Each stage is heavily shaped by the local chromatin state³⁴² and

the activity of DNA-bound regulators such as TFs³⁴¹. While the outlines of these stages are well established, the contribution of auxiliary protein “cofactors” that bridge Pol II, chromatin, and RNA processing remains an area of active investigation.

Initiation requires Pol II access to the promoter region at the transcription start site (TSS)³⁴⁰. Nucleosomes represent dense DNA-chromatin structures^{343–345} that impede Pol II^{346,347} and compete with TFs for DNA binding^{348–351}. TFs can counteract this by binding distal enhancers^{352–354}, by directly binding nucleosomal DNA³⁵⁵, and by recruiting histone modifiers that relax the chromatin structure³⁵⁶. TFs also recruit additional key factors³⁵⁷, including the Mediator complex that phosphorylates Pol II to enable productive initiation³⁵⁸. Although these principles are widely accepted, initiation is highly gene- and context-specific, raising open questions about how different cofactors dictate promoter selectivity and enhancer-promoter communication.

During elongation, Pol II frequently pauses ~50 base pairs (bp) downstream of the TSS^{359–361}. Release from this promoter-proximal pause is a major regulatory checkpoint³⁶² that can restrict how often transcription is initiated and thus regulate the rate of RNA production^{363,364}. For example, oncogenic MYC can stimulate pause release and elongation, with significant consequences for gene expression³⁶⁵. Chromatin accessibility also modulates elongation rate, with faster elongation altering splicing decisions. Notably, high accessibility and rapid elongation can lead to the inclusion of introns in the final transcript^{366,367}. This intimate coupling of elongation and RNA processing suggests that proteins historically described as “elongation factors” may in fact exert their influence through RNA-binding or RNA-processing activities.

Lastly, termination ensures the release and efficient recycling of Pol II³⁶⁸, and is closely coordinated with 3'-end processing such as polyadenylation^{368,369}. Models such as the “allosteric”³⁷⁰, “torpedo”³⁷¹, and unified models^{372,373} converge on the idea that termination is tightly linked to RNA maturation and export, making it less of a clean endpoint and more of a regulatory node that integrates transcription with downstream RNA fate^{368,374}.

Despite decades of work, distinguishing direct transcriptional regulators from indirect co- and post-transcriptional factors remains challenging. Proteins detected at chromatin or Pol II complexes, and those shown to influence RNA abundance, are sometimes classified as transcription-regulatory factors, even when their primary functions may lie in RNA processing. This blurring of categories underscores that transcription is not three discrete stages but a continuum dynamically shaped by

chromatin, regulatory factors, and tightly coupled RNA processing events. A central open question is how factors at the transcription-RNA processing interface truly exert their function: do they regulate transcription directly, or are they mis-annotated RNA regulators acting co/post-transcriptionally?

This question is particularly relevant for Enhancer of Rudimentary Homolog (ERH), one of the central factors investigated in this thesis. ERH has been proposed to act in transcription elongation, but much evidence points instead towards functions in RNA processing. In the following section, I will review what is known about ERH's involvement in transcription and discuss how its activities fit within this broader framework of transcription-RNA regulation.

4.2.2 Enhancer of Rudimentary Homolog (ERH) – a transcriptional regulator?

A major focus in this PhD was to investigate novel RNA regulators of IFN γ signalling, among which ERH was a central factor. ERH was originally discovered in *Drosophila melanogaster* as e(r)³⁷⁵, and has since been recognised as highly conserved from yeast³⁷⁶ to humans³⁷⁷. Although it cannot bind DNA or RNA directly, ERH has been implicated in diverse RNA regulatory processes, seemingly through interactions with other RNA-binding proteins (RBPs) and protein complexes³⁷⁸.

In human cells, ERH was initially implicated in transcriptional elongation. Affinity purification followed by mass spectrometry (AP-MS) identified ERH as an interactor of the Pol II C-terminal domain phosphatase FCP1³⁷⁹ and the elongation factor SPT5³⁸⁰. However, no direct functional involvement in elongation has been demonstrated, and these associations remain unsubstantiated by additional interaction evidence³⁷⁸. Instead, this model has mostly fallen in favour of ERH being involved in other co- and post-transcriptional processes such as splicing^{381–383} (covered later on) or in chromatin regulation³⁸⁴.

Through chromatin regulation, ERH may indirectly influence transcriptional output. It was identified among 172 proteins enriched in sonication resistant chromatin (srHC) marked by H3K9me3, a model of dense heterochromatin characterised by low transcriptional output³⁸⁵. Notably, ERH was one of the most critical factors for maintaining H3K9me3 heterochromatin and thereby repression of srHC-associated genes, including both protein-coding genes and repetitive elements (particularly

endogenous retroviruses, ERVs)³⁸⁴. However, the underlying molecular mechanism remains unresolved.

One candidate functional protein partner is scaffold attachment factor B (SAFB), which was also enriched in srHC^{384,385}, is known to stabilise the heterochromatin architecture³⁸⁶ and repress repetitive elements³⁸⁷, and directly interacts with ERH³⁸⁸. This makes SAFB a plausible mediator of ERH's effect on chromatin. On the other hand, chromatin changes observed in ERH-depleted cells could represent secondary effects rather than a direct role. Paradoxically, a different proteomic study identified ERH among the most enriched proteins in euchromatin³⁸⁹, raising the possibility that ERH functions across distinct chromatin contexts, or that these associations reflect indirect or context-dependent effects.

Taken together, current data suggest that ERH significantly contributes to transcriptional repression of specific genes and repetitive elements, likely through chromatin-associated mechanisms. Yet whether this represents a direct transcriptional role or an indirect consequence of RNA processing remains an open question.

4.2.3 Regulation of the major transcription factor IRF1

Another focus in this PhD was to investigate how IRF1, a key TF in IFN immune responses, is regulated. As previously described, the transcriptional upregulation of ISGs downstream of IFN γ critically relies on activated STAT1 and IRF1^{115–118}. Whereas post-translational regulation of upstream signalling factors has been rather well characterised, for example the negative feedback of SOCS proteins on JAKs¹⁶⁷, the dynamic regulation of the IRF1 protein is less understood.

IRF1 is a short-lived protein³⁹⁰, a feature considered particularly important for immune homeostasis, in which rapid turnover allows resolution of inflammatory responses to prevent chronic activation and auto-inflammation^{207,391}. At the same time, IRF1 protein levels must be maintained at sufficient basal levels to exert its tumour-suppressive functions at homeostasis^{392,393}. This balance is seemingly mediated by proteasomal degradation^{394,395}. Some candidate E3 ligases, including FBXW7³⁹⁶, MDM2³⁹⁷, and CHIP³⁹⁷, have been described to target IRF1 for ubiquitination, identified through targeted experimental investigations. Their relevance, however, varies between studies, suggesting that IRF1 turnover may depend on cellular context, IRF1 localisation, and the functional engagement of IRF1 in transcription^{396,397}.

Importantly, no unbiased cell-based screens have been performed to identify regulators of IRF1 protein stability. Given the precedent from other short-lived TFs such as MYC^{398–401} and p53⁴⁰², which are regulated by multiple E3 ligases depending on context, it seems likely that additional, possibly critical, E3 ligases for IRF1 remain to be discovered. Thus, despite substantial work on IRF1's transcriptional functions, the molecular mechanisms that govern its own regulation remain open for further investigation.

4.2.4 RNA pre-processing – how splicing forms the mature transcript

Together with and after transcription, pre-mRNAs undergo extensive processing to become stable and functional mature mRNAs. The major changes include 5'-end capping, 3'-end processing, and splicing. I will briefly outline these processes with emphasis on splicing, as it emerged highly relevant to factors investigated in this thesis.

At the 5'-end, capping occurs early, often in coordination with the transition from transcription initiation to elongation⁴⁰³. This process involves recruitment of several capping enzymes to Pol II^{404,405} and produces a 7-methylguanosine (m⁷G) cap that protects both pre- and mature mRNAs from 5' to 3' exonucleolytic degradation^{406,407}. Beyond stability, the cap is recognised by cap-binding proteins such as the cap-binding complex (CBC)⁴⁰⁷ and eIF4E^{408,409}, which recruit splicing and translation initiation factors^{407,410}, thereby bridging these processes. This coordination underscores that RNA maturation is not a group of isolated steps but rather regulated interconnected layers spanning splicing, stability, and translation.

At the 3'-end, cleavage and subsequent polyadenylation occur in close coordination with termination of transcription as Pol II reaches gene ends^{368,411}. Following Pol II recognition of polyadenylation signals (PASs)⁴¹², the RNA is cleaved and polyadenylated at polyadenylation sites, often within the 3' untranslated region (3'UTR)^{413,414}. This processing requires a large protein complex^{415–418} and produces a 3' poly(A) tail of variable length⁴¹⁹ that, similar to the 5' cap, protects the transcript from degradation^{420,421} while facilitating downstream nuclear export⁴²² and translation⁴²³. The usage of alternative PASs (alternative polyadenylation, APA) adds further complexity by generating transcript isoforms that differ in coding regions or 3'UTRs⁴²⁴.

Importantly, 3'UTRs harbour binding sites for numerous RBPs, such as ARE-binding RBPs (AU-RBPs), that shape mRNA stability and fate⁴²⁵.

Splicing is the most sequence-altering step, in which introns are removed and exons joined to generate correctly coded, functional mRNAs⁴²⁶. Although introns likely stem from limited autocatalytic self-splicing sequences^{427,428}, they are now a defining feature of complex eukaryotic transcriptomes, including that of humans^{429,430}. On the surface, splicing appears inefficient, with cells spending much energy to transcribe lengthy sequences only to then remove them. However, splicing generates critical proteomic diversity through alternative splicing^{426,427,431–434} and introns contribute additional regulatory functions through sequence motifs^{435,436}. From an evolutionary perspective, introns also likely facilitated exon shuffling^{437,438}, further illustrating how splicing adds a fundamental layer of regulatory capacity and complexity.

Most introns are excised by the spliceosome, a highly dynamic machinery composed of small nuclear ribonucleoprotein (snRNP) complexes (U1/2/4/5/6) and over 150 additional proteins^{439–441}, that recognises intronic 5' and 3' splice sites (5'ss and 3'ss). Recognition of the 5'ss is initiated by U1, whose binding affinity depends on conserved but variable sequence motifs that determine the 5'ss strength^{442,443}. Nearby sequence motifs and their corresponding RBPs can also aid 5'ss recognition, particularly for weak 5'ss^{444–446}. Recognition of the 3'ss is initiated by splicing factor 1 (SF1) and the U2AF heterodimer, and later reinforced by U2, SF3B1, and PHF5A^{447–450}. Collectively, these factors identify the 3'ss, the branch point, and the polypyrimidine tract^{449,450}. Importantly, splice site recognition and subsequent splicing are extensively modulated by regulatory sequences and their RBPs^{451–454}, transcription kinetics such as Pol II elongation rates^{455–457}, and the RNA structure^{458,459}. Moreover, splice site pairing is not restricted to adjacent sites but can also occur across internal introns and exons^{460,461}. Once paired, U4/5/6 snRNPs and additional factors stabilise the connection^{441,462}, allowing the intron to be excised as a lariat and the exons ligated to generate an uninterrupted coding sequence^{439,441}.

While the biochemical outlines of splicing are well established, critical questions remain about specificity. Different splicing factors preferentially regulate distinct mRNA subsets, but the underlying principles are not always clear. Intrinsic RNA features, such as weak splice sites, auxiliary motifs, or RNA secondary structures, may create dependencies on specific factors. At the same time, evolutionary diversification of splicing machineries may have enabled fine-tuned, context-specific control of

transcript subsets. Addressing these questions is essential to explain why some genes are highly sensitive to disruption of specific splicing regulators, whereas others are unaffected.

The importance of fine-tuned splicing is highlighted by alternative splicing, occurring in up to 95% of multi-exon human genes^{463–465} to generate proteomic diversity through mechanisms such as exon skipping, alternative splice site usage, mutually exclusive exons, and intron retention (IR)⁴⁶⁶. Among these, IR, which is the inclusion of one or more introns in the mature polyadenylated mRNA, also has other interesting functions.

Originally considered merely an error of splicing⁴⁶⁷, IR is now recognised as widespread⁴⁶⁸ and functionally versatile. It contributes to transcriptome plasticity and gene expression control^{367,469–471}, plays key roles in cell development and differentiation^{195,471–473}, shapes cell type-specific programmes^{196,474,475}, and adds to disease complexity by diversifying cancer transcriptomes^{476,477}. In immune contexts, IR has emerged as a key mechanism for stage-specific regulation. For example, coordinated IR in inflammatory regulators modulates macrophage development and activation, tuning their responsiveness to stimuli^{195,196}. Similarly, B-cell maturation is accompanied by dynamic IR programmes enriched in splicing regulators, suggesting that IR acts as a developmental checkpoint⁴⁷¹. These examples illustrate how dynamic IR can serve as a key regulatory mechanism of immune cell development and activity. Mechanistically, the fate of IR-containing mRNAs varies. Some are retained and degraded in the nucleus^{478–480}, while others persist as stable “detained introns” that can be rapidly spliced in response to stress, DNA damage, or infections, thereby serving as transcript reservoirs for fast activation^{475,481–484}. Exported IR-containing mRNAs often harbour premature termination codons (PTCs) and are degraded by nonsense-mediated decay (NMD) in the cytoplasm, thereby downregulating gene expression^{485–490}. Yet others escape cytoplasmic degradation and encode novel protein isoforms^{491–493}.

Despite these advances, major questions remain about how IR is specified for different mRNA subsets. Is retention determined primarily by local intronic/exonic sequences and structural RNA features, or imposed contextually by transcription kinetics and RBP availability? Emerging high-resolution sequencing and computational methods now provide the means to dissect these layers, offering the

potential to clarify how IR is regulated. Such insights will be particularly important for immune pathways, where IR can act as a key regulatory mechanism.

4.2.5 ERH – a potential splicing regulator

Both phenotypic studies and interaction assays have implicated ERH in splicing. ERH was first linked to this process in 2012, with decreased *ERH* expression impairing splicing of the mRNA encoding the mitotic motor protein CENPE, significantly reducing viability in KRAS-mutant cancer cells³⁸¹. Subsequent studies reported widespread splicing defects upon *ERH* knockdown, affecting hundreds to over a thousand transcripts, particularly those encoding DNA replication and repair factors such as ATR^{382,383}. However, these studies investigated specific transcripts³⁸³ or relied on rRNA-depleted, whole-cell RNA sequencing comparing total intronic and exonic levels³⁸², which cannot clearly distinguish splicing changes from altered transcription or nuclear export. Moreover, since cytoplasmic mRNAs dominate over nuclear species, splicing changes in transcripts restricted to the nucleus could have been missed. The precise nature of ERH-dependent splicing defects, and their consequences for transcript fate, thus remain unresolved.

At the mechanistic level, it is unclear how ERH engages with the splicing machinery. The spliceosome component SNRPD3^{494,495} was initially suggested to mediate ERH's role in *CENPE* splicing³⁸¹, but no direct interaction has since been confirmed. Through short cross-linking mass spectrometry (XL-MS), ERH was instead identified in complex with THRAP3 and BCLAF1, together forming the proposed TEB complex⁴⁹⁶. Since THRAP3 and BCLAF1 have described roles in splicing and nuclear export of a subset of mRNAs⁴⁹⁷, ERH could act here as a stabilising or scaffolding factor. ERH also directly binds SAFB, with proposed effects on phosphorylation of serine/arginine-rich splicing factor (SRSF) proteins³⁸⁸, though the functional significance of this remains unclear. Lastly, ERH was found in the interactome of the exon junction complex (EJC)⁴⁹⁸, a key regulator of RNA processing that participates in splicing, discussed in detail below. In this context, ERH can directly interact with DNA polymerase delta interacting protein 3 (POLDIP3)⁴⁹⁹, a multifunctional RNA regulator implicated in splicing⁵⁰⁰.

Taken together, ERH associates with multiple splicing-related proteins, but whether these interactions are redundant, context-specific, or functionally critical

remains an open question. Equally unclear are the defining features of ERH-sensitive transcripts, such as sequence motifs for specific RBPs or structural elements, that could help explain their selective dependence. Ultimately, clarifying whether ERH acts through specific molecular partners or indirectly via its integration into broader RNA processing complexes will be essential to understand how it mechanistically regulates splicing.

4.2.6 Regulators of splicing – SR proteins and hnRNPs

A wide range of RBPs and additional protein cofactors regulate both conventional and alternative splicing. Two major RBP families that emerged in connection with ERH during my research were the SRSFs and the hnRNPs. Both families can act as either positive enhancers or negative repressors depending on their binding position relative to the splicing event and the broader RNA context^{451,453,501–503}.

Several SRSF proteins play key roles in regulating IR. Retained introns typically possess weak splice sites, rendering them susceptible to IR^{367,504,505}, but they also often exhibit elevated GC content^{367,470,506} and are enriched for SRSF-binding motifs, including those recognised by SRSF1 and SRSF7^{507,508}. Knockdown of these factors results in increased IR, suggesting that SRSF proteins aid splicing and normally prevent IR⁵⁰⁷. Moreover, both SRSFs and retained introns are highly enriched in nuclear speckles^{482,509–513}, biomolecular condensates that play a key role in processing and quality control of mRNAs⁵¹⁴, suggesting a specialised role for SRSFs in this type of IR. Beyond splicing, SRSFs also act in downstream processes such as mRNA nuclear export⁵¹⁵ and NMD⁵¹⁶. This broader involvement complicates the mechanistic interpretation of their contribution to transcript fate, as observed effects may reflect not only splicing changes but also altered RNA export or decay.

The hnRNP family also plays diverse roles in alternative splicing, including IR, but with highly context-dependent outcomes⁵¹⁷. For example, hnRNPLL can induce retention of certain introns⁵¹⁸, whereas hnRNPK was found to be one of the most impactful non-core splicing factors preventing IR, similarly to SRSF1⁴⁷¹. Binding motifs for multiple hnRNPs are enriched within retained introns and flanking exons^{505,507}, underscoring their great potential as both inhibitors and facilitators of splicing.

Together, SRSFs and hnRNPs exemplify how splicing regulators can act in multifaceted and sometimes opposing ways, raising key questions about what

determines transcript-specific dependencies. Disentangling their central roles in splicing from other functions in RNA export or decay will be essential to fully understand how they control IR and other alternative splicing events in various contexts.

4.2.7 The splicing-deposited mastermind – the exon junction complex (EJC)

Another potential IFN γ -regulating factor implicated alongside ERH in my work was the EJC. The EJC is a multi-protein complex deposited on mRNAs during splicing and functions as a central hub that coordinates several RNA processing steps, including further splicing regulation, nuclear export, and NMD^{519,520}. The EJC effectively acts as a molecular “mark” of successful splicing, linking pre-mRNA processing to post-splicing fate decisions. In this section, I discuss the EJC’s composition and its major functional roles in splicing, export, and NMD.

The core EJC is composed of three invariable factors: eukaryotic initiation factor 4A-3 (EIF4A3), RNA binding motif protein 8A (RBM8A), and mago-nashi homolog (MAGOH, or its homologous protein MAGOHB)^{521,522}. EIF4A3 is a DEAD-box RNA-binding protein and serves as the primary RNA-interacting component, while RBM8A and MAGOH form a stable heterodimer that locks EIF4A3 onto RNA^{523,524}. Although CASC3 was initially also considered a core subunit^{521,525}, much evidence now points toward a more defined role of CASC3 in cytoplasmic EJC-related processes: it mainly localises to the cytoplasm⁵²⁶, it has a lower protein abundance⁴⁹⁸, EJCs without CASC3 were described⁵²⁷, and loss of CASC3 did not affect EJC assembly⁵²⁸. EJCs are canonically deposited 20-24 nucleotides upstream of exon-exon junctions^{529,530}. Non-canonical EJCs at other locations have been described^{498,530}, but their relative presence differs between studies⁵³¹ and their functional relevance is debated. EJC recruitment is tightly coupled to splicing^{532–535}, solely occurring with splicing *in vivo*⁵¹⁹, making the EJC a reliable mark for successful intron removal^{519,520}.

Once deposited, the EJC is not just a passive mark, but it can also influence other splicing events within the same transcript⁵³⁶. Importantly, it interacts with the ACIN1-RNPS1-SAP18 (ASAP) and PNN-RNPS1-SAP18 (PSAP) splicing complexes^{536–539}. The EJC also suppresses aberrant re-splicing that would otherwise cause cryptic splice sites, with EIF4A3 and RNPS1 being critical in this safeguard

mechanism⁵⁴⁰. This illustrates how EJC deposition provides a form of quality control to ensure splicing fidelity.

The EJC also couples successful splicing to mRNA export. The transcription-export complex (TREX) is key for nuclear export and is in part recruited by the 5' cap-associated CBC⁵⁴¹. However, this might just be an intermediate step⁵⁴², and the EJC seems to be key for final TREX recruitment by acting as a critical recruitment platform for export adaptors like ALYREF^{542–544}. This coupling ensures that incompletely processed transcripts are not prematurely exported, thereby linking nuclear quality control to downstream gene expression.

Lastly, the first discovered and the most well-known role of the EJC is coupling IR and dysfunctional splicing to cytoplasmic RNA degradation via NMD^{545,546}. When a translating ribosome encounters a PTC with an EJC located downstream, NMD is triggered⁵¹⁹ with multiple NMD factors recruited^{547,548}. Core NMD proteins such as UPF3A/UPF3B^{545,546,549}, as well as EJC-associated RNPS1^{550–552} and CASC3⁵²⁸, play key roles in this process that first halts translation⁵⁵³ and subsequently recruits decay factors to degrade the mRNA⁵⁵⁴. This ensures that aberrant or potentially deleterious proteins are not synthesised.

Although the EJC is deposited onto virtually all spliced mRNAs^{519,529,530}, its loss does not impact all transcripts equally⁵⁴⁰. Why certain mRNAs are disproportionately EJC-dependent remains unclear and may involve specific sequence motifs, intron-exon architecture, or interactions with other RBPs and protein cofactors. Clarifying which EJC functions and interactions are essential for regulating distinct transcript subsets, such as those involved in the IFN γ pathway, will be critical for understanding how the EJC selectively shapes gene expression.

4.2.8 Nuclear-cytoplasmic mRNA export via TREX, ALYREF, SRSFs, the EJC, and potentially ERH

Once pre-mRNAs have undergone 5' capping, 3' polyadenylation, and splicing, they are ready for nuclear export. The resulting messenger ribonucleoprotein (mRNP) contains a combination of RNA modifications and bound proteins that together ensure recognition by the TREX complex. In this way, TREX serves as a quality control for correctly processed mRNAs, linking transcriptional completion to mRNA export⁵⁴².

The two key recognised features during this process are the 5' CBC and splice junction-bound EJCs⁵⁵⁵. These are identified by the TREX helicase UAP56 and the essential mRNA export adaptor ALYREF^{541,543,556}. TREX then recruits the export receptor NXF1-NXT1⁵⁵⁷, allowing mRNP export through the nuclear pore complex to the cytoplasm⁵⁵⁸.

A critical yet incompletely understood feature of export is mRNP organisation and compaction^{555,559–561}. The mRNP must be compacted into export-competent particles to facilitate nuclear transport and passage through the nuclear pore complex (NPC)^{544,559,562}. This packaging is thought to be mediated by multivalent RNA-binding proteins, with ALYREF and SRSFs playing key roles^{515,544,562}. ALYREF binds EJCs through MAGOH and EIF4A3^{523,544,563} and appears to facilitate EJC multimerisation, thereby contributing to mRNP compaction^{498,544,561}. SRSFs can act as additional export adaptors that recruit NXF1-NXT1⁵¹⁵ and contribute to mRNP compaction during nucleoplasmic travel. Together, these proteins likely form cooperative networks that closely link mRNA segments and thus ensure mRNP integrity and efficient export⁵⁴⁴.

Interestingly, XL-MS has revealed interactions between ALYREF, the export adaptor CHTOP, and ERH⁵⁴⁴, raising the possibility that ERH contributes to mRNP assembly or stability during mRNA export. Given its small size and multiple binding pockets, ERH could possibly assist in bridging protein components of the mRNP and promote higher-order multimerisation and compaction. However, apart from interaction assays that place ERH together with export adaptors^{496,544,564} and the EJC⁴⁹⁸, no functional studies have yet investigated whether ERH is required for efficient mRNA export.

Notably, mRNA export is not merely a passive endpoint of RNA processing but an actively regulated checkpoint. TREX recruitment, ALYREF binding, and SRSF activity can be modulated by post-translational modifications^{515,565}, changes in binding occupancy⁵⁶⁶, or their recruitment to specific nuclear compartments^{543,567}. Such regulation could likely affect which transcripts are prioritised for export under different cellular states, adding a further layer of gene expression control.

Taken together, TREX, ALYREF, SRSFs, and the EJC form the central framework of mRNP export, but how these factors cooperatively package transcripts – and whether auxiliary proteins like ERH are critical contributors – remains an open question. Clarifying this interplay will be key to understanding how transcript

maturation is coupled to export and how selective export may fine-tune pathway-specific responses, including IFN γ signalling.

4.2.9 Nuclear degradation – a key quality control mechanism

Nuclear decay is not merely a passive clean-up system, but a key quality control step that is tightly interwoven with all steps of pre-mRNA processing. By continuously surveying nascent and processed transcripts, it minimises the production of low-quality transcripts and actively regulates gene expression by controlling mRNA abundance⁵⁶⁸.

The two main nuclear RNA decay pathways act via XRN2 or the RNA exosome^{568–571}. XRN2 is a 5'→3' exoribonuclease that degrades 5' monophosphorylated transcripts, such as decapped or cleaved mRNAs^{568,572}. XRN2 mostly targets long nascent RNAs while they are still associated with Pol II, and thus serves as a co-transcriptional quality control process^{568,571,573}. In contrast, the RNA exosome is a multiprotein 3'→5' decay machinery that targets RNAs throughout their nuclear life cycle^{568,574,575}. Its ribonucleolytic activity is carried out by DIS3 and RRP6⁵⁷⁶ and guided by the MTR4 helicase in cooperation with one of two specialised adaptor complexes: the nuclear exosome-targeting (NEXT) complex or the poly(A) exosome-targeting (PAXT) complex^{568,569}. Through this modular targeting system, the exosome can eliminate diverse classes of faulty mRNAs⁵⁶⁸.

Aberrant transcripts targeted for nuclear decay can arise from defects in any pre-mRNA processing step, including premature or late termination^{577–579}, excessive polyadenylation⁵¹⁴, or IR⁴⁸⁰. In eukaryotes, IR-containing transcripts are recognised by unspliced 5'ss⁵⁶⁸ and are rapidly degraded by the RNA exosome⁴⁸⁰. Whether XRN2 can also degrade IR transcripts remains debated, with evidence supporting^{580,581} or refuting⁵⁶⁸ such a role, possibly depending on whether the transcript is still Pol II-associated. Interestingly, in yeast, IR surveillance and degradation is mediated by Mmi1 and the ERH orthologue Erh1, which form the EMC complex^{582,583}. Mmi1 binds near retained introns to further suppress their splicing⁵⁸⁴ and recruits MTREC (PAXT orthologue), leading to exosomal degradation in Mmi1 decay foci^{584,585}. However, no Mmi1 orthologue has been identified in mammals³⁷⁸, raising the possibility that human ERH may engage in distinct, yet-to-be-characterised IR-regulatory pathways.

Whereas most aberrant mRNAs are efficiently targeted for nuclear decay, properly processed transcripts and those with regulated detained introns are normally

not degraded. This selectivity is achieved through two major mechanisms: (i) competition with mRNP-bound RBPs and export machinery that shields the mRNA, (ii) spatial segregation to protective nuclear compartments⁵⁶⁸. For example, the TREX complex has been shown to coat mRNP globules, likely shielding them from exosome targeting^{544,566,586}. Moreover, ALYREF directly competes with MTR4, PAXT, and NEXT for mRNP-binding via the mRNP component ARS2⁵⁶⁶, and it associates with other exosome-competing factors^{410,587–591}. Similarly, the EJC indirectly promotes transcript stability by providing a RBP platform for TREX, and by facilitating mRNP packaging^{523,544,563}. Additionally, properly processed and IR-containing transcripts localise to protective nuclear speckles^{509,510,514}, which exclude the RNA exosome^{512,513,581} and contain the MTR4-inhibitory regulator NRDE2^{581,592}, thereby stabilising transcripts and allowing time for slower^{514,581} or dynamic signal-dependent splicing^{475,481–484}.

Taken together, nuclear decay emerges as a highly integrated layer of gene regulation, dynamically balancing transcript surveillance with preservation of transcripts destined for productive export. Its central role in RNA fate means that observed changes in mRNA abundance may reflect altered processing and tightly coupled decay rather than transcription, which is an important consideration when interpreting abundance phenotypes. Defining the molecular rules that distinguish transcripts targeted for degradation from those protected for processing remains an active area of investigation.

4.2.10 Cytoplasmic mRNA control by tristetraprolin (TTP)

Once exported to the cytoplasm, mRNAs can be translated up to hundreds of times, making regulation of their stability essential to avoid excessive protein production⁵⁹³. A major post-transcriptional regulator of innate immune mRNAs during inflammatory resolution is the ARE-binding protein TTP⁵⁹⁴.

TTP binds AREs in the 3'UTR of target mRNAs, including those encoding major pro-inflammatory cytokines such as TNF^{198,201,595} and IL6⁵⁹⁶, and promotes their degradation^{198–201,595}. Loss-of-function mutations in its two RNA-binding zinc finger domains abrogate RNA binding¹⁹⁹ and phenocopy complete TTP deficiency⁵⁹⁷, underscoring the requirement of RNA binding for its decay function. Unlike RBPs with intrinsic endonuclease activity that directly remove 5' or 3' mRNA modifications^{598,599},

TTP acts indirectly by recruiting the CCR4-NOT deadenylase complex and the DCP1/DCP2 decapping machinery^{600–603}. In parallel, TTP delivers its target transcripts to processing bodies (P-bodies), stress-inducible cytoplasmic mRNP granules⁶⁰⁴, whose assembly and composition are dynamically shaped by cell type and stress context^{605,606}. P-bodies are enriched for decay factors, translational repressors, and unstable mRNAs^{607–609}, and TTP promotes degradation and suppresses translation of its targets within these structures^{610–613}, partly through interactions with translation repressors⁶¹⁴.

Interestingly, recent evidence suggests that TTP loading may occur co-transcriptionally, with pre-mRNA engagement in the nucleus “licensing” transcripts for subsequent cytoplasmic decay⁶¹⁵. This model links nuclear processing to cytoplasmic turnover and implies that decay selectivity may be predetermined early in an mRNA’s life. However, not all 3’UTR-bound mRNAs are destabilised^{201,610–612}, and TTP frequently binds introns^{201,610–612}, with unclear biological significance^{201,594}. These findings suggest that post-binding regulation, potentially through unknown functional partners or post-translational TTP modifications, ultimately determines whether a targeted mRNA is degraded or spared.

The physiological importance of properly regulated TTP activity is underpinned by the phenotype of TTP-deficient mice, which develop systemic inflammation (TTP deficiency syndrome)⁶¹⁶ as numerous cytokine and chemokine mRNAs become stabilised^{198,616–619}. Conversely, overexpression of TTP in mice provides dominant repression of induced inflammation⁶²⁰, highlighting that its activity must be tightly balanced to avoid either uncontrolled cytokine production or impaired immune responses.

TTP expression is kept low at homeostasis and is transcriptionally induced by inflammatory stimuli such as LPS-activated TLR4 signalling or IFN γ signalling^{198,621–624}, and is then majorly regulated through phosphorylation and proteasomal turnover. TTP is extensively phosphorylated at over 30 sites by stress-activated kinases^{614,625–627}, though only a few have been functionally characterised. The best characterised events are phosphorylation of murine S52 and S178, which are mediated by p38/MAPK signalling and the MK2 kinase^{625–627}. This phosphorylation decreases TTP’s RNA-binding capacity and activity^{628–631} while simultaneously stabilising TTP^{625,627}. Conversely, loss of these sites renders TTP unstable yet functionally active⁶³². This has prompted a model in which TTP is initially stabilised but inactive

early in inflammation and only promotes mRNA decay upon subsequent dephosphorylation^{201,596}. Curiously, TTP degradation has been proposed to be proteasome-dependent yet ubiquitination-independent, potentially through its intrinsically disordered N- and C-terminal domains^{625,633}. However, many aspects of this model remain unresolved, including the importance of other phosphorylation sites and the identity of factors controlling TTP turnover.

Overall, TTP exemplifies how cytoplasmic RNA decay is both selective and tightly regulated. Its activity appears to be shaped by nuclear “licensing” events, extensive post-translational regulation, and its dynamic localisation to P-bodies. Clarifying how these layers of control intersect will be crucial to fully understand how TTP shapes inflammatory gene expression.

4.3 Aims

The overarching aim of this PhD thesis was to investigate the regulation and functional role of key RNA processing factors in innate immunity, centring on IFN γ signalling. I addressed this aim by contributing to research that focused on: **(i)** identifying and characterising novel IFN γ -regulatory factors involved in post-transcriptional processing, **(ii)** uncovering regulatory protein degradation mechanisms of IRF1, a major TF for IFN γ -driven inflammatory responses, and **(iii)** elucidating the complex interplay between protein degradation, phosphorylation, and functional activity of TTP, a key regulator of cytoplasmic RNA decay for innate immune transcripts.

(i) The primary focus of my PhD was to identify and characterise novel RNA processing factors regulating IFN γ signalling. Previously, post-transcriptional RNA regulatory factors have typically been overlooked for IFN γ signalling, likely due to the challenges associated with investigating such cell-essential factors. We aimed to overcome this problem by utilizing an inducible and genome-wide CRISPR-Cas9 screening approach, and in this way identify novel RNA regulators. Furthermore, I aimed to characterise their specific RNA processing mechanism in IFN γ signalling, including which factors they regulate. Lastly, by explaining their regulatory role in IFN γ signalling, I aimed to further characterise their general molecular function through different experimental and analytical RNA sequencing approaches.

(ii) I also contributed to a study that aimed to identify the regulatory degradation mechanism of the short-lived IRF1 protein. Balanced protein regulation of IRF1 is an important yet poorly understood mechanism that prevents auto-inflammation but also allows for positive IRF1 functions like tumour suppression. We aimed to uncover regulatory factors in this process, such as novel E3 ligases that can mediate IRF1 proteasomal turnover, by utilising a genetic cell screen. Importantly, we also wanted to determine how any identified degradation mechanisms affect the functional output of IRF1-dependent transcription of IFN γ response genes.

(iii) Lastly, I also contributed to research investigating the regulatory mechanisms of TTP protein degradation. TTP is a key negative regulator of innate immune responses as it destabilises mRNAs encoding pro-inflammatory mediators. Its timely activity and proteolytic removal are important yet poorly understood mechanisms. We aimed to understand this regulatory process better by identifying novel regulators of TTP protein abundance through a genetic screen. By investigating

the mechanism of such regulatory factors, we aimed to dissect the intricate interplay between phosphorylation, degradation, and functional activity of TTP.

5. Results

5.1 Results 1

Publication “ERH regulates type II interferon immune signalling through post-transcriptional regulation of *JAK2* mRNA”

5.1.1 Preamble

How co- and post-transcriptional RNA processing regulates IFN γ signalling and major downstream transcription factors like IRF1 remains unclear, including which specific factors may be involved. Through an inducible CRISPR/Cas9 screening approach, this study identified multiple novel RNA regulatory factors for IFN γ signalling, including ERH, the splicing and export factor SRSF1, the EJC complex, and the export adaptor ALYREF. The removal of these factors impairs post-transcriptional processing of the mRNA encoding the IFN γ signalling kinase JAK2, resulting in loss of this key protein and the inability to launch an effective immune response. Furthermore, through different RNA sequencing approaches we uncover a critical role of ERH in preventing IR of AU-rich regions, like those present in *JAK2*, and describe the relative similarities between ERH and ALYREF depletion on the RNA transcriptome. In this way, we further characterise the functional role of ERH in RNA splicing and export. Overall, this research exemplifies and highlights how post-transcriptional RNA regulation can play a key and rate-limiting role in innate immune responses.

5.1.2 Authors and status

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5.1.3 Contributing statement

My contributions to this study were conceptualisation, methodology, software, formal analysis, investigation, data curation, writing, review and editing, and visualisation.

5.1.4 Publication

ERH regulates type II interferon immune signaling through post-transcriptional regulation of *JAK2* mRNA

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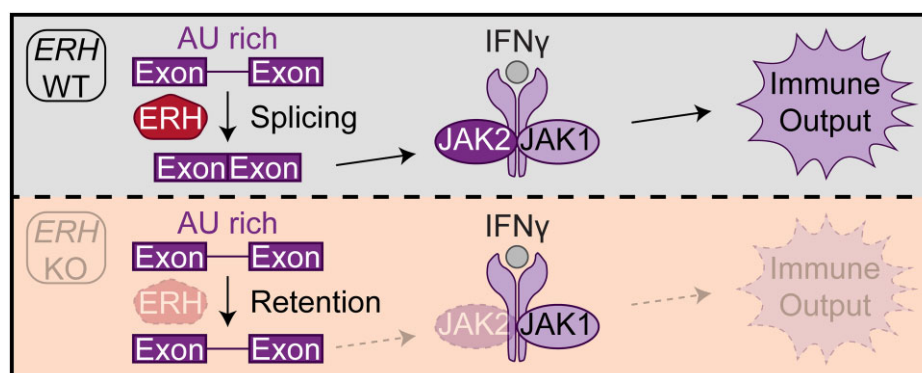
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Abstract

Type II interferon (IFN γ) signaling is essential for innate immunity and critical for effective immunological checkpoint blockade in cancer immunotherapy. Genetic screen identification of post-transcriptional regulators of this pathway has been challenging since such factors are often essential for cell viability. Here, we utilize our inducible CRISPR/Cas9 approach to screen for key post-transcriptional regulators of IFN γ signaling, and in this way, we identify ERH and the ERH-associated splicing and RNA export factors MAGOH, SRSF1, and ALYREF. Loss of these factors impairs post-transcriptional mRNA maturation of *JAK2*, a crucial kinase for IFN γ signaling, resulting in abrogated *JAK2* protein levels and diminished IFN γ signaling. Further analysis highlights a critical role for ERH in preventing intron retention in AU-rich regions in specific transcripts, such as *JAK2*. This regulation is markedly different from previously described retention of GC-rich introns. Overall, these findings reveal that post-transcriptional *JAK2* processing is a critical rate-limiting step for the IFN γ -driven innate immune response.

Graphical abstract



Introduction

Interferons (IFNs) are critical cytokines in the innate immune system, mediating the first line of defense against diverse pathogens [1, 2]. These cytokines fall into three main categories: type I (IFN α and IFN β), type II (IFN γ), and type III (IFN λ). Upon binding specific cell surface receptors, IFNs

trigger signaling cascades that activate Janus kinase (JAK) and signal transducer and activator of transcription (STAT) pathways [3–10]. This activation results in the transcription of hundreds of interferon-stimulated genes (ISGs) that encode proteins with antiviral, antiproliferative, and immunomodulatory properties [11, 12].

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Similar to type I and type III IFNs, IFN γ signaling can drive antiviral gene expression but is primarily involved in antibacterial responses [13]. IFN γ signaling is critically dependent on the JAK/STAT pathway composed of two receptor heterodimers (IFNGR1 and IFNGR2) and activation of the associated kinases (JAK1 and JAK2) that phosphorylate the transcription factor STAT1, which in turn drives ISG expression (Fig. 1A). In this way, IFN γ activates macrophages, thus promoting phagocytosis and the production of reactive oxygen and nitrogen species – critical components for eliminating intracellular bacterial pathogens [13].

Moreover, IFN γ signaling is known to be key for effective checkpoint inhibition that is used in cancer immunotherapy to relieve immune cells of negative signals from checkpoint proteins such as CTLA4 and PD1. In this context, non-responders to anti-CTLA4 therapy were found to have defects in IFN γ signaling within their tumors [14]. In addition, failure to respond to IFN γ and anti-PD1 melanoma therapy was associated with loss-of-function mutations in *JAK1* and *JAK2* in tumor biopsy samples [15]. Despite their numerous host-beneficial immunomodulatory functions, JAK1/JAK2 activity resulting from IFN γ signaling requires proper regulation. This is exemplified by the fact that mutations in exon 14 of *JAK2* (V617F) result in its constitutive activation and leads to myeloproliferative neoplasms [16–18].

To prevent aberrant activation, innate immune signaling is well-recognized to be tightly controlled at the post-translational level. For example, the phosphorylation of receptor-activated kinases critically regulates the key transcription factors driving type I IFN transcription (IRF3 and IRF7), as well as STAT1-containing transcription factor complexes driving ISG expression in both type I and type II IFN signaling [19].

However, evidence is emerging that key factors of these pathways, as well as individual ISGs, are also post-transcriptionally regulated [20–23]. For example, alternative splicing and intron retention (IR) are critical mechanisms that regulate mRNAs encoding key immune factors that modulate inflammatory responses [20, 23], and control macrophage development and function [21, 24]. Furthermore, mRNA export rates have been shown to complement mRNA decay rates to properly regulate innate immune responses [22]. Overall, these examples highlight components of the post-transcriptional RNA processing machinery as critical factors for innate immunity.

RNA processing factors comprise a significant proportion of genes that are important for cellular viability, likely since they regulate large groups of mRNAs, including those needed for fundamental biological processes [25, 26]. Their essential nature has made it difficult to systematically screen for these regulators. Here we set out to identify novel factors controlling IFN γ signaling, with a focus on post-transcriptional regulators. Given that many of these genes are essential for cell viability, we hypothesized that previous gene trap-mediated genetic screening [27] may have overlooked critical post-transcriptional factors that function in IFN γ signaling. Therefore, we utilized our established inducible CRISPR/Cas9 platform [28], which allows identification of cell-essential genes.

Using this approach, we identified Enhancer of Rudimentary Homolog (ERH) and its interacting splicing and export factors as novel positive regulators of IFN γ -induced signaling. These factors act through post-transcriptional regulation of *JAK2*: in their absence, *JAK2* protein was lost,

thereby abrogating IFN γ signaling. Our data suggest that post-transcriptional regulation of *JAK2* is rate-limiting and disproportionately affected by the disruption of these RNA regulatory factors. Furthermore, ERH was critical for preventing intron retention in *JAK2* and multiple other mRNAs, including several involved in DNA replication and the DNA damage response. Our results specifically reveal that ERH is essential for efficient splicing in AU-rich regions, contrasting with previously described intron retention of GC-rich introns. Overall, these findings highlight the importance of post-transcriptional *JAK2* processing by ERH and interacting factors in mounting an effective type II IFN response.

Materials and methods

Vectors

All plasmids and sgRNAs used in this study are listed in Tables 1 and 2, respectively. The lentiviral human genome-wide sgRNA library (six sgRNAs/gene) and the lentiviral vectors driving the expression of single sgRNAs (U6 promoter) and eBFP2 or iRFP (PGK promoter) were described previously [28]. The lentiviral *JAK2* or sgRNA-resistant ERH expression vectors were obtained by cloning *JAK2* cDNA (Addgene plasmid 23 915) or *ERH* cDNA into a modified pLX303 vector that co-expressed myc-tagged mCherry through a P2A site. The ERH expression vector with C-terminally tagged mTurquoise2 used in co-IP-MS/MS was cloned in the pEGFP-C1 backbone (Clontech).

Cell culture and reagents

All cell lines used in this study and their applications are listed in Table 3, reagents in Table 4, and antibodies in Table 5. Cells tested negative for mycoplasma contamination and the used cell lines in this study are not on the current International Cell Line Authentication Committee (ICLAC) list of commonly misidentified cell lines (version 13). RKO and RAW 264.7 parental cells were authenticated by short tandem repeat analysis. Dox-inducible Cas9 single-cell-derivative clones were generated previously [28, 29], and active Cas9 function was validated to solely occur in the presence of dox through knockout of essential genes and competitive proliferation assays. To obtain empty vector-, *JAK2*- or ERH-expressing cells, the indicated plasmids were transduced into RKO-Dox-Cas9 cells, and cells expressing mCherry were bulk sorted with a FACSaria III (BD Biosciences). Similarly, to obtain sgRNA expressing cells, sgRNA-fluorophore expressing plasmids were transduced into RKO-Dox-Cas9 or RAW 264.7-Dox-Cas9 cells, and fluorophore expressing cells were bulk sorted by FACS. All cells were cultured at 37°C and 5% CO₂ in a humidified incubator.

FACS-based CRISPR–Cas9 screen

The genome-wide FACS-based screen and generation of next-generation sequencing (NGS) libraries were performed as previously described for RKO cells [28]. In short, Cas9-mediated knockouts were induced by treatment with dox for 2.5 or 5 days, IRF1 expression was stimulated for 24 h pre-harvesting with human IFN γ at 10 ng/mL (Enzo Life Sciences, ENZ-PRT141-0100). IRF1 levels were determined through intracellular staining with a PE-conjugated anti-IRF1 antibody (Cell Signaling Technology, 12 732). The top 1% of IRF1^{high} cells, and 4% IRF1^{low} cells were collected by flow cytometry for

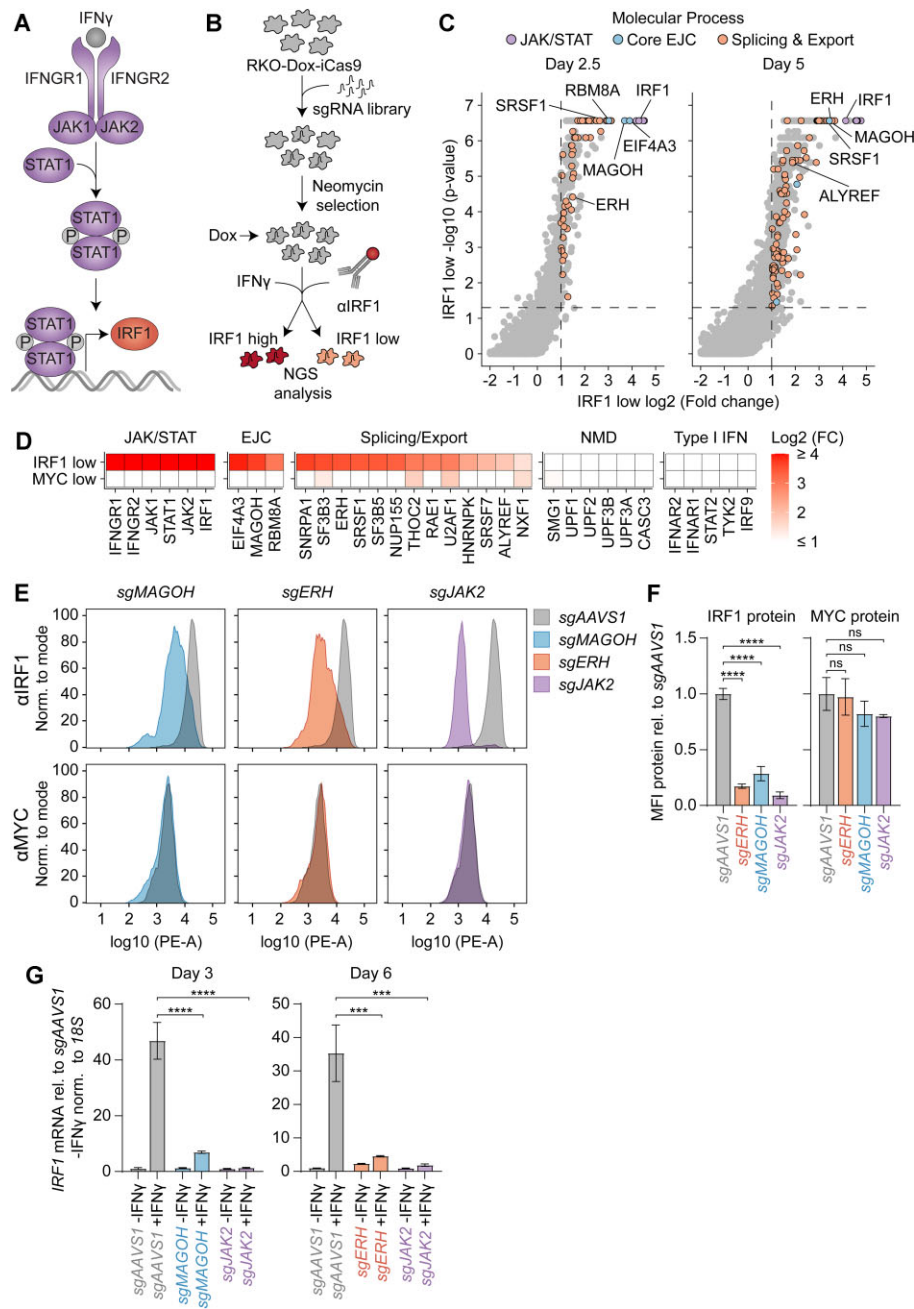


Figure 1. Identification of ERH and other novel positive regulators of IFN γ signaling by genome-wide genetic screening. **(A)** Schematic of the IFN γ -induced JAK/STAT signaling pathway, which stimulates expression of many genes, including IRF1. **(B)** Overview of FACS-based CRISPR-Cas9 knockout screen. Human RKO cells with dox-inducible iCas9 were transduced with a lentiviral genome-wide sgRNA library. Cas9 expression was induced for 2.5 or 5 days, after which cells were treated with IFN γ , and IRF1 induction detected by intracellular staining. Cells with the lowest or highest IRF1 levels were collected by FACS, and disrupted genes were identified by analyzing sgRNA-targeted coding sequences. **(C)** sgRNA enrichment in the IRF1^{low} cell population was plotted. Dashed lines indicate significance ($P \leq 0.05$) and enrichment ($\log_2$ fold change ≥ 1). Significantly enriched genes involved in the JAK/STAT pathway, the exon junction complex, or RNA splicing and export are highlighted. **(D)** Heatmap of selected IRF1 regulators as in (C) or MYC regulators [28] involved in JAK/STAT signaling, the EJC, splicing and export, nonsense mediated decay, or type I interferon signaling. For each gene, the time point with the strongest enrichment is plotted. **(E)** RKO-iCas9 cells were transduced with vectors expressing the indicated sgRNAs. After 5 days of dox-induced Cas9 expression, cells were stimulated with IFN γ , after which endogenous IRF1 or MYC were detected by intra-cellular staining and flow cytometry. Representative samples from four (sgMAGOH) or five (sgAAVS1, sgERH, and sgJAK2), $n = 4$ or 5 biological replicates, are shown. **(F)** Quantification of median fluorescence intensity (MFI) from panel E. Data represent means and sd; $n = 4$ or 5 biological replicates. One-way ANOVA with Bonferroni's multiple comparison correction ($*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$; $****P \leq 0.0001$). **(G)** In parallel, IRF1 mRNA levels were measured by RT-qPCR. Data represent the mean and sd; $n = 3$ biological replicates. Two-tailed t-test with Benjamini-Hochberg correction ($*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$; $****P \leq 0.0001$).

Table 1. Plasmids

In plasmid names, “-” denote separate elements		
Plasmid	Purpose	Reference or source
pRRL-TRE3G-Cas9-P2A-GFP-PGK-IRES-rtTA3	Dox inducible Cas9	Scinicariello S <i>et al.</i> , 2023
pRRL-PBS-U6-sgRNA-EF1as-Thy1-P2A-NeoR (sgETN)	Library sgRNA	de Almeida M, Hinterndorfer M <i>et al.</i> , 2021
pLentiv2-U6-PGK-iRFP720-P2A-Neo	Single sgRNA	de Almeida M, Hinterndorfer M <i>et al.</i> , 2021
pLentiv2-U6-PGK-eBFP2-P2A-Neo	Single sgRNA	de Almeida M, Hinterndorfer M <i>et al.</i> , 2021
pLX303-SFFV-MYC-mCherry-P2A	Vector expression	This study
pLX303-SFFV-MYC-mCherry-P2A-JAK2	JAK2 expression	This study
pLX303-SFFV-MYC-mCherry-P2A-ERH	ERH expression	This study
C1-CMV-ERH-mTurquoise2	ERH expression	This study
pCRV1-Gag-Pol	Lentiviral expression	Hatziiioannou <i>et al.</i> , 2004
pHCMV-VSV-G	Lentiviral expression	Yee <i>et al.</i> , 1994

Table 2. sgRNA sequences

Gene	Species	Sequence (5' to 3')
AAVS1	Human	GCTGTGCCCGCATGCACAC
ERH	Human	GGTAGGCTGTACCAGCAAAA
MAGOH	Human	GTACATAAAAGCGTGATGG
JAK2	Human	GTGGAATTCAGTGGTCAAGA
STAT1	Human	GAGCTGCTGGAAAAGACTGA
ROSA	Mouse	AAGATGGGCGGGAGTCTTC
Erh	Mouse	GGGTAGGCTGTACCAGCAAAA
Stat1	Mouse	GGGCTGTCTGTTCTACCACGA
ACIN1-1	Human	GATGATGGGACCCATGACAA
ACIN1-2	Human	GGGACAGCAGCTACCAAGG
ALYREF-1	Human	TGAATTTGGAACGCTGAAGA
ALYREF-2	Human	GCCGCGCGAGTGAATCGAGG
BCLAF1-1	Human	AAGGCCAGGCCAAATATCAC
BCLAF1-2	Human	ATTCTAGAAAAGAAGCGATAC
CASC3-1	Human	GAACCGGGGCCCTTCATCTG
CASC3-2	Human	GACTGGAGAGAGGCCAAAGTG
CHTOP-1	Human	GGAGAATAGACCCTCTGTCC
CHTOP-2	Human	CTCATTTAGAGACATCTTGG
DROSHA-1	Human	TGAGTGCATCGAATCCACAC
DROSHA-2	Human	TAGGCGGGGAGACTGTGATC
MAGOH-1	Human	CGACAGTGAATTAACCAAG
MAGOH-2	Human	GAGTTTGAGTTTCGACCGGA
POLDIP3-1	Human	TGAGAGGCCAATCTTCTGCC
POLDIP3-2	Human	CGAACTCATCAGGAAGCGCG
SRSF1-1	Human	CGGGTCTCTGAACTCAACGA
SRSF1-2	Human	AACGATTGCCGCATCTACGT
THRAP3-1	Human	GTTGGGCAAACAGTCTGAG
THRAP3-2	Human	GAGGAGCAGCCTATACAAAG
UPF1-1	Human	GGTATTACAGTAAACACGC
UPF1-2	Human	GGCCAAAGCTGACTCAGTGG

analysis by next generation sequencing. The used gating strategy has been previously described [28].

CRISPR screen analysis was performed with MAGeCK [30], as previously described [28]. The workflows for quantifi-

cation of raw sequencing reads (<https://github.com/ZuberLab/crispr-process-nf>) and enrichment/depletion analysis of sgRNAs using MAGeCK (<https://github.com/ZuberLab/crispr-mageck-nf>) are available online. Molecular process labeling was guided by literature research and GO term annotations accessed with AmiGO 2 version 2.5.17 [31, 32], for JAK/STAT pathway (GO:0 060 333), RNA splicing (GO:0 008 380), RNA export (GO:0 006 405), NMD (GO:0 000 184), EJC (GO:0 035 145), and type I IFN (GO:0 060 337).

Lentivirus-like particle production and transduction

Semi-confluent Lenti-X cells were transfected with mixes composed of a lentiviral transfer plasmid of interest, pCRV1-Gag-Pol [33], and pHCMV-VSV-G [34], by using polyethylenimine (PEI, Polysciences, 23 966) in a 1:3 ratio (μg DNA/μg PEI) in DMEM without supplements. Virus-like particle (VLP)-containing supernatant was cleared of cellular debris by filtration (0.45 μm) and were kept at 4°C or –80°C for short-term or long-term storage, respectively. Target cells were transduced in the presence of 5 μg/ml (RKO) or 6 μg/ml (RAW 264.7) of polybrene (Sigma-Aldrich, TR1003G).

Intracellular staining for flow cytometry

Cell fixation, permeabilization, and staining were performed as previously described [29]. Permeabilized cells were blocked with Human BD Fc Block (BD Biosciences, 564 220). Samples were analyzed on an LSRFortessa (BD Biosciences) with BD FACSDiva software (v8.0). FACS data were further analyzed using FlowJo (v10.8).

Western blotting

Cells were lysed in RIPA lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% SDS, 0.5% Sodium deoxycholate, 1% Triton X-100), rotated for 30 min at 4°C and centrifuged at 16 000 x g for 20 min at 4°C. Protein concentrations of the supernatants were determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, 23 225). Lysates were boiled at 95°C for 5 min in Laemmli sample buffer with 10% β-mercaptoethanol. Lysates (20 μg) were loaded on SDS polyacrylamide gels with varying percentages based on molecular weight of proteins of interest. Proteins were blotted on nitrocellulose membranes at 4°C for 75 min at 300 mA in Towbin buffer (25 mM Tris-HCl pH 8.3, 192 mM glycine, and 20% ethanol). Membranes were blocked in 5% BSA in PBS-T for 1 h at room temperature, and subsequently incubated with primary antibody overnight at 4°C while shaking. Membranes were washed five times with PBS-T and incubated with HRP-coupled secondary antibody for 1 h at room temperature and imaged with the Chemi-Doc Imaging System from Bio-Rad. Relative protein levels were quantified with Image Lab (BioRad) on non-saturated exposures.

Total RNA isolation, cDNA synthesis, and qPCR

Total RNA was harvested from 1–2 × 10⁶ RKO-iCas9 or RAW 264.7-iCas9 cells by Trizol lysis (Thermo-Fisher Scientific, 5596–018) and total RNA was isolated per the manufacturer's recommendations. The RNA was treated with Turbo DNase (Thermo Fisher Scientific, AM2238), and cDNA was

Table 3. Cell lines

Cell lines and primary cells	Type	Reference or source	Parental	Purpose	Media	Supplements
HEK293T	Human kidney neural tissue	CRL-3216		ERH expression	Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, D6429)	10% FBS (Sigma-Aldrich, F7524) and 1% penicillin/streptomycin (Sigma-Aldrich, P4333)
Lenti-X 293T	Human kidney neural tissue	Takara, Cat# 632180		VLP production	Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, D6429)	10% FBS (Sigma-Aldrich, F7524) and 1% penicillin/streptomycin (Sigma-Aldrich, P4333)
RKO	Human colon carcinoma	CRL-2577		Parental cell line	RPMI 1640 (Thermo Fisher Scientific, 21875)	10% FBS (Sigma-Aldrich, F7524), L-glutamine (4 mM, Sigma-Aldrich, G7513), sodium pyruvate (1 mM, Sigma-Aldrich, S8636), and 1% penicillin/streptomycin (Sigma-Aldrich, P4333)
RKO-Dox-Cas9-P2A-GFP	Human colon carcinoma	de Almeida M, Hinterdorfer M <i>et al.</i> , 2021	RKO	Dox-inducible Cas9	RPMI 1640 (Thermo Fisher Scientific, 21875)	10% FBS (Sigma-Aldrich, F7524), L-glutamine (4 mM, Sigma-Aldrich, G7513), sodium pyruvate (1 mM, Sigma-Aldrich, S8636), and 1% penicillin/streptomycin (Sigma-Aldrich, P4333)
RKO-DOX-Cas9-P2A-GFP_MYC-mCherry-P2A	Human colon carcinoma	This study	RKO-DOX-Cas9-P2A-GFP	Vector expression	RPMI 1640 (Thermo Fisher Scientific, 21875)	10% FBS (Sigma-Aldrich, F7524), L-glutamine (4 mM, Sigma-Aldrich, G7513), sodium pyruvate (1 mM, Sigma-Aldrich, S8636), and 1% penicillin/streptomycin (Sigma-Aldrich, P4333)
RKO-DOX-Cas9-P2A-GFP_MYC-mCherry-P2A-JAK2	Human colon carcinoma	This study	RKO-DOX-Cas9-P2A-GFP	JAK2 expression	RPMI 1640 (Thermo Fisher Scientific, 21875)	10% FBS (Sigma-Aldrich, F7524), L-glutamine (4 mM, Sigma-Aldrich, G7513), sodium pyruvate (1 mM, Sigma-Aldrich, S8636), and 1% penicillin/streptomycin (Sigma-Aldrich, P4333)
RKO-DOX-Cas9-P2A-GFP_MYC-mCherry-P2A-ERH	Human colon carcinoma	This study	RKO-DOX-Cas9-P2A-GFP	sgRNA resistant ERH expression	RPMI 1640 (Thermo Fisher Scientific, 21875)	10% FBS (Sigma-Aldrich, F7524), L-glutamine (4 mM, Sigma-Aldrich, G7513), sodium pyruvate (1 mM, Sigma-Aldrich, S8636), and 1% penicillin/streptomycin (Sigma-Aldrich, P4333)
RAW264.7	Murine macrophages	ATCC TIB-71		Parental cell line	Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, D6429)	10% FBS (Sigma-Aldrich, F7524) and 1% penicillin/streptomycin (Sigma-Aldrich, P4333)
RAW264.7-Dox-Cas9-P2A-GFP	Murine macrophages	ATCC TIB-71	RAW264.7	Dox-inducible Cas9	Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, D6429)	10% FBS (Sigma-Aldrich, F7524) and 1% penicillin/streptomycin (Sigma-Aldrich, P4333)

Table 4. Reagents

Description	Manufacturer/supplier	Catalogue number	Abbreviation	Application	Dilution/concentration
Cycloheximide	Sigma-Aldrich	C1988	CHX	Cell culture	25 µg/mL or 40 µg/mL
Doxycycline hyclate	Sigma-Aldrich	D9891	Dox	Cell culture	100 or 500 ng/mL
G418 disulfate salt	Sigma-Aldrich	A1720	G418	Cell culture	0.5–1 mg/mL
Actinomycin D	Sigma-Aldrich	A1410	ActD	Cell culture	5 µg/mL
Benzonase Nuclease	Merck	70 746	Benzonase	Cell lysis	250 U/µL
SYBR TM Gold Nucleic Acid Gel Stain (10,000X)	Thermo Fisher Scientific	S11494	SYBR TM Gold	nucleic acid detection	0.5–1X
Polybrene	Sigma-Aldrich	TR1003G		Cell culture	5 or 6 µg/mL
Polyethylenimine	Polysciences	23 966	PEI	Cell culture	3 µL PEI / 1 µg DNA
cOmplete TM Protease Inhibitor Cocktail	Roche	11 697 498 001	PI	Cell lysis	1X
Hoechst	Thermo Fisher Scientific	H3569		IF	0.4X
Luna Universal qPCR Master Mix	New England Biolabs	M30035		qPCR	1x
Pierce TM Protein A/G Magnetic Beads	Thermo Fisher Scientific	88 803		IP	
TRIzol TM Reagent	Invitrogen	15 596 018		RNA extraction	
Invitrogen TM TURBO TM DNase (2 U/µL)	Thermo Fisher Scientific	10 792 877		RNA extraction	
RevertAid Reverse Transcriptase (200 U/µL)	Thermo Fisher Scientific	EP0441		RNA extraction	
Bovine Serum Albumin > 96%	Sigma-Aldrich	A2153	BSA	SDS-page	5% (W/V)
ProLong TM Gold Antifade Mountant	Invitrogen	P36934		IF	
Phenylmethylsulfonyl fluoride	Sigma	P7626	PMSF	Cell lysis	0.1 mM
2-Mercaptoethanol	Carl Roth	4227.3		SDS-Page	
Human BD Fc Block	BD Biosciences	564 220		Intracellular staining	
Dulbecco's modified Eagle's medium (DMEM)	Sigma-Aldrich	D6429		Cell culture	
RPMI 1640	Thermo Fisher Scientific	21 875		Cell culture	
Trypsin-EDTA	Sigma-Aldrich	T4049		Cell culture	
FBS	Sigma-Aldrich	F7524		Cell culture	
L-Glutamine solution	Sigma-Aldrich	G7513		Cell culture	
Sodium pyruvate solution	Sigma-Aldrich	S8636		Cell culture	
Penicillin-Streptomycin	Sigma-Aldrich	P4333		Cell culture	
MEM NEAA (100X)	Gibco	11140-050		Cell culture	
Human interferon gamma	Enzo Life Sciences	ENZ-PRT141-0100	IFNγ	Cell culture	25 ng/mL
Mouse interferon gamma	Gift from G. Adolf, Boehringer Ingelheim, Vienna		IFNγ	Cell culture	10 ng/mL
Human Interleukin-1 beta	Peprotech	200-01B-10µG	IL-1β	Cell culture	10 ng/mL
Mouse Interleukin-1 beta	Peprotech	211-11b	IL-1β	Cell culture	50 ng/mL
Epoxomicin	Gentaur Molecular Products	607-A2606	EPX	Cell culture	10 µM
4-Thiouridine	BIO SYNTH	NT06186	4sU	Cell culture	1000 µM
NEBNext Magnesium RNA Fragmentation Module	New England Biolabs	E61505		RNA fragmentation	
Biotin-XX MTSEA	biotium	90066-1		4sU biotinylation	5 µg/mL
ROTI@Aqua-P/C/I, 100 ml (phenol-chloroform-isoamylalcohol, pH 4,5-5)	ROTH	X985.3	PCI	RNA extraction	
µMACS TM Streptavidin Kit, for 20 isolations	Miltenyi Biotec	130-133-282		4sU pulldown	

prepared with Oligo dT18 primers (Thermo Fisher Scientific, S0132) or random hexamer primers (Thermo Fisher Scientific, S0142) using RevertAid Reverse Transcriptase (Thermo Fisher Scientific, EP0441) as per the manufacturers' recommendations. qPCR samples were analyzed in technical triplicates or duplicates and with H₂O and no reverse transcriptase controls on a Mastercycler (Biorad) using 1x qPCR MM (10 mM Tris pH 8.5, 50 mM KCl, 0.15% Triton X-100, 2 mM MgCl₂, 200 µM dNTPs (Promega, U1515), 200 mM Trehalose, 2.5% Formamide (Applchem, A2156), 0.5x SYBR Green I (Thermo-Fisher Scientific, S7567), and 25 U/mL Taq polymerase (Promega, M7484B), in nuclease free H₂O (Ambion, AM9930)). Relative changes in gene expression were calculated based on the 2^{-ΔΔCT} method [35]. All qPCR primers are listed in Table 6.

Immune stimulation and 3' mRNA QuantSeq

FACS sorted RKO-iCas9 or RAW 264.7-iCas9 cells harboring constitutive sgRNA expression (*sgAAVS1/sgROSA* or *sgERH*) were treated with 100 or 500 ng/mL dox (Sigma-Aldrich, D9891) for 5 days to induce Cas9-mediated knock-outs. Subsequently, they were treated for 4 h with 25 or 10 ng/mL IFNγ (Enzo Life Sciences, ENZ-PRT141-0100 and a gift from G. Adolf, Boehringer Ingelheim, Vienna), 10 or 50 ng/mL IL-1β (Peprotech, 200-01B-10µG and 211-11b), or MQ (non-treated), respectively. Total RNA was isolated as previously described using Trizol, and 3' mRNA sequencing libraries were generated according to manufacturer's recommendations (Lexogen, 015-QuantSeq 3' mRNA-Seq Library Prep Kit FWD for Illumina and 020-PCR Add-on Kit for Illumina). Samples were analyzed on an Illumina HiSeqV4 SR50

Table 5. Antibodies

Target	Application	Dilution	Conjugate	Manufacturer	Catalogue number	Name	Type
Rabbit IgG	WB	1:3500	HRP	Cell Signaling Technology	7074S		Secondary
Mouse IgG	WB	1:3500	HRP	Cell Signaling Technology	7076S		Secondary
Beta-Actin	WB	1:20 000	HRP	Abcam	ab49900	AC-15	Primary
JAK2	WB	1:1000		Cell Signaling Technology	3230	D2E12 XP	Primary
IRF1	WB	1:1000		Cell Signaling Technology	8478	D5E4 XP	Primary
ERH	WB/IP/IF	1:2000/ 1:100/ 1:500		Abcam	ab166620	EPR10830(B)	Primary
phospho-STAT1	WB	1:1000		Cell Signaling Technology	7649	D4A7	Primary
JAK1	WB	1:1000		Abcam	ab133666	EPR349(N)	Primary
STAT1	WB	1:1000		Santa Cruz Biotechnology	SC-346	E23	Primary
Vinculin	WB	1:1000		Sigma-Aldrich	V9131	hVIN-1	Primary
ALYREF	WB	1:500		Santa Cruz Biotechnology	sc-32 311	11G5	Primary
MAGOH	WB	1:1000		Santa Cruz Biotechnology	sc-56 724	21B12	Primary
SRSF1	WB	1:1000		Thermo Fisher Scientific	PA5-30 220		Primary
Lamin A/C	WB	1:1000		Santa Cruz Biotechnology	sc-376 248	E-1	Primary
Alpha-Tubulin	WB	1:1000		Sigma-Aldrich	T9026	DM1A	Primary
IRF1	FACS	1:100	PE	Cell Signaling Technology	12 732	D5E4 XP	Primary
MYC	FACS	1:100	PE	Cell Signaling Technology	35 876	D3N8F	Primary
Rabbit IgG	IF	1:1000	Alexa 594	Invitrogen	A-11 012		Secondary
IgG Isotype Control	IP	1:300		Cell Signaling Technology	2729		Primary
CD90.1/Thy1.1	FACS	1:500	APC	BioLegend	202 526		Primary
Myc-tag	WB	1:5000		MILLIPORE	05-724	4A6	Primary
c-Myc	WB	1:1000		Cell Signaling Technology	5605	D84C12	Primary

platform by the NGS Facility at Vienna BioCenter Core Facilities (VBCF), Austria.

For RNA-seq analysis, quality control was performed with fastqc (v0.11.8) and pre-processing (adapter and quality trimming) with trim_galore (v0.6.2) for RKO and fastp (v0.20.1) for RAW 264.7 cells, respectively. Cell-line specific 3'-ends were determined with 3-GamES (<https://github.com/poojabhat1690/3-GamES>) using human (GRCh38) and mouse (GRCm38) RefSeq and Ensembl annotations for RKO and RAW 264.7 cells, respectively. QuantSeq reads were quantified with SlamDunk v0.3.4 [36] using the counting Windows output from 3-GamES, and 3'-end read counts were collapsed per gene.

Differential expression analysis was done in R [37] using DESeq2 [38]; genes with $\log_2\text{FC} \geq 1$ and $\text{padj} \leq 0.05$ were considered to have significantly different expression. Drastically different outliers from PCA analysis were excluded from the analysis (RKO samples: replicate 1 – sgAAVS1 non-treated, replicate 2 – sgERH IFN γ treated, and replicate 3 – sgAAVS1 IL-1 β treated). Differentially expressed genes were analyzed for enriched GO terms by Enrichr [39–41].

Protein stability determination

FACS sorted RKO-iCas9 cells with constitutive sgRNA expression (sgAAVS1 or sgERH) were treated with 100 ng/mL dox (Sigma-Aldrich, D9891) for 5 days to induce Cas9-mediated knockouts. To determine JAK2 protein stability, the cells were subsequently treated for 4 h with 10 μM of the proteasome inhibitor epoxomicin (EPX, Gentaur Molecular Products, 607-A2606) or DMSO as a control. In parallel, cells were treated with 40 $\mu\text{g/mL}$ of the translation inhibitor cycloheximide (CHX, Sigma-Aldrich, C1988) for up to 6 h. Total protein was extracted and analyzed by western blotting.

Transcription rate determination

FACS sorted RKO-iCas9 cells harboring constitutive sgRNA expression (sgAAVS1 or sgERH) were treated with 100 ng/mL dox (Sigma-Aldrich, D9891) for 5 days to induce Cas9-mediated knockouts. To determine JAK2 transcription rates, cells were subsequently treated for 15 min with 1 mM 4-Thiouridine (4sU, BIOSYNTH, NT06186) or water as a control. All subsequent steps were performed under minimal light conditions. Cells were washed in cold PBS and RNA isolation was performed with High Performance RNA Isolation kit (Vienna, VBC Molecular Tools Shop, RNAisomag) and a King-Fisher Flex robot (Thermo Fisher Scientific). For each sample, 70 μg RNA was precipitated overnight at -20°C with 2.5x volume EtOH containing 0.1 mM DTT, 0.1x volume 3M NaOAc (pH 5.2), and 1 μL glycogen. The pellet was washed twice with 75% EtOH containing 0.1 mM DTT and resuspended in H $_2$ O with 0.1 mM DTT. RNA fragmentation was performed with the NEBNext Magnesium RNA Fragmentation Module (New England Biolabs, E6150S) according to the manufacturer's recommendation with 1 min fragmentation time, and precipitation was performed as previously described with finally resuspending the RNA in 350 μL water.

To biotinylate 4sU labeled RNA, 50 μL 10x biotinylation buffer (200 mM HEPES pH 7.8 and 10 mM EDTA pH 8.0) and 100 μL MTSEA-biotin (biotium, 90066–1) in DMF (50 $\mu\text{g/mL}$) were added, and the samples were rotated at room temperature for 2 h. The RNA was then isolated with acidic phenol chloroform isoamyl alcohol (PCI, ROTH, X985.3) according to the manufacturer's instructions. The RNA was precipitated with 1x volume of isopropanol instead of EtOH, and ultimately resuspended in 100 μL water.

Pulldown of biotinylated RNA was then performed by first adding 100 μL of μMACS Streptavidin MicroBeads (Miltenyi Biotec, 130–133-282) and rotating the samples for 10 min. The μMACS columns were equilibrated with 100 μL nucleic

Table 6. qPCR primers

Target	Primer	Sequence (5' to 3')
18s	Fwd	GTAACCCGTTGAACCCCAT
	Rev	CCATCCAATCGGTAGTAGCG
GAPDH	Fwd	GAAGGTGAAGGTCGGAGTC
	Rev	GAAGATGGTGATGGGATTT
Gapdh	Fwd	ATGGTGAAGGTCGGTGTGA
	Rev	TGAAGGGGTCGTTGATGG
RPLP0	Fwd	CCAGGCTTTAGGTATCAC
	Rev	AGCACTTCAGGGTTGTAG
TBP	Fwd	GAGTTCTGGGATGTACC
	Rev	GTTCTGGGCTCTCTTATC
HSPA4L (IR)	Fwd	TTGTAGGAGGAGCAACAC
	Rev	TTATACCTGTAACGCACATC
HEATR1 (IR)	Fwd	TGAGTGTGGTGACATAAG
	Rev	CACCGATTAGTTTTCTCG
JAK2 (IR)	Fwd	GAACATACTAAATGCTCCAG
	Rev	TACATTCCCAGTAATAAGG
PIK3C2A (IR)	Fwd	GACAAAGAGGTTAATGTGG
	Rev	GTTGCTGTATAATGCTTCTG
TOP2B (IR)	Fwd	TGTAGTGGACAGAACACAG
	Rev	CTTAATACACTGCCCTTCC
RAD50 (IR)	Fwd	ACAGCGAGGTAAGTTGTC
	Rev	AGAGCCAGGACTGAAATC
JAK2 (cytoplasm)	Fwd	GGAAGATTACCAAGACCAG
	Rev	CCTTATTTGATCCACTCG
pre-IRF1	Fwd	AGGAGCCAGAAATGACAGC
	Rev	TCAGTACCCAGATGCTTTACC
JAK2	Fwd	GACTGGACTGTATGTACTTCG
	Rev	CCACTGAGGTTGTACTCTTC
JAK1	Fwd	ATTACGGTGCTGTCTAGG
	Rev	ACACTCAGGTTCTTGGAG
STAT1	Fwd	GTGATCTCCAACGTCAGCCA
	Rev	ACATGTTCAAGTGGTCCACA
IFNGR1	Fwd	TATGATCCCCGAAACTACC
	Rev	CTAACTGGCACTGAATCTC
IFNGR2	Fwd	CTGTTGTCTACCAAGTGC
	Rev	CTGTTGCTGTGATCTGTG
Jak2	Fwd	AGTGGAGAGTATGTTGCAG
	Rev	GACTCGTCTATGTGGAAGAC
Jak1	Fwd	CTACACAGTCAAGGACGAC
	Rev	GAGTCACAGTAAGTGAGCAG
Stat1	Fwd	AGATCCCGTACAGATGTC
	Rev	TTACTGTCCAGCTCCTTC
Ifngr1	Fwd	TGGGGAGATCCTACATAC
	Rev	GATGAGATTCCGCTACTG
Ifngr2	Fwd	CACCCATTACAGTCTTC
	Rev	GAGGTCCAACAGTAACATTC
pre-JAK2	Fwd	AAGAAAGCAGGTAATCAGAC
	Rev	GACATACTAACCTGCAGACG
pre-Jak2	Fwd	AGTGGTCAAGAGGAAAC
	Rev	TCTACCAGCAGAGAATGG
JAK2 (cryptic splicing)	Fwd	GACAGAAATGGAGGGAAC
	Rev	GATACACCTGAAGAACTGG

acid buffer (Miltenyi Biotec, 130–133–282), and then twice with 100 μ L 1x biotinylation buffer. Samples were applied to columns and washed three times with 900 μ L washing buffer (100 mM Tris-HCl pH 7.5, 10 mM EDTA pH 8.0, 1 M NaCl, 0.1% Tween-20), after which 4sU labeled RNA was eluted two times with 100 μ L of 100 mM DTT, and subsequently precipitated with isopropanol.

The RNA was treated with Turbo DNase (Thermo Fisher Scientific, AM2238), isolated with PCI (ROTH, X985.3), and precipitated with isopropanol. Preparation of cDNA was performed as described above, and qPCRs were performed using Luna Universal qPCR Master Mix (NEB,

M3003). Input samples were included for normalization purposes.

ERH co-IP and mass spectrometry

Twenty-four hours prior to transfection, 6×10^6 HEK-293T cells were seeded in eight 10-cm dishes (four negative controls, four for ERH-transfection). The cells were transfected with 6 μ g plasmid per plate with FuGENE HD (Promega, E2311) as per the manufacturer's instructions. The other four plates were left untransfected. Media were exchanged 6 h post-transfection. After transfection, cells were grown for 48 hours and harvested by incubation with 1 ml of trypsin/DPBS solution. Trypsin was deactivated by addition of 10 mL of DMEM with FCS and antibiotics, and cells were washed three times with DPBS. Cells lysed by addition of 250 μ L 2x lysis buffer (50 mM Tris HCl pH 7.5, 300 mM NaCl, 3.0 mM MgCl₂, 2 mM DTT, 0.2% Triton X-100, 1 tablet cOmplete Mini, Roche) and 150 μ L MQ, and by bioruptor sonication (three cycles, high intensity, 30 s). GFP-trap beads were prepared as previously described for the Llama antibody against GFP 16 (LaG16) [42]. The lysate was incubated with the GFP-trap beads for 2 h, washed five times with 500 μ L wash buffer (25 mM Tris HCl pH 7.5, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM DTT, 1 tablet cOmplete Mini, Roche), and boiled at 95°C for 10 min in resuspension buffer (per reaction: 7.5 μ L of 4X LDS sample Buffer, 3 μ L DTT, 14.5 μ L MQ).

Peptides were separated on a 20-cm self-packed column with 75 μ m inner diameter filled with ReproSil-Pur 120 C18-AQ (Dr. Maisch GmbH) mounted to an EASY HPLC 1000 (Thermo Fisher) and sprayed online into an Q Exactive Plus mass spectrometer (Thermo Fisher). We used a 94-min gradient from 2 to 40% acetonitrile in 0.1% formic acid at a flow of 225 nl/min. The mass spectrometer was operated with a top 10 MS/MS data-dependent acquisition scheme per MS full scan. Mass spectrometry raw data were searched using the Andromeda search engine [43] integrated into MaxQuant suite 1.5.2.8 [44]. In both analyses, carbamidomethylation at cysteine was set as fixed modification, while methionine oxidation and protein N-acetylation were considered as variable modifications. Match-between-run option was activated. Prior to bioinformatic analysis, reverse hits, proteins only identified by site, protein groups based on one unique peptide, and known contaminants were removed. For further bioinformatic analysis, the LFQ values were log₂-transformed and the median across replicates was calculated. This enrichment was plotted against the – log₁₀- transformed *P* values (Welch's t-test) using the ggplot2 package in the R environment.

Immunoprecipitation

Cells were lysed in Frackelton buffer (10 mM Tris-HCl pH 7.4, 50 mM NaCl, 30 mM Na₄P₂O₇, 50 mM NaF, 2 mM EDTA, 1% Triton X-100, 1 mM DTT, 0.1 mM PMSE, and 1x protease inhibitor cocktail). Lysates were rotated for 5 min at 4°C and then centrifuged for 10 min at 20 000 $\times$ g at 4°C. Supernatant protein concentrations were determined by Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, 23 225), and 500 μ g total protein was used for each IP (20 μ g; 4% was taken for input). IPs were incubated rotating overnight at 4°C with either IgG Isotype control (1:300, Cell Signaling Technology, 2729) or anti-ERH (1:100, Abcam, ab166620). Magnetic beads (Pierce Protein A/G Magnetic Beads, Thermo Fisher Scientific, 88 803) were washed 2x in Frackelton buffer,

blocked by rotating for 1 h at 4°C in 3% BSA in Frackelton buffer, washed 3x in Frackelton buffer, and 25 µL beads were then added to each IP and left rotating for 2 h at 4°C. After five washes in Frackelton buffer, the IPs were boiled at 95°C for 10 min in Laemmli sample buffer with 10% β-mercaptoethanol, and the whole IP/input was analyzed by WB.

Immunofluorescence microscopy

Around 2.5×10^5 cells were seeded onto coverslips and fixed with 4% paraformaldehyde (PFA) for 15 min, 48 h post-seeding. Cells were permeabilized with 0.25% Triton X-100 in PBS for 5 min, and blocked in 1% BSA for 30 min at RT. Coverslips were first incubated with anti-ERH antibody (Abcam, ab166620) 1:500 in 1% BSA for 1 h at RT, then with anti-rabbit IgG Alexa Fluor 594 (Invitrogen, A-11012) 1:1000 in 1% BSA for 1 h at RT, and finally with 0.4X Hoechst (Thermo Fisher Scientific, H3569) in PBS for 5 min at RT. Coverslips were mounted using ProLong Gold Antifade Mountant (Invitrogen, P36934) and images were collected using a modified Imager.Z2 microscope (Zeiss) at 63X magnification. Images were modified uniformly across the image using Fiji [45].

Subcellular fractionation and 3' mRNA QuantSeq

FACS-sorted RKO-iCas9 cells with constitutive sgRNA expression (*sgAAVS1*, *sgERH*, *sgALYREF*, *sgSRSF1*, *sgMAGOH*, or *sgPOLDIP3*) were treated with 100 ng/mL dox (Sigma-Aldrich, D9891) for 5 days. For subcellular fractionation, 2×10^6 cells were gently lysed in REAP buffer (0.1% NP-40 in 1x PBS) at 4°C, and $\geq 50\%$ of the lysate was taken for the whole cell fraction. Nuclei and cytoplasmic fractions were separated twice through centrifugation at 3000 x g for 1 min at 4°C. The cellular lysate fractions were analyzed by WB as described previously or used for RNA extraction. RNA isolation was performed with High Performance RNA Isolation kit (Vienna, VBC Molecular Tools Shop, RNAisomag) and a KingFisher Flex robot (Thermo Fisher Scientific). Preparation of cDNA was performed as described above, and qPCRs were run with Luna Universal qPCR Master Mix (NEB, M3003). To assess cryptic splicing, RT-qPCR amplicons were loaded on 2% agarose gels, imaged with a blue-light Safe Imager (Thermo Fisher Scientific), and analyzed with Fiji [45].

Generation of 3' mRNA sequencing libraries was done according to manufacturer's recommendations (Lexogen, 015-QuantSeq 3' mRNA-Seq Library Prep Kit FWD for Illumina, 081-UMI Second Strand Synthesis Module for QuantSeq FWD, and 020-PCR Add-on Kit for Illumina). Samples were analyzed on a NovaSeq SP SR100 platform by the NGS Facility at VBCF, Austria.

For RNA-Seq analysis, reads were pre-processed with fastp (v0.20.1) which was configured to extract 6 nt UMIs and trim 4 nt from the read 5'-ends along standard quality and adapter trimming settings. Quality control was performed with fastqc (v0.11.8). Reads were mapped to 3'-ends with the nf-core/slamseq pipeline v0.1. For the 3'-end annotations, we used the previously published set from [46] but extended it with GENCODE annotations for missing genes. Read and UMI counts were collapsed per gene and counted with a custom Python script. Differential expression analysis was done in R [37] with the RStudio IDE [47] and by using DESeq2 [38] with apeglm shrinkage option [48]. Genes with $\log_2FC \geq 1$ and $p_{adj} \leq 0.05$ were considered significantly differentially

expressed. Biomart (v2.50.3) [49, 50] was used to retrieve the number and lengths of exons and introns of canonical transcripts. Hierarchical clustered distance heatmaps and PCA plots were generated with ggplot2 [51] based on the variance stabilizing transformed (vst) mean mRNA expression of sample groups [38]. Enriched GO terms were obtained as described above.

Poly(A)-enriched RNA-seq

FACS-sorted RKO-iCas9 cells with constitutive sgRNA expression (*sgAAVS1* or *sgERH*) were treated with 100 ng/mL dox (Sigma-Aldrich, D9891) for 5 days to induce Cas9-mediated knockouts. Cells were treated for 4 h with 25 µg/mL CHX (Sigma-Aldrich, C1988) in DMSO, or DMSO only. Poly(A)-enriched RNA-Seq libraries were generated using the NEBNext Ultra II RNA Library Prep Kit for Illumina (New England Biolabs, E7770), and analyzed on a NovaSeq S4 PE150 XP platform by the NGS Facility at VBCF, Austria.

RNA-Seq analysis was performed using the nf-core/rnaseq pipeline (v3.13.2) [52, 53], which was run on the CLIP Batch Environment (CBE, VBC, Vienna) high performance cluster with Singularity [54]. Briefly, adapter and quality trimming was done with fastp [55], removal of rRNA reads with SortMeRNA [56], alignment and quantification of reads with STAR [57] and salmon [58], sorting and indexing with SAMtools [59], duplicate read marking with Picard, transcript assembly and quantification with StringTie [60], assembly of coverage files with BEDTools [61], and extensive QC with RSeQC [62], Qualimap [63], dupRadar [64], preseq [65], and DESeq2 [38]. The genome used for the analysis was built from the latest Ensembl version (GRCh38, v110). Differential gene expression analysis was done as previously described using DESeq2 [38, 41].

IR events were detected and analyzed for differential expression with IRFinder-S (v. 2.0.0) and DESeq2 [66, 67] using standard settings. $\log_2FC (\geq 1)$ and $p_{adj} (\leq 0.05)$ cut-offs were used to determine significance. Specific IR examples were retrieved with the integrative genomics viewer (IGV) [68]. IR events were considered adjacent if separated by one exon, and a random control was included that selected the same number of introns for each gene as reported previously [66]. Hierarchical clustered distance heatmaps, enriched GO terms, and canonical transcript information were obtained as described above.

For comparison of intrinsic features, IR events caused by the *ERH* KO were compared to the same number of randomly sampled introns in other genes without intron retention in either knockout (mean IR ratio ≤ 0.05 in both *sgAAVS1* and *sgERH*) or with intron retention in both knockouts (mean IR ratio ≥ 0.1 and absolute $\log_2FC \leq 0.2$ between knockouts). GC-content was analyzed with the Biostrings package [69]. The Maximum Entropy Score was used to calculate 5' and 3' splice site strengths using MaxEntScan [70]. Motif enrichment analysis was performed using the MEME suite's simple enrichment analysis (SEA) [71, 72] and the CISBP RNA motif database [73]. The first 200 bp of the 5' and 3' intronic regions (excluding the 6 and 30 bp parts of splice sites) and the first 100 bp of the flanking 5' and 3' exons (excluding the 3 and 1 bp parts of splice sites) were used for the analysis; introns and exons shorter than 200/100 bp were excluded from the analysis. All plotting was done in R using ggplot2 [51].

Results

Identification of novel positive regulators of IFN γ signaling by genome-wide genetic screening

To identify novel regulatory factors of the type II interferon signaling pathway, we performed a genome-wide genetic screen using our previously established time-controlled CRISPR/Cas9 screening approach [28, 74]. RKO cells are human diploid colon carcinoma cells, which can respond to several cytokines, and are amenable for forward genetic screening [28]. We used an optimized doxycycline (dox)-inducible Cas9 (iCas9) human RKO cell line [28], where functional editing by Cas9 only occurs in the presence of dox (Supplementary Fig. S1A). This allows for temporal control of gene knockouts and thus the possibility to assess the effects of disrupting cell-essential genes [28].

A genome-wide lentiviral sgRNA library [74] was transduced into the RKO-dox-iCas9 line at low multiplicity of transduction to ensure one integration per cell. Cells with single-gene disruptions were treated with IFN γ , and endogenous protein levels of the ISG IRF1 were used as a proxy for IFN γ signaling (Fig. 1A). IFN γ -treated knockout cells with the lowest or highest IRF1 protein levels were then collected by fluorescence-activated cell sorting (FACS) for further analysis (Fig. 1B). Two timepoints (2.5 and 5 days after Cas9 induction by dox) were used to allow the identification of factors independent of their turnover rate and whether they were essential for viability. The relative abundances of sgRNAs in collected cells were analyzed by NGS and compared to day-matched mean-distribution cell pools representing pre-enrichment library sgRNA frequencies (Fig. 1B). This comprehensive assay thus measures the effects of transcriptional to post-translational regulation on IFN γ signaling.

To identify factors required for IFN γ signaling, and hence IRF1 induction, we analyzed enriched sgRNAs in the IRF1^{low} cell population. As expected, IRF1 itself and all well-known JAK/STAT factors involved in propagating IFN γ signaling (Fig. 1A) scored as top hits (Fig. 1C and D and Supplementary Fig. S1B), attesting to the validity of the screen. In contrast, factors which are critical for type I IFN signaling, but not for IFN γ -dependent responses, were not enriched (Fig. 1D), demonstrating its specificity.

To discriminate specific from more general effects, we compared our hits with those of a similar screen that successfully identified regulators of the transcription factor MYC [28]. We reasoned that MYC would be a good control to filter out general RNA- and protein regulatory factors, since MYC mRNA and protein are rapidly turned over in cells [75–77], similar to IRF1 mRNA and protein [78, 79].

Interestingly, our screen specifically identified the essential exon junction complex (EJC) factors *RBM8A*, *MAGO*H, and *EIF4A3* as strong and specific positive regulators of IFN γ -induced IRF1 after 2.5 days of dox induction (Fig. 1C and D, and Supplementary Fig. S1B). Furthermore, multiple splicing and export factors that cooperate with the EJC, including *SRSF1* and *ALYREF*, scored as hits in our screen (Fig. 1D). In contrast, factors involved in other EJC-related processes, including *CASC3* and *UPF1* which are connected to translation-coupled nonsense-mediated decay (NMD), were not identified as hits in the screen (Fig. 1D).

In addition to these well-known splicing and export factors, the enigmatic RNA regulatory factor ERH was identified as a strong determinant of IFN γ -induced IRF1 expression at day 5

post-Cas9 induction (Fig. 1C and D and Supplementary Fig. S1B). ERH is not reported to have nucleic acid binding capacities but is instead thought to assist in forming protein complexes in diverse RNA regulatory processes [80]. Notably, it has been linked to the EJC [81], pre-mRNA splicing [82–84], and RNA export [85–87]. Consistent with a role as a facilitating factor, it has also been reported to control other RNA-related processes in cells, including piRNA processing [88, 89], miRNA maturation [90, 91], transcription [92, 93], and repressive heterochromatin formation [94].

ERH and the EJC are involved at multiple steps in post-transcriptional mRNA regulation and the EJC is loaded onto all spliced mRNAs [95–97]. Therefore, the possibility of a specific role in IFN γ -dependent IRF1, but not MYC, expression was surprising (Fig. 1D). To confirm this specificity, the core EJC component *MAGO*H, the post-transcriptional integrator *ERH*, and the positive control *JAK2* were knocked out individually, and intracellular IRF1 and MYC levels were determined by FACS (Fig. 1E and F). Consistent with the screens, inducible *MAGO*H and *ERH* knockouts reduced IFN γ -induced IRF1 levels without affecting MYC under comparable experimental conditions (Fig. 1E and F). From these results we concluded that EJC- and ERH-related splicing and mRNA export are more critical for IFN γ -induced expression of ISGs, such as IRF1, than for global mRNA regulation of other factors like MYC.

Given the functional association of the EJC and ERH with RNA processing, we hypothesized that *IRF1* levels were transcriptionally or post-transcriptionally regulated. To test this, we used RT-qPCR to investigate whether ERH and *MAGO*H regulated *IRF1* mRNA levels, and whether this was dependent on IFN γ .

ERH or *MAGO*H ablation significantly reduced IFN γ -induced *IRF1* mRNA levels, but not *IRF1* baseline levels (Fig. 1G). Furthermore, ERH depletion similarly reduced *IRF1* pre-mRNA levels (Supplementary Fig. S1C) yet did not affect *IRF1* mRNA turnover as determined by actinomycin D chase and RT-qPCR analysis (Supplementary Fig. S1D). Thus, our data indicate that loss of *ERH* or *EJC* components specifically reduces IFN γ -dependent signaling and consequently prevents the transcriptional induction of ISGs such as IRF1.

ERH maintains *JAK2* mRNA levels, and consequently, IFN γ signaling

Since ERH was critical for IFN γ -induced *IRF1* transcription, we hypothesized that ERH regulates IFN γ signaling and hence global ISG expression. Moreover, as ERH is highly conserved [98], we reasoned that its role in IFN γ -induced transcription would also be conserved across cell types and species. To investigate whether loss of *ERH* abolishes ISG induction across species and cell types, we performed 3' mRNA QuantSeq in IFN γ -treated or non-treated RKO-iCas9 cells (human colon carcinoma) [28] and RAW 264.7-iCas9 cells (murine macrophage) [29].

Principle component analysis showed that the three different stimulatory conditions clustered together (Supplementary Fig. S2A). Three samples were identified as outliers and were excluded from further analyses (Supplementary Fig. S2A).

Under non-treated baseline conditions, ERH depletion affected expression of less than 5% of all genes (Source data Fig. 2A and Source data Supplementary Fig. S2B,C), consistent with the notion that ERH is specific and only affects a distinct

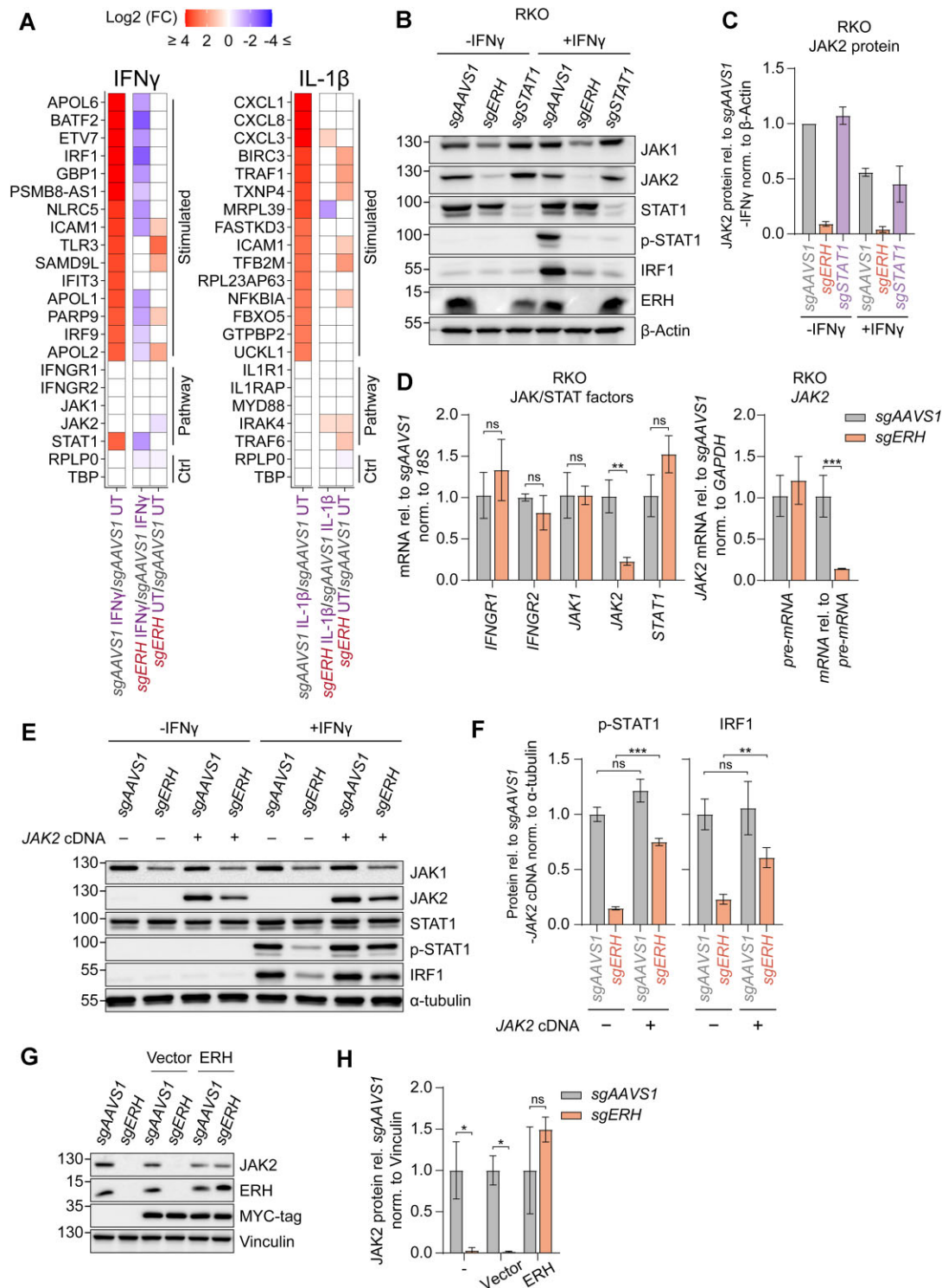


Figure 2. ERH is essential for maintaining JAK2 mRNA and JAK2 protein levels, and consequently critical for IFN γ signaling. **(A)** RKO-iCas9 cells expressing the indicated sgRNAs were treated with dox for 5 days to induce Cas9 editing. The cells were then stimulated with IFN γ or IL-1 β , after which RNA levels were quantified by 3' QuantSeq. The top 15 stimulated genes and pathway components are shown. Non-significant changes ($p_{adj} \leq 0.1$) were given a 0-fold change difference. $n = 3$ or $n = 2$ biological replicates; replicate outliers were excluded from the analysis (Supplementary Fig. S2A). **(B)** Dox-induced RKO-iCas9 cells expressing the indicated sgRNAs were analyzed by WB, and **(C)** quantified. Data represent means and sd; $n = 2$ biological replicates. **(D)** RKO-iCas9 cells with the indicated genes targeted for 5 days were analyzed by RT-qPCR. Data represent means and sd; $n = 3$ biological replicates. Two-tailed t-test with Benjamini-Hochberg correction (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$). **(E)** RKO-iCas9 cells expressing the indicated sgRNAs were transduced with a JAK2 cDNA expressing vector, stimulated with IFN γ for 4 h, after which protein lysates were analyzed by WB, and **(F)** quantified. Data represent means and sd; $n = 3$ biological replicates. Unpaired t-test with Welch correction and with Benjamini-Hochberg correction (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$). **(G)** RKO-iCas9 cells expressing the indicated sgRNAs for 5 days and transduced with a sgRNA resistant *ERH* cDNA expressing vector or empty vector were lysed and subsequently analyzed by WB (representative blot), and **(H)** quantified. Data represent means and sd; $n = 3$ biological replicates. Unpaired t-test with Welch correction (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$).

group of mRNAs (Fig. 2A and Supplementary Fig. S2B,C). *ERH* ablation significantly affected expression (absolute fold change ≥ 2 and $\text{padj} \leq 0.05$) of 809 (548 up & 261 down) genes in RKO cells, and 545 (361 up & 184 down) in RAW 264.7 cells. These changes correspond well with a previous study that reported 785 (526 up & 262 down) or 422 (243 up and 179 down) mRNA changes after siRNA-mediated *ERH* knockdown in non-differentiated or differentiated human fibroblasts [94]. In both RKO and RAW 264.7 cells, *ERH* ablation reduced expression of multiple mRNAs involved in DNA replication and DNA repair (Supplementary Fig. S2D), consistent with previous observations following *ERH* depletion [83, 94].

As predicted, *ERH* ablation specifically abolished IFN γ -induced but not baseline mRNA levels of ISGs, including *IRF1* and *BATF2*, in RKO cells (Fig. 2A), indicating that *ERH* is indeed crucial for IFN γ -induced ISG transcription. Likewise, in RAW 264.7 cells, *ERH* ablation significantly reduced IFN γ -induced expression of ISGs, including *Irf1*, *Batf2*, and *Cxcl10* (Supplementary Fig. S2C). To assess whether *ERH* was specifically important for IFN γ signaling or has a more general effect on rapid cytokine-induced transcription of response genes, we treated cells in parallel with IL-1 β as an alternative cytokine stimulus. In contrast to IFN γ -induced ISGs, *ERH* depletion did not reduce IL-1 β -induced mRNA expression of response genes such as CXCL factors in neither RKO (Fig. 2A, Supplementary Fig. S2B), nor RAW 264.7 cells (Supplementary Fig. S2B,C). In summary, *ERH* plays a highly specific regulatory role in IFN γ -induced gene expression across cell types and species.

Given the specific role of *ERH* in controlling the type II IFN axis, we reasoned that *ERH* may regulate the levels of JAK/STAT factors, which are essential for IFN γ signaling (Fig. 1A). To systematically test how *ERH* ablation impacts JAK/STAT pathway components, their protein levels were analyzed by WB in lysates from sg*ERH* cells. Loss of *ERH* strongly reduced STAT1 activation, as evidenced by its reduced phosphorylation upon IFN γ treatment, without major effects on total STAT1 levels (Fig. 2B). This indicated that the effect of *ERH* loss lies upstream of STAT1. In line with this hypothesis, *ERH* targeting led to an almost complete loss of JAK2 under both stimulated and unstimulated conditions (Fig. 2B and C), arguing that *ERH* maintains baseline JAK2 levels.

Given that *ERH* functions in various RNA regulatory processes, we investigated whether reduced JAK2 mRNA levels explained the loss of JAK2 protein. *ERH* ablation reduced JAK2 mRNA baseline levels in 3' mRNA QuantSeq data without affecting the expression of other JAK/STAT factors (Fig. 2A and Supplementary Fig. S2C). RT-qPCR measurements of mRNAs encoding the individual JAK/STAT pathway components (IFNGR1, IFNGR2, JAK1, JAK2, and STAT1) further confirmed that *ERH* knockout exclusively reduced JAK2 transcript levels, which were decreased by approximately 10-fold (Fig. 2D).

Next, we examined JAK2 pre-mRNA levels using intron-exon junction primers. While *ERH* ablation reduced mature JAK2 mRNA levels, it did not significantly affect JAK2 pre-mRNA levels (Fig. 2D). Thus, *ERH* is important for post-transcriptional JAK2 processing yet has minimal effects on JAK2 transcription. These effects were also conserved in RAW 264.7 cells, as *ERH* ablation similarly reduced JAK2 protein levels (Supplementary Fig. S2E,F) and JAK2 mature-mRNA

levels relative to pre-mRNA in these cells (Supplementary Fig. S2G). The effect size in RAW 264.7 was lower than in RKO cells, consistent with less efficient *ERH* depletion after 5 days of Cas9-induced editing (Fig. 2B and Supplementary Fig. S2E).

To test whether the reduced JAK2 levels caused abrogated IFN γ signaling in *ERH*-targeted cells, we rescued JAK2 expression in these cells by inserting a cDNA JAK2-P2A-mCherry expression vector (Supplementary Fig. S2H). As predicted, exogenous expression of JAK2 largely rescued JAK/STAT signaling and IFN γ -driven ISG transcription in *ERH* KO cells (Fig. 2E) as determined by significantly increased p-STAT1 and IRF1 levels (5-fold increase, $\text{padj} \leq 0.001$, and 2.7-fold increase, $\text{padj} \leq 0.01$, respectively) (Fig. 2F).

Lastly, we determined whether expression of exogenous *ERH* could rescue JAK2 levels in sg*ERH*-targeted cells. To this end, RKO-iCas9 cells harboring sgAAVS1 or sg*ERH* constructs were transduced with a lentiviral vector constitutively expressing sgRNA-resistant *ERH* (Supplementary Fig. S2H). Subsequently, Cas9 was induced, and protein levels analyzed by WB (Fig. 2G and H). Indeed, exogenous expression restored *ERH* protein levels close to their endogenous concentrations in sgAAVS1 controls (Fig. 2G and H). As expected, exogenous *ERH* expression also rescued JAK2 protein levels (Fig. 2G,H). From this we concluded that the effects from *ERH*-targeting on JAK2 were specific, and not the result of off-target effects.

Taken together, our data show that *ERH* has a conserved role in post-transcriptional processing of JAK2 mRNA under homeostatic conditions, and consequently is critical for IFN γ -induced JAK/STAT-driven ISG expression.

ERH associates with the EJC and EJC-associated splicing and export factors that are critical for JAK2 production

To elucidate the function of *ERH*, we analyzed its associated proteins. To this end, we exogenously expressed ERH-mTurquoise2 in HEK-293T cells and employed co-IP tandem mass spectrometry (MS/MS) (Supplementary Fig. S3A). As expected, *ERH* itself and its previously described direct protein-protein interaction partners such as POLDIP3 [99] were significantly enriched in the pull-down (Fig. 3A), validating our experimental setup.

Consistent with previous data showing that EJC components [81] and related splicing and export factors such as the TREX complex [85, 87] pull down *ERH*, we detected a significant enrichment of peptides for MAGOH/MAGOHB, ALYREF, and SRSF1 in our *ERH* pulldown (Fig. 3A). Independent co-IPs of endogenous *ERH* from RKO cells further confirmed these interactions (Fig. 3B). These results suggest that the EJC and its associated factors may function with *ERH* in the same mRNP complexes.

Several of the identified interacting factors also scored well in our genetic screen for regulators of IFN γ signaling (Fig. 1C). Factors that both physically interacted with *ERH* by co-IP and genetically phenocopied the effect of *ERH* loss tended to be linked to nuclear post-transcriptional RNA processing, particularly splicing and export (Fig. 3C,D – left side). Additional nuclear factors that regulate splicing and export were solely identified by co-IP MS/MS, including BCLAF1, THRAPP3, and ACIN1 (Supplementary Fig. S3B and Fig. 3D – right side), consistent with the predominantly nuclear

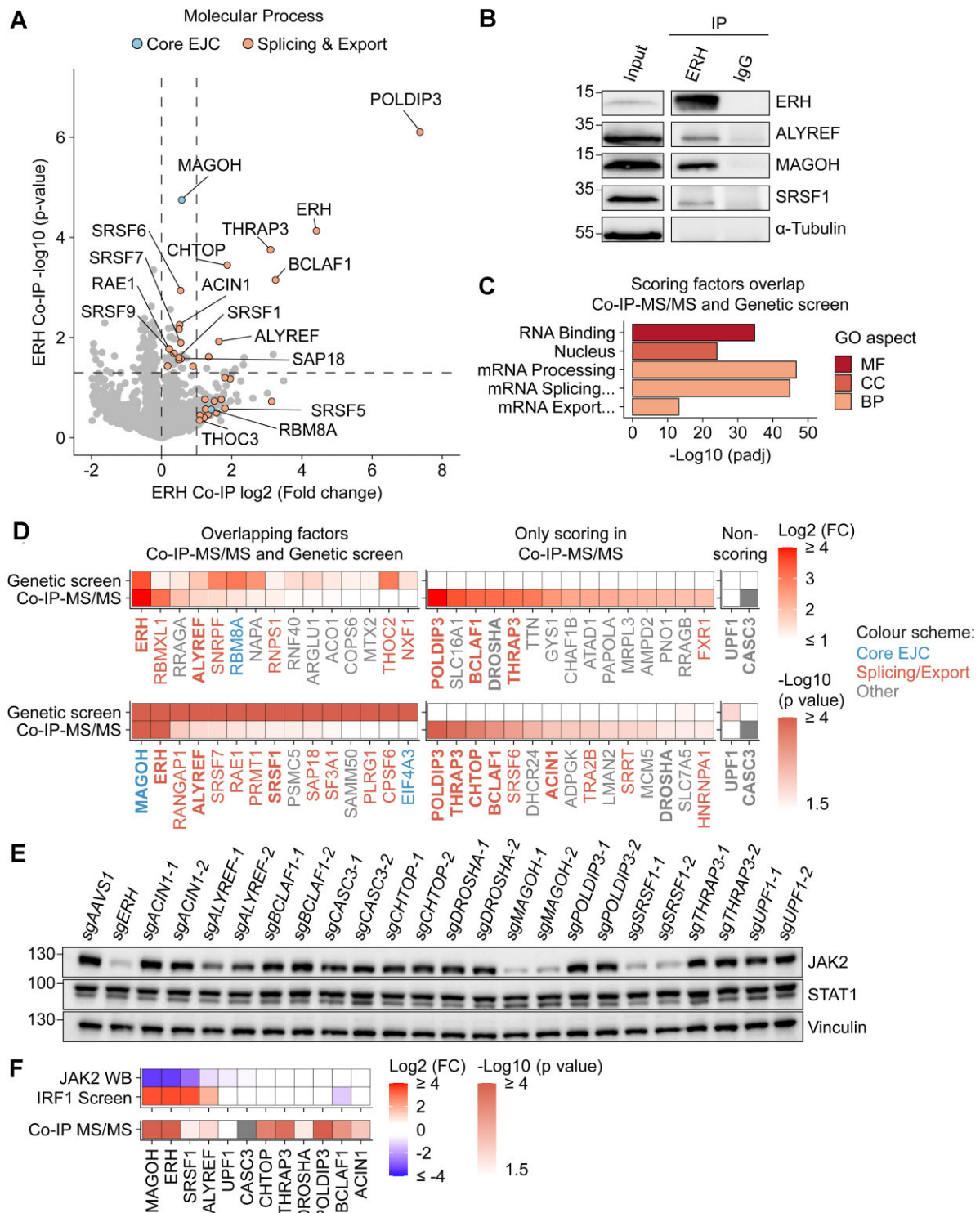


Figure 3. EJC-associated splicing and export factors interact with ERH and are critical for JAK2 production. **(A)** ERH-mTurquoise2 was isolated from HEK-293T cells by immunoprecipitation (IP), and interacting proteins identified by tandem mass spectrometry (MS/MS). Cells not expressing the ERH-mTurquoise2 construct were used as comparative controls. Dashed lines indicate enriched proteins based on adjusted P value ($P \leq 0.05$ and log2 fold change > 0), or fold change (log2 fold change ≥ 1). $n = 3$ biological replicates. **(B)** Endogenous ERH IP from RKO-iCas9 cells, and indicated interactors analyzed by WB. Representative immunoblot of $n = 2$ biological replicates. **(C)** Selected top GO terms for overlapping genes enriched in ERH co-IP-MS/MS and the genetic screen of IFN γ signaling regulators. MF, molecular function; CC, cellular component; BP, biological process. **(D)** Heatmaps showing the top 15 enriched proteins in ERH co-IP-MS/MS based on fold change or adjusted P value, that either scored (left) or did not score (right) in the genetic screen for IFN γ regulators. For each factor, the timepoint post Cas9 induction with lowest P value or highest fold change from the genetic screen is shown. Factors are labeled based on their involvement in: EJC, splicing and export, or other processes. Non-scoring cytoplasmic factors and other factors used for validation in panel **(E)** are indicated in bold. **(E)** RKO-iCas9 cells with the indicated genes targeted for 5 days were analyzed for JAK2 and STAT1 protein levels by WB. Two different sgRNAs targeting each gene are indicated with "1" or "2". Representative blot from four ($n = 4$) biological replicates. **(F)** Heatmaps showing from top to bottom: mean JAK2 protein levels for sgRNAs targeting the same gene as quantified from panel **E**, enrichment in the genetic screen for IFN γ regulators at 5 days of Cas9 induction (Fig. 1C), and adj. P value in ERH co-IP-MS/MS from panel **E**.

localization of endogenous ERH in RKO (Supplementary Fig. S3C) and other cells [100]. Together, the ERH interactome and the identified IFN γ -regulatory factors support a critical role of ERH in *JAK2* post-transcriptional mRNA processing.

To determine if nuclear factors interacting with ERH also regulate *JAK2*, each factor was targeted with two independent sgRNAs, after which *JAK2* protein levels were measured by WB (Fig. 3E). In addition, total STAT1 levels were analyzed as a proxy for global cellular effects caused by the knockouts. WB measurements confirmed the effective and specific depletion of ERH, MAGOH, ALYREF, and SRSF1 in targeted cells (Supplementary Fig. S3D). We chose three groups of factors to investigate based on their interaction with ERH and how they scored in the genetic screen: (i) factors interacting by co-IP-MS/MS and scoring in the genetic screen (i.e. factors that might facilitate *JAK2* processing: ERH, ALYREF, MAGOH, and SRSF1), (ii) factors interacting by co-IP-MS/MS but not scoring in the genetic screen (i.e. potentially cooperative, but not functionally limiting factors: ACIN1, BCLAF1, THRAP3, CHTOP, DROSHA, and POLDIP3), and (iii) non-interacting and non-scoring factors (i.e. negative controls such as cytoplasmic RNA regulatory factors: CASC3 and UPF1).

As predicted, the first group of factors (interacting by co-IP-MS/MS and scoring well in the genetic screen), significantly and specifically reduced *JAK2*, but not STAT1, protein levels (Fig. 3F and Supplementary Fig. S3E). *MAGOH* and *SRSF1* ablation, and to a lesser extent *ALYREF* loss, reduced *JAK2* levels similarly to *ERH* ablation (~7-fold and ~2 fold, respectively). In contrast, depletion of non-scoring factors (groups two and three) did not affect or only mildly reduced *JAK2* (~23% reduction), and did not affect STAT1 levels (Fig. 3F and Supplementary Fig. S3E). In conclusion, our results indicate that ERH and its interacting splicing and export factors such as MAGOH, SRSF1, and ALYREF together regulate *JAK2* mRNA processing.

Defects in *JAK2* mRNA processing prevent its subsequent nuclear export

Since ERH interacted with nuclear mRNA processing factors, we hypothesized that *ERH* controls the nuclear/cytoplasmic mRNA localization of *JAK2* and a subset of other cellular mRNAs that are likewise regulated.

To determine how ERH influenced the relative subcellular mRNA concentrations of *JAK2* and to identify similar mRNAs that are post-transcriptionally affected, we performed subcellular fractionation and 3' mRNA QuantSeq in RKO-iCas9 cells. To identify functional similarities and differences between ERH and its interactors, we also targeted *MAGOH*, *SRSF1*, and *ALYREF*. In addition, *POLDIP3* was targeted as a negative control, since it interacts with ERH but does not affect *JAK2* levels (Fig. 3F). Robust fractionation was confirmed by WB using the cytosolic marker Vinculin and the nuclear marker Lamin A/C (Supplementary Fig. S4A). The sequencing experiments were performed without spike-ins for general mRNA abundance, and thus measured relative changes in mRNA levels.

There is extensive cross-talk between transcriptional, post-transcriptional, and mRNA degradation machineries, reviewed in [101–103]. Therefore, the knockouts could affect sub-cellular mRNA distributions that are indicative of transcriptional or post-transcriptional effects in three major

ways: (i) mRNAs with cytoplasmic depletion and increased or unaffected nuclear retention, indicative of disrupted post-transcriptional mRNA processing connected to either splicing, export, translation, or mRNA degradation by NMD, (ii) mRNAs with increased nuclear and unaffected cytoplasmic levels, indicative of increased transcriptional output and altered post-transcriptional processing, and (iii) mRNAs with both decreased or increased nuclear and cytoplasmic abundance, indicative of altered transcription.

Consistent with ERH having a post-transcriptional role, QuantSeq analysis of the fractions revealed that *ERH* ablation specifically reduced cytoplasmic levels and/or increased nuclear levels of multiple mRNAs (Fig. 4A). Depletion of ERH significantly reduced cytoplasmic (log2FC -1.2 and padj 7.1×10^{-8}) and increased nuclear (log2FC 1.2 and padj 1.9×10^{-8}) *JAK2* mRNA abundance (Fig. 4A), indicating that *ERH* ablation disrupted *JAK2* post-transcriptional mRNA processing, resulting in *JAK2* nuclear retention.

Faulty transcripts retained in the nucleus can be targeted for degradation [104–106]. Therefore, we reasoned that mRNAs with considerably reduced cytosolic levels, yet minimally affected nuclear concentrations, likely also represent transcripts that are post-transcriptionally regulated by ERH. Other mRNAs that phenocopied *JAK2* and were likewise reduced in the cytosol upon *ERH* KO included multiple DNA repair and DNA replication factors (Supplementary Fig. S4B), consistent with previous reports of ERH regulating splicing of such mRNAs [83, 84]. In contrast to mRNAs reduced in the cytoplasm, only minor similarities and enriched GO terms were identified for mRNAs only affected in the nucleus (Supplementary Fig. S4B), suggesting that these mRNAs are regulated through a different mechanism.

To identify features that could explain the post-transcriptional effect of ERH on *JAK2*, we analyzed mRNAs that phenocopied *JAK2* behavior in *ERH* KO cells. Since *JAK2* is a distinctly large mRNA (comprised of 28 exons, and a 7 kb mature mRNA derived from a 146 kb gene), we analyzed the number of exons and length of mRNAs/genes of other candidate ERH-targets. Interestingly, mRNAs depleted in the cytoplasm, but not mRNAs accumulating in the nucleus, had significantly more exons and introns than non-affected mRNAs (Fig. 4B). However, these mRNAs and their corresponding genes were not significantly longer than average (Fig. 4B), suggesting that the number but not the length of exons/introns was an important feature for ERH-regulated mRNAs. We reasoned that a higher number of exons/introns could increase the cumulative probability for mis-splicing and result in non-exported transcripts. Taken together, these data indicated that mRNAs that decreased in the cytoplasm, but not those increased in the nucleus, exhibited *JAK2*-like features, supporting a model of altered splicing and subsequent decreased export upon ERH depletion.

Next, we compared how ERH affected overall subcellular mRNA localization compared to its interactors (*MAGOH*, *SRSF1*, *ALYREF*, and *POLDIP3*). Interestingly, distance heatmaps (Fig. 4C) and unsupervised principal component analysis (PCA) (Supplementary Fig. S4C) showed that ERH depletion affected cytoplasmic mRNA expression most similar to loss of the RNA export factor *ALYREF*. Furthermore, when comparing subsets of mRNAs with reduced cytoplasmic expression in either knockout, *ERH* and *ALYREF* ablations behaved most similarly to one another (Fig. 4C). In contrast to cytoplasmic transcript levels, nuclear

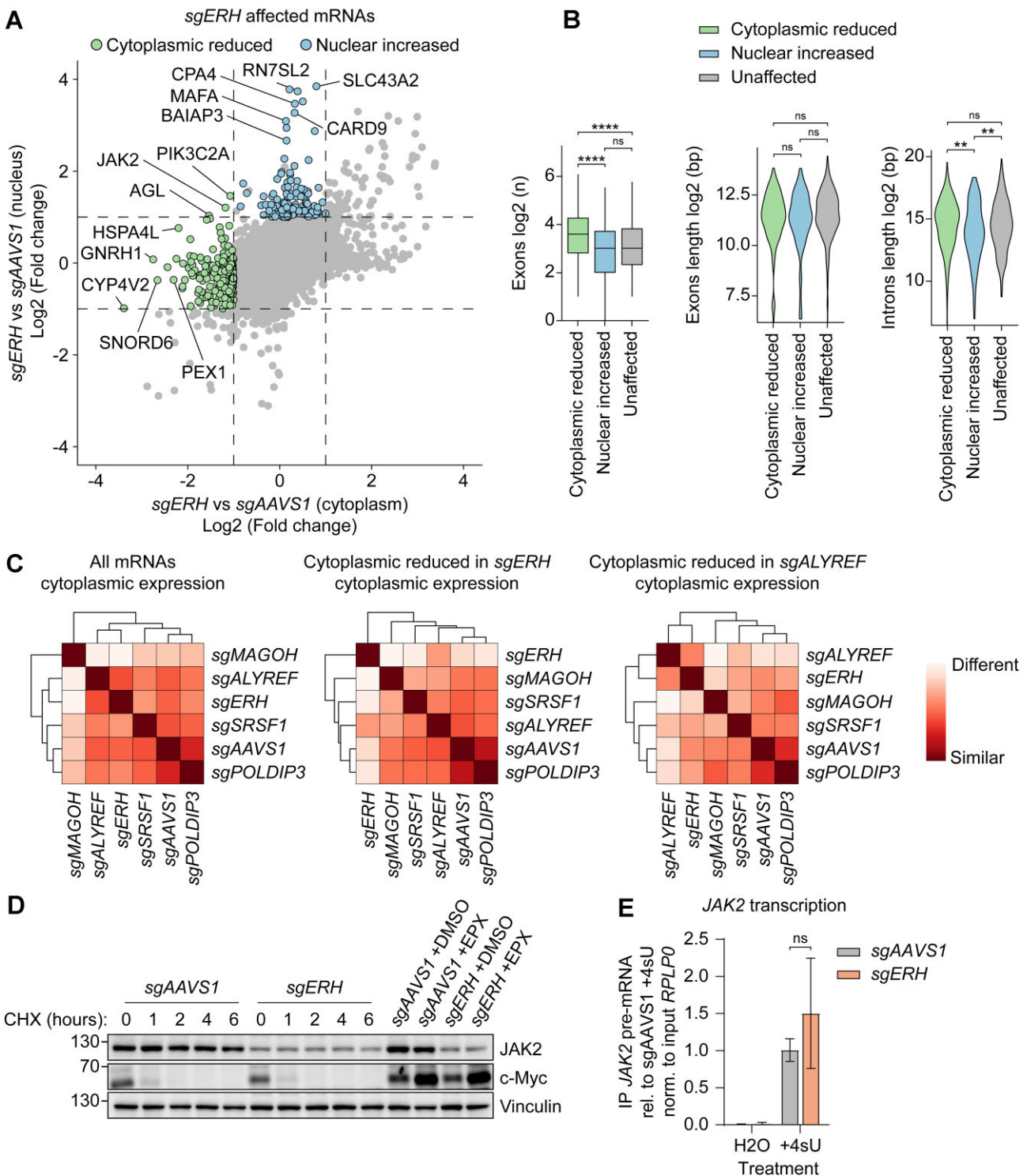


Figure 4. ERH prevents faulty post-transcriptional JAK2 mRNA processing. **(A)** RKO-iCas9 cells expressing Cas9 for 5 days and *sgAAVS1* or *sgERH* were fractionated and their mRNA levels quantified by 3' QuantSeq. Dashed lines indicate absolute log2 fold expression change ≥ 1 . $n = 3$ biological replicates. **(B)** Log2 quantification of number of exons (left), or length of exons (middle) and introns (right) in mRNA subsets from **(A)** shown by box plot or violin plots, respectively. Number of mRNAs (n) for each group from left to right: 244, 121, and randomly sampled 365. Two-sided Wilcoxon Rank Sum test ($*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$; $****P \leq 0.0001$). **(C)** Hierarchical clustered heatmap showing the distance between RKO sample groups with indicated knockouts in subcellularly fractionated 3' QuantSeq based on cytoplasmic variance stabilizing transformed (vst) mean mRNA expression from biological triplicates ($n = 3$). Subsets used for analysis from left to right: all mRNAs ($n = 17\,657$), mRNAs with reduced cytoplasmic expression in *sgERH* as in **(A)**, $n = 248$, and mRNAs with reduced cytoplasmic expression in *sgALYREF*, $n = 117$. **(D)** RKO-iCas9 cells expressing the indicated sgRNAs were treated with cycloheximide (CHX) for the indicated time points, or for 4 h with DMSO or epoxomicin (EPX). Subsequently, protein lysates were analyzed by WB. Data show a representative WB from $n = 3$ biological replicates. **(E)** RKO-iCas9 cells with the indicated genes targeted for 5 days were treated with either water or 4-Thiouridine (4sU) for 15 min, after which newly transcribed 4sU labeled transcripts were isolated by pull-down, and analyzed by RT-qPCR. Data represent means and sd; $n = 3$ biological replicates. Unpaired t-test with Welch correction ($*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$; $****P \leq 0.0001$).

affected mRNAs differed markedly between the two knockouts (Supplementary Fig. S4C and D). Consistent with a general role in mRNA export, *ALYREF* knockout resulted in strong nuclear accumulation of nearly 900 mRNAs. In comparison, less than 150 mRNAs accumulated in the nuclear fractions of *sgERH* cells.

These results indicated that ERH affects JAK2 abundance by affecting its encoding mRNA at a post-transcriptional level, which ultimately reduces its cytosolic pool for translation. Consistent with this notion, rescue of *sgERH*-targeted cells with an sgRNA-resistant ERH cDNA restored cytosolic JAK2 levels (Supplementary Fig. S4E).

However, it remained a possibility that ERH additionally affected JAK2 protein stability. To test this, *sgAAVS1* and *sgERH* cells were chased with the translation inhibitor CHX, or incubated with the proteasome inhibitor EPX. Translation inhibition rapidly chased out the unstable cMYC protein, whereas JAK2 was stable in both *sgAAVS1* and *sgERH* cells through the time course (Fig. 4D, Supplementary Fig. S4F). In line with these findings, the cMYC protein was stabilized by EPX treatment, whereas JAK2 remained unaffected. These findings indicate that JAK2 is a stable protein, and its stability is not affected by ERH ablation.

Subsequently, we tested whether changes in transcription contributed to the diminished JAK2 mRNA levels in *sgERH* cells. To this end, *sgAAVS1* or *sgERH* cells were pulse labeled with 4sU for 15 min, after which labeled *de novo* synthesized RNA was isolated by pull-down, and analyzed for JAK2 mRNA levels by RT-qPCR. Only samples incubated with 4sU yielded a signal, indicating that the detected signal reflects newly transcribed JAK2 mRNA (Fig. 4E). Moreover, there was no significant difference between *sgAAVS1* and *sgERH* samples, indicating that loss of ERH does not affect JAK2 mRNA transcription (Fig. 4E).

In conclusion, our results suggest that ERH directly or indirectly, for example by regulating splicing, facilitates export of a subset of mRNAs.

ERH is critical to prevent intron retention and subsequent export disruption of JAK2 mRNA

Our data so far indicated that ERH is critical for proper accumulation of cytosolic JAK2 mRNA. We thus hypothesized that ERH plays a functional role in either: (i) stabilizing cytosolic JAK2 mRNA by preventing NMD, (ii) exporting JAK2 mRNA to the cytoplasm, or (iii) proper splicing of JAK2 mRNA, as a prerequisite for its subsequent nuclear export.

To determine which of these processes was affected in the absence of ERH, RNA-Seq was performed on fractionated and poly(A)-enriched RNA from *sgERH* RKO-iCas9 cells. Parallel samples treated with the translation inhibitor CHX were included to determine if translation-dependent NMD plays a role in JAK2 mRNA regulation by ERH. Changes in relative cytoplasmic and nuclear mRNA levels correlated well between the poly(A)-enriched RNA-Seq samples and our previous fractionated 3' mRNA QuantSeq data, underpinning the reproducibility and comparability of the two experiments (Supplementary Fig. S5A).

To assess whether ERH loss resulted in mis-splicing and IR, we utilized IRFinder and DESeq2 [66, 67] for differential enrichment analysis of IR in *sgERH* samples compared to the *sgAAVS1*-targeted controls. Consistent with being required for proper JAK2 splicing, ERH ablation caused significant IR

in nuclear JAK2 mRNA (introns 9–14 and intron 16) and in other mRNAs with reduced cytoplasmic expression in the absence of ERH (Fig. 5A and B and Supplementary Fig. S5B), hence referred to as ‘JAK2-like’ mRNAs. Approximately 28% of these mRNAs had at least one increased IR event (in the nucleus or cytoplasm) in comparison to 3% in non-affected mRNAs (Fig. 5C). This was likewise the case for mRNAs with concurrently reduced nuclear expression (Fig. 5A), suggesting that these affected mRNAs were efficiently recognized and degraded by the nuclear RNA regulatory machinery. Moreover, multiple mRNAs with IR following ERH ablation were involved in DNA repair or replication (Supplementary Fig. S5C), confirming that ERH plays a role in splicing of these transcripts. In summary, ERH is required for proper splicing and prevention of IR in a specific subset of mRNAs, including JAK2.

ERH ablation often caused multiple IR events in ‘JAK2-like’ transcripts. These transcripts had a median of three IR events compared to one in unaffected mRNAs (Fig. 5D). Taking these factors into account, cytosolically depleted mRNAs had on average fifteen times more ERH-dependent IR events compared to unaffected mRNAs (Fig. 5E). Moreover, many ‘JAK2-like’ transcripts, such as *PIK3C2A*, retained multiple adjacent introns in *sgERH* samples (Fig. 5B and Supplementary Fig. S5B). We determined that these IR events were more often adjacent than can be predicted by random chance (Supplementary Fig. S5D), similar to what has been previously described for general IR [66]. It has been suggested that connected IR events could depend on their splicing factors and other RBPs [66], in line with ERH being involved in multiple RNA-related processes such as splicing and determining Pol-II elongation rates [107] resulting from opening of chromatin [94].

To determine whether NMD contributed to reduced cytoplasmic mRNA levels in intron-retained mRNAs following ERH ablation, we compared non-treated and CHX treated samples. CHX treatment was confirmed to effectively inhibit NMD by the increase of *GADD45A* (Supplementary Fig. S5E), an mRNA that is constantly turned-over by NMD [108, 109]. In contrast, CHX treatment stabilized only a small fraction of the ERH-regulated cytosolically depleted mRNAs with IR (Supplementary Fig. S5E) and did not significantly increase cytoplasmic IR in these mRNAs. Thus, ERH-regulated mRNAs are not reduced by cytoplasmic control mechanisms such as NMD but undergo nuclear retention as commonly described for inefficiently spliced transcripts [104, 110, 111]. In line with this notion, most IR events (~64%) caused by ERH ablation and their corresponding RNAs (~59%) were only detected in the nucleus (Fig. 5F). Moreover, the degree of IR, as measured by the IR ratio, caused by ERH ablation was significantly higher in nuclear IR events compared to cytoplasmic IR events (median IR ratio 0.01 or 0.08 in cytoplasm, and 0.03 or 0.16 in nucleus, in *sgAAVS1* or *sgERH*, respectively) (Fig. 5G). In conclusion, our results indicate that in the absence of ERH, most ‘JAK2-like’ mRNAs are not exported and consequently retained in the nucleus.

To determine if ERH-interacting factors identified by co-IP MS/MS (Fig. 3A) regulate IR similarly to ERH, we analyzed by RT-qPCR whether their knockouts affected IR in the same nuclear RNA samples analyzed by 3' mRNA QuantSeq (Fig. 4C). We analyzed six different mRNAs with the strongest ERH-regulated IR events (including JAK2) that had altered

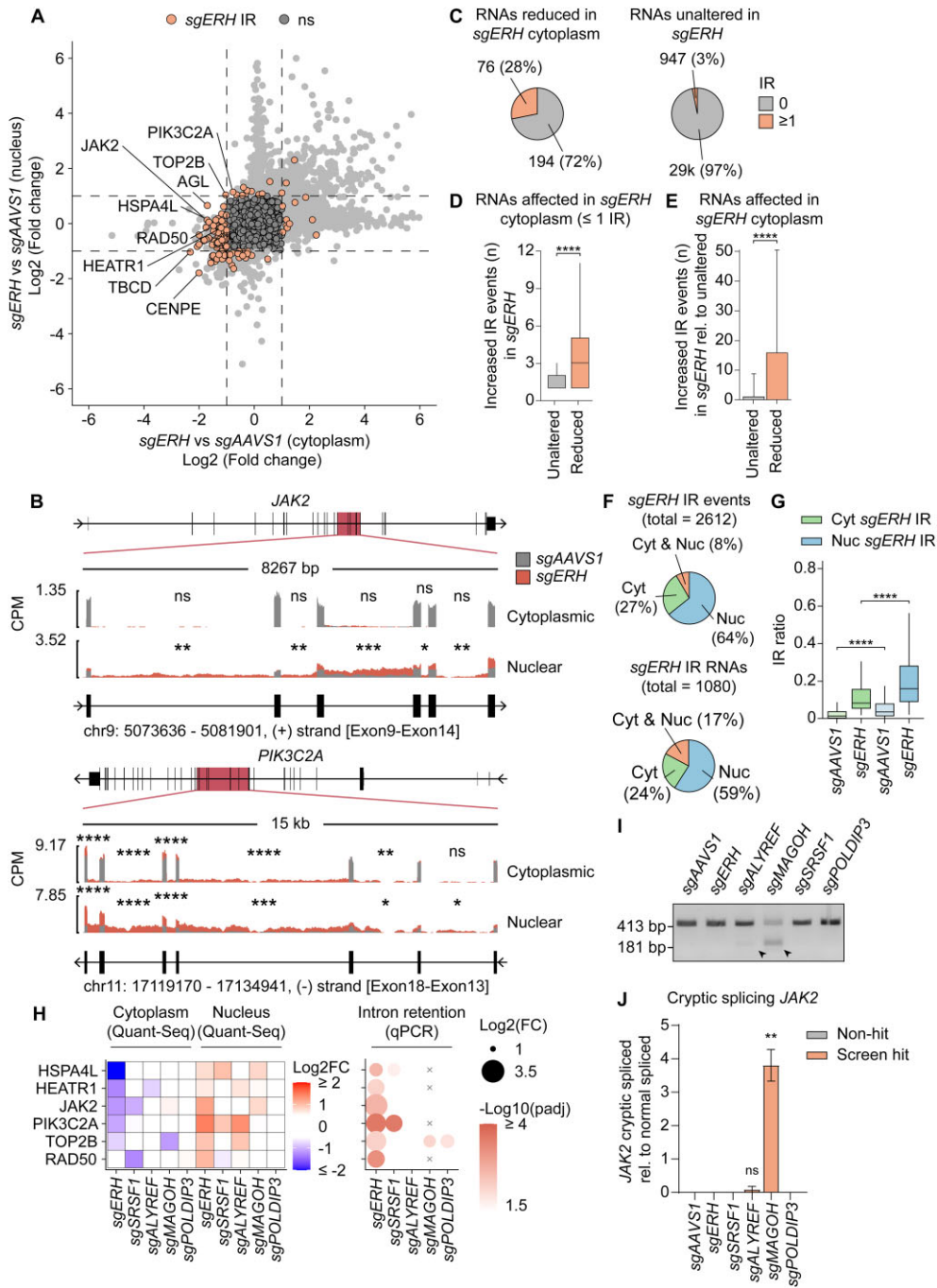


Figure 5. ERH prevents intron retention and disrupted export of *JAK2* and other select mRNAs. RKO-iCas9 cells with the indicated knockouts after 5 days of Cas9 induction were fractionated and subjected to poly(A)-enriched RNA-Seq analysis. **(A)** mRNAs with expression difference and at least one increased intron retention (IR) event ($\text{padj} \leq 0.05$ and $\log_2$ fold change ≥ 1 , nucleus or cytoplasm) in *sgERH* over *sgAAVS1* determined by IRFinder are highlighted. $n = 3$ biological replicates. **(B)** *JAK2* and *PIK3C2A* mRNA regions with IR in *sgERH*; mean reads per million (CPM) in *sgAAVS1* or *sgERH* are plotted. Total (top) and zoomed in (bottom) gene structures are displayed with horizontal lines indicating introns and blocks indicating exons. Significance based on adjusted P value for increased IR in *sgERH* is indicated (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$). **(C)** Pie charts depicting the number of RNAs with at least one increased IR event regulated by ERH, comparing cytosolically reduced and unaltered RNAs. **(D)** Box plots comparing number of increased IR events in *sgERH* between RNA subsets containing at least 1 increased *sgERH*-regulated IR event. Two-sided Wilcoxon Rank Sum test (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$). **(E)** Bar graph comparing the number of increased IR events in *sgERH* between RNA subsets relative to unaltered RNAs. Data represent means and sd. Two-sided Wilcoxon Rank Sum test (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$). **(F)** Pie charts depicting number of retained introns (top) or their corresponding RNAs (bottom) caused by *ERH* ablation (mean IR ratio ≥ 0.05 , $\text{padj} \leq 0.05$ and $\log_2$ fold change ≥ 1 between knockouts) in the cytoplasm, nucleus, or both. **(G)** Boxplots comparing mean IR ratio of retained introns in RKO-iCas9 cells with indicated knockouts. Two-sided Wilcoxon Rank Sum test with Holm-Bonferroni correction (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$). **(H)** Heatmaps of subcellularly fractionated 3' QuantSeq (left), and intron retention measured by RT-qPCR in the same RNA samples (right.) ($n = 3$ biological replicates. x; not measured). **(I)** RKO-iCas9 cells expressing the indicated sgRNAs were treated with dox for 5 days to induce Cas9 expression. Total RNA was isolated and subjected to *JAK2* splicing analysis by end-point RT-PCR and agarose gel electrophoresis, and **(J)** bands were quantified. Cryptically spliced *JAK2* mRNA (181 bp amplicon) and correctly spliced *JAK2* mRNA (413 bp amplicon) are indicated. Data represent means and sd; $n = 3$ biological replicates. Welch's two-tailed t-test (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$).

cytoplasmic or nuclear mRNA levels in at least one other knockout (as measured by 3' mRNA QuantSeq) (Fig. 5H).

Consistent with our poly(A)-enriched RNA-Seq results, *ERH* ablation caused IR in all selected mRNAs, including *JAK2*. In contrast, none of the other knockouts caused IR in *JAK2*, suggesting that these factors likely act in different RNA processing steps of *JAK2* and similarly affected “*JAK2*-like” mRNAs. Notably, only *SRSF1* ablation reduced cytoplasmic *JAK2* like in the *ERH* KO, however, we could not measure similar IR in the *SRSF1* KO. Interestingly, *SRSF1* ablation did show IR in mRNAs with nuclear retention, namely *PIK3C2A* and to a lesser extent *HSPA4L* (Fig. 5H), suggesting that a smaller subset of the ERH-regulated splicing events could be accomplished together with *SRSF1*.

We further compared the knockouts by analyzing the mean sample distance based on mRNA expression levels for mRNAs with or without ERH-regulated IR. In line with these factors likely acting at different RNA processing steps, the similarity between the knockouts did not increase when comparing mRNAs with ERH-regulated IR to those without (Supplementary Fig. S5F).

Previously, we confirmed that loss of ERH or ERH-interacting factors like *MAGO*H and *ALYREF* reduces *JAK2* protein levels (Fig. 3F). Therefore, we hypothesized that these factors regulate different steps of *JAK2* post-transcriptional processing, yet – if this is a rate-limiting step – ultimately affect *JAK2* protein levels in a similar way.

Knockdown of the core EJC factor *EIF4A3* has previously been shown to cause mis-splicing of cryptic splice sites as analyzed by total RNA-Seq [112]. This faulty splicing could result in non-functional and degraded proteins and could potentially explain lower *JAK2* protein levels in *MAGO*H knockout cells. Indeed, erroneous splicing in exon3 of *JAK2* in the absence of EJC components has been previously reported [112]. We further validated that *MAGO*H ablation caused faulty splicing of *JAK2* by RT-qPCR (Fig. 5I), where nearly four times more mis-spliced *JAK2* was present than normally spliced *JAK2* (Fig. 5J). Similar splicing changes were also detected in the *ALYREF* knockout, which had lower (~10%) faulty spliced *JAK2* levels (Fig. 5J). This reduced effect is in line with *ALYREF* loss having a smaller effect on *JAK2* protein and IFN γ -induced IRF1 levels (Fig. 3F).

From these results we conclude that ERH-interacting factors do not affect IR in “*JAK2*-like” transcripts as ERH does, but are rather critical for different post-transcriptional steps of *JAK2* mRNA processing, and in this way affect *JAK2* protein and IFN γ signaling.

ERH-regulated retained introns are AU-rich

To determine if ERH-regulated IR events had unique characteristics, we analyzed four characteristics known to influence IR [107, 113]: (i) lengths of the introns and flanking exons, (ii) 3' and 5' splice site strengths, (iii) intronic and exonic GC content, and (iv) motif-abundances for RBPs. We compared retained introns caused by *ERH* ablation with a similar number of randomly sampled introns that were similarly retained in both *sgAAVS1* and *sgERH* under homeostatic conditions, hence referred to as general IR, and with non-retained introns.

We determined that ERH-regulated introns were shorter (Fig. 6A) and had weaker 3' splice site strength (Fig. 6B) than non-retained introns, similar to general IR and as previously reported for regularly retained introns [107, 113]. Interest-

ingly, the 5' splice site was not weaker in ERH-regulated introns whereas the 3' splice site was even weaker than generally retained introns (Fig. 6B), suggesting that the 3' splice site predisposes introns to ERH-mediated regulation. Furthermore, ERH-regulated introns had longer flanking exons than other introns, while total gene length was comparable to non-retained ones (Supplementary Fig. S6A). From these data we concluded that ERH-regulated introns have multiple features that have been previously reported to predispose introns for retention, such as weaker 3' splice sites and shorter intron length.

Previously, several types of retained introns that exhibit different features have been proposed [107]. However, all retained intron types have been reported to contain increased GC content in comparison to their non-retained counterparts [107, 113]. Strikingly, ERH-regulated introns (Fig. 6C) and their flanking exons (Supplementary Fig. S6B) had significantly reduced, rather than increased, GC content compared to non-retained introns, which was to a lesser extent also identified for their corresponding genes (Supplementary Fig. S6B). In line with lower GC content, ERH-dependent introns, and to an even greater degree their flanking exons, were enriched for AU-rich motifs compared to non-retained ones (Fig. 6D). The AU-rich element-binding proteins (AUBPs) linked to these motifs included multiple hnRNPs, KHDRBSs, and other RNA processing factors primarily involved in splicing, but also RNA export, supporting a regulatory role of ERH in splicing of AU-rich transcripts. This is markedly different from previous reports on regularly retained GC-rich introns that have an enrichment of SR-protein binding sites [66].

In summary, our study identified ERH as a functionally critical factor for correct splicing of a select set of mRNAs, including *JAK2*. Loss of ERH-associated factors implemented in post-transcriptional processing, such as *SRSF1*, *MAGO*H, and *ALYREF*, likewise disrupted *JAK2* mRNA maturation. Our data indicate that these factors affect *JAK2* at different post-transcriptional steps, yet ultimately lead to the same outcome of reduced *JAK2* protein levels. These findings indicate that ERH only functionally overlaps with the global splicing and export machineries for a smaller set of transcripts, which critically rely on ERH to prevent IR. Specifically, our results indicate that ERH is required for efficient splicing of introns in AU-rich regions, which differs from previously described regular GC-rich IR. Together, these findings position ERH and post-transcriptional processing as critical regulatory factors for mounting effective innate immune responses by type II IFN signaling.

Discussion

In recent years, there have been multiple examples for genome-wide CRISPR screens that use inducible Cas9 expression to successfully capture regulatory factors regardless of their impact on cellular viability [28, 29, 79]. We applied this approach to identify novel positive regulatory factors of IFN γ signaling, aiming to complement previous gene-trap screens that could not identify essential factors [27]. In this study, we successfully identified multiple cell-essential post-transcriptional RNA regulatory factors, namely ERH and other EJC-related splicing and export factors, as novel and specific regulators of IFN γ -induced ISG expression. Overall, we determined that these factors were critical for expression of the signaling kinase *JAK2*, and in this way regulated overall

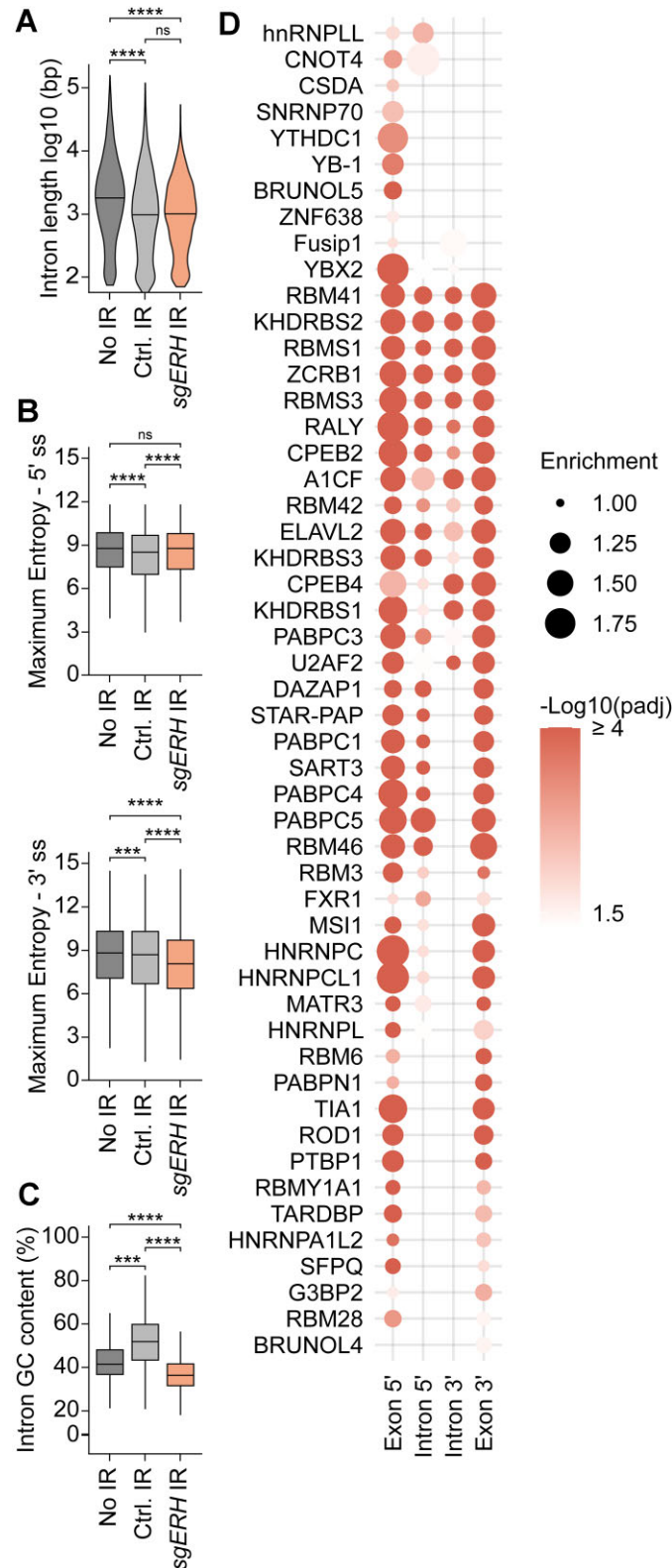


Figure 6. ERH-regulated retained introns and flanking exons are AU-rich. RKO-iCas9 cells with the indicated knockouts were fractionated and subjected to poly(A)-enriched RNA-Seq analysis. **(A)** Violin plots comparing length distributions between non-retained introns ("No IR"; mean IR ratio ≤ 0.05 in both *sgAAVS1* and *sgERH*), retained introns in both knockouts ("Ctrl. IR"; mean IR ratio ≥ 0.1 and absolute log2 fold change ≤ 0.2 between knockouts), or retained introns caused by *ERH* ablation ("*sgERH* IR"; mean IR ratio ≥ 0.05 , padj ≤ 0.05 and log2 fold change ≥ 1 between knockouts). $n = 2178$ in each group (randomly sampled for control groups). **(B)** Box plots comparing average 5' and 3' splice site strength measured by Maximum Entropy Score [70]. **(C)** Box plots comparing GC content. **(A-C)** Two-sided Wilcoxon Rank Sum test with Holm-Bonferroni correction (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$). **(D)** Heatmap of enriched (padj ≤ 0.05) RNA binding protein (RBP) motifs located near ERH-regulated retained introns compared to non-retained introns measured by simple enrichment analysis (SEA). 5' and 3' boundaries of flanking exons (100 bp) and introns (200 bp), excluding splice sites, were analyzed. For each RBP, the motif with the lowest padj is shown.

IFN γ -induced JAK/STAT signaling and subsequent ISG transcription.

To determine the mechanism of *JAK2* regulation we found that ERH was critical to prevent IR in *JAK2* mRNA and a subset of other similarly ERH-dependent transcripts. A significant fraction of these cytosolically reduced mRNAs could be explained by an increase in IR in *ERH* KO cells. Interestingly, the interactors of ERH investigated in this study were important for different steps of *JAK2* processing. MAGOH, and to a lesser extent ALYREF, regulated separate cryptic splicing events. Similarly, SRSF1 regulated seemingly other steps in *JAK2* mRNA processing, potentially through different IR events [66] or by acting as an export adaptor [114]. Overall, this highlights post-transcriptional *JAK2* processing as a critically susceptible process.

This raises the question as to what makes *JAK2* and similar mRNAs disproportionately sensitive to disruption of core RNA processing, such as splicing and export. It is clear from this study and previous ones that ablation of general acting factors, like core EJC factors or the TREX export adaptor ALYREF, disproportionately affects mRNA abundance of a subset of transcripts [112, 115, 116]. One potential explanation for this could be the lack of compensatory and redundant mechanisms for processing of these mRNAs. Moreover, splicing and export could be relatively more important and potentially rate-limiting compared to other RNA processes such as transcription for these mRNAs. Overall, the complex interplay between different RNA processes and how this affects specific mRNAs presents an exciting avenue for future research. In particular, exploration of the molecular mechanisms by which individual RNA processing factors affect subsets of transcripts will be of high interest.

ERH acts by bridging proteins and aids in protein complex formation [80]. Thus, understanding the functional interactions with its binding partners, and the functional importance of these interactions for generating specific biological output, will be key for understanding the molecular mechanisms by which ERH regulates various cellular processes. We and other groups have shown that ERH interacts with multiple EJC-associated factors. Indeed, cross-linking mass spectrometry revealed close-range interactions between ERH and the export adaptors ALYREF and CHTOP [85] and the splicing/export factors BCLAF1 and THRAP3 [86].

We found that loss of *ERH* or *ALYREF* mimicked each other most closely in terms of cytoplasmic mRNA abundance, and *SRSF1* loss resulted in similar IR as ERH for some mRNAs. However, the overall effect of *ERH* loss, or loss of its interactors was not greater for intron-retained mRNAs, suggesting that ERH might affect these mRNAs through multiple means with its interactors. Indeed, our results indicate that intron-retained mRNAs were not extensively degraded in the cytoplasm, but rather poorly exported. The functional similarity between ERH and the export adaptor ALYREF may indicate that ERH has a role in both splicing and export of these mRNAs as a scaffolding/compaction factor in their RNP complexes. Further studies are required to explore the mechanism of ERH-dependent effects on mRNA export, for example by investigating non-spliced histone RNAs that depend on ALYREF [117], and in this way separate splicing from export-dependent outcomes. Despite a substantial decrease in mature target transcripts in the cytoplasm in the absence of *ERH*, their nuclear counterparts did not substantially increase in

most experiments, indicating that the retained nuclear target transcripts were likely degraded in the nucleus.

We found that in the absence of *ERH*, multiple adjacent introns tended to be retained. This behavior has been previously reported [66], yet its underlying reason has remained unclear. Moreover, introns retained in the absence of *ERH* were substantially more AU-rich than classically retained introns, a feature reported for mRNAs spliced at the nuclear periphery [118]. In line with this, we speculate that ERH could play a more dominant functional role in the nuclear periphery, and its absence could disproportionately affect transcripts spliced in that part of cellular three-dimensional space.

It should be noted that *ERH* ablation could potentially cause IR through secondary effects. An important factor shown to facilitate IR is increased chromatin accessibility, as this accelerates Pol II elongation rates resulting in alternative-splicing [113]. Previous work showed that ERH can be important to maintain repressive chromatin modifications [94], and we cannot exclude that increased chromatin accessibility by *ERH* ablation could have influenced IR. Our identified ERH-regulated IR events differed from conventional retained introns in having increased proximal AU-rich motifs with many corresponding AUBPs being involved in splicing and export, indicating that chromatin accessibility is unlikely to be a critical aspect for ERH-specificity. The fact that these factors were not identified in our co-IP-MS/MS could suggest that they are only transiently associated to the RNP complex during splicing. Some of the factors, such as HNRNPL, also scored in our genetic screen, but most did not, indicating they might act redundantly or that only some of them are functionally involved in these splicing events. Taken together, our results are in favor of a model in which ERH prevents AU-rich IR in a splicing dependent manner, thereby ensuring faithful export of mRNAs, including *JAK2*.

ERH is highly evolutionary conserved in eukaryotes, ranging from yeast to humans [80]. In contrast, the *JAK2* gene arose much later in evolution in jawed-vertebrates [119]. Moreover, ERH is constitutively expressed at similar high levels across all human tissue types [99], and is not IFN γ -induced (Fig. 2B). This makes it unlikely that ERH has adapted to directly control innate immunity. Instead, it suggests that intrinsic *JAK2* mRNA features make it critically dependent on ERH-dependent processing. Nevertheless, an increasing number of studies underpins the importance of post-transcriptional processing for generating immune output [20, 21, 24, 120]. Therefore, it could be that some immune transcripts have evolved to be processed by core post-transcriptional machineries to ensure proper immune output.

In this context, some retained intronic transcripts (referred to as detained intronic transcripts) can serve as reservoirs that can be activated and spliced in response to dynamic stimuli such as cellular stress, DNA damage, and viral infection [104, 121, 122]. Such a dynamic role makes sense for ERH as the IR-regulated mRNAs are involved in innate immune signaling (*JAK2*) and the DNA damage response. However, mRNAs with conventional GC-rich detained introns are seemingly resistant to exosomal decay and accumulate in nuclear speckles [104, 123, 124] – nuclear domains that play a role in retention and quality control of detained mRNAs [125]. In contrast, ERH-regulated mRNAs with AU-rich IR did not generally accumulate in the nucleus, indicating that they may be cleared by exosomal decay, as has been reported for inefficiently spliced

transcripts [110, 111]. Determining whether ERH always aids in proper splicing and export of these mRNAs or if it dynamically influences IR in response to different stimuli will be an exciting avenue for future research.

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Supplementary data

Supplementary data is available at NAR online.

Conflict of interest

S.L.A. is a co-founder, shareholder, scientific advisor, and member of the board of QUANTRO Therapeutics.

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Data availability

The RNA-seq data in this publication have been deposited in NCBI’s Gene Expression Omnibus [126] and are accessible through GEO Series accession numbers: GSE274069, GSE274070, GSE274071, and GSE274074. Code used for downstream RNAseq analysis and plotting in this study is available in Zenodo (<https://doi.org/10.5281/zenodo.15229803>). Proteomics data is deposited at PRIDE (PXD055858). All other data generated or analyzed during this study are included in the manuscript and supporting files.

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5.2 Results 2

Publication “SPOP targets the immune transcription factor IRF1 for proteasomal degradation”

5.2.1 Preamble

A couple of E3s that target IRF1 in specific cell contexts have been described previously, but how IRF1 protein abundance is properly regulated – which is critical for preventing both autoimmunity and tumourigenesis – overall remains an important and largely unanswered question. Through a genetic screen we identified the E3 ligase receptor speckle type BTB/POZ protein (SPOP) as a novel and direct mediator of IRF1 protein degradation. Furthermore, we identify four serine/threonine (S/T) rich degrons in IRF1 that are important for IRF1-SPOP binding through the protein-protein interacting MATH domain in SPOP. Finally, we show that SPOP-dependent IRF1 degradation regulates IRF1-mediated transcription of inflammatory response genes stimulated by IFN γ . Overall, this study highlights the importance of balanced regulation of key RNA processing factors like IRF1 for proper innate immune responses.

5.2.2 Authors and status

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5.2.3 Contributing statement

My contributions to this study were formal analysis and investigation.

5.2.4 Publication

SPOP targets the immune transcription factor IRF1 for proteasomal degradation

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Abstract Adaptation of the functional proteome is essential to counter pathogens during infection, yet precisely timed degradation of these response proteins after pathogen clearance is likewise key to preventing autoimmunity. Interferon regulatory factor 1 (IRF1) plays an essential role as a transcription factor in driving the expression of immune response genes during infection. The striking difference in functional output with other IRFs is that IRF1 also drives the expression of various cell cycle inhibiting factors, making it an important tumor suppressor. Thus, it is critical to regulate the abundance of IRF1 to achieve a 'Goldilocks' zone in which there is sufficient IRF1 to prevent tumorigenesis, yet not too much which could drive excessive immune activation. Using genetic screening, we identified the E3 ligase receptor speckle type BTB/POZ protein (SPOP) to mediate IRF1 proteasomal turnover in human and mouse cells. We identified S/T-rich degrons in IRF1 required for its SPOP MATH domain-dependent turnover. In the absence of SPOP, elevated IRF1 protein levels functionally increased IRF1-dependent cellular responses, underpinning the biological significance of SPOP in curtailing IRF1 protein abundance.

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Editor's evaluation

IRF1 is a key transcription factor with important roles during pathogenic infection and in some cancers. The regulation of IRF1 protein abundance is critical, but the underlying mechanisms are not known. Using a CRISPR-based knockout screen, the authors identify SPOP as an E3 ligase that binds IRF1 and enforces its degradation via the proteasome. The study represents an important advance backed by solid evidence that may have implications in immunology, virology, and cancer fields.

Introduction

Adaptation of the functional proteome is essential to counter pathogens during infection, yet precisely timed degradation of these response proteins after pathogen clearance is likewise key to preventing autoimmunity and hyperinflammation (**Crow and Stetson, 2022**).

Recognition of pathogen-associated molecular patterns and subsequent secretion of cytokines known as interferons (IFNs) are particularly critical for conferring an anti-pathogen response (**Takeuchi and Akira, 2010**). Type I interferons (IFN α/β) can be secreted by most cells upon infection, whereas

type II IFNs (IFN γ) are secreted by specific cell types such as natural killer cells and T helper 1 cells. Binding of these cytokines to their receptors initiates cell signaling driving transcription of hundreds of response genes, which collectively mediate the anti-pathogen response (**Schoggins, 2019**).

Interferon regulatory factors (IRFs) play essential roles as transcription factors in the IFN system. Some IRFs, such as IRF3, are constitutively expressed. Their activity is predominantly regulated through phosphorylation, and they are essential for induction of type I IFNs (**Tamura et al., 2008**). In contrast, some other IRFs, including IRF1, are transcriptionally induced during infection (**Feng et al., 2021**).

IRF1 is a key family member for the induction of the innate immune response (**Forero et al., 2019; Kimura et al., 1994; Langlais et al., 2016**). The importance to precisely control IRF1 levels and its functional output is underpinned by the fact that aberrant IRF1 expression is linked to inflammatory syndromes, such as Behcet's disease, arthritis, and systemic lupus erythematosus (**Donn et al., 2001; Govind et al., 2014; Lee et al., 2007; Uddin et al., 2011; Zhang et al., 2015**). The striking difference in functional output with other IRFs is that IRF1 also drives the expression of various cell cycle inhibiting factors (**Armstrong et al., 2012; Tanaka et al., 1996**), making it an important tumor suppressor (**Nozawa et al., 1999; Xie et al., 2003**). Thus, it is critical to regulate the abundance of IRF1 to achieve 'Goldilocks' levels at which there is sufficient IRF1 to prevent tumorigenesis, yet not too much which could cause excessive immune activation.

In addition to its transcriptional regulation, the other main means of IRF1 regulation is at a protein level through rapid degradation by the proteasome (**Nakagawa and Yokosawa, 2000; Pion et al., 2009**), which seems to in turn be critical to limit transcriptional output and reduce the IRF1 protein pool once IRF1 transcription is shut off during immune resolution. In line with this notion, proteasome inhibitors have been shown to block IRF1 degradation (**Attard et al., 2005**). Several factors have thus far been reported to mediate poly-ubiquitination of IRF1, and direct its proteasomal degradation.

The importance of these individual regulators for IRF1 turnover is to a degree determined by their cellular context (**Liu et al., 2021; Remoli et al., 2020**). This draws an interesting parallel to other transcription factors which can be ubiquitinated by various different E3 ligase complexes, depending on cellular context. cMYC is a prime example of such a cell context-regulated transcription factor. A Cullin E3 ligase complex using the FBXW7 substrate receptor module (SCF^{FBXW7}) has been reported as a key factor in cMYC degradative ubiquitination in many cell types (**Welcker et al., 2004; Yada et al., 2004**). In contrast, various other E3s, including TRIM32, CHIP, and several other Cullin F-box complexes, have been shown to be important for cMYC degradation, yet in more cell-specific contexts (**Sun et al., 2021**). Identification and characterization of such E3 ligases is critical for ultimately understanding how their action controls transcriptional output in various cell types, within the context of whole organisms.

As for other transcription factors, turnover mechanisms can be different for distinct cellular IRF1 protein pools, dependent on their localization and functional engagement in transcription. As such, understanding which functional IRF1 domains determine its turnover is important. IRF1 has an N-terminal DNA binding domain, followed by a nuclear localization signal (NLS), IRF1 transactivation IRF-association domain (IAD2), and a C-terminal intrinsically disordered enhancer region (IDR). This C-terminal IDR is an important determinant of IRF1 turnover (**Nakagawa and Yokosawa, 2000**). On the one hand, the IDR is stabilized by the chaperones HSP70 and HSP90 (**Landré et al., 2013; Nakagawa and Yokosawa, 2000; Narayan et al., 2009; Narayan et al., 2015; Narayan et al., 2011a; Narayan et al., 2011b**), whereas it is targeted for degradative ubiquitination by the protein quality control E3 ligase CHIP (**Narayan et al., 2015; Narayan et al., 2011b**). This has suggested that cells may, at least in part, turn over IRF1 co- or post-translationally using their general protein quality control pathways.

In addition, the N-terminal DNA binding domain has been reported to determine IRF1 stability (**Landré et al., 2013**). Consistent with a model in which IRF1 is ubiquitinated by the E3 ligase MDM2 in an unbound state, DNA binding competes with E3 ligase interaction, thereby stabilizing IRF1 in a functionally engaged state (**Landré et al., 2013**).

Lastly, the IAD2 domain has been implicated in driving IRF1 ubiquitination and degradation (**Garvin et al., 2019; Pion et al., 2009**). In part, this has been attributed to generation of a GSK3 β -dependent phospho-degron on DNA-bound IRF1, and subsequent recognition by the SCF^{FBXW7} E3 ligase complex, which drives its degradation.

In this study, we set out to identify novel factors that control IRF1 abundance in an unbiased manner. Genome-wide genetic screens were performed to identify novel IRF1-specific E3 ligases.

This identified the Cullin ubiquitin E3 ligase receptor SPOP to functionally mediate IRF1 degradation. Follow-up analyses identified four degrons in, or near the IRF1 IAD2 enhancer domain responsible for SPOP binding, and subsequent IRF1 ubiquitination and degradation. Consequently, loss of *SPOP* increased transcriptional output of IRF1-stimulated genes.

Results

Establishment of an IRF1 protein abundance reporter cell line for genetic screening

The IRF1 protein is ubiquitinated and turned over by the proteasome. However, the cellular E3 ligases contributing to targeting IRF1 for degradation have remained poorly understood. The overarching goal of this study was to identify and characterize novel regulators of IRF1 protein abundance. For the identification of IRF1 stability regulators, we characterized IRF1 degradation properties in the human RKO colon carcinoma cell line, as it is highly amenable for genetic screening.

IRF1 is transcriptionally induced upon type I or type II IFN stimulation in a wide variety of cells. Likewise, stimulation of RKO cells with IFN γ strongly induced endogenous IRF1 protein levels (**Figure 1A**; 11.2-fold). Subsequent treatment with the translation inhibitor cycloheximide (CHX) rapidly chased out the induced IRF1 protein (**Figure 1A**). In the absence of de novo protein synthesis, IRF1 levels returned to pre-stimulation protein levels within 4 hr, indicating that it is highly unstable. Simultaneous inhibition of lysosomal degradation by Bafilomycin A (Baf) did not affect endogenous IRF1 stability and half-life (**Figure 1—figure supplement 1A–B**), indicating that IRF1 is predominantly turned over by the proteasome. In line with this finding, endogenous IRF1 protein levels measured by intracellular staining were also induced by IFN γ and depleted upon translation inhibition (**Figure 1B**; left panel), whereas inhibition of proteasomal degradation by MG132 increased IRF1 levels (**Figure 1B**; right panel). Together, these results indicate that IRF1 is induced by IFN γ and degraded by the proteasome in RKO cells, as has been described for other cell types, making it a suitable model for identification of IRF1 stability regulators.

Endogenous IRF1 protein levels are influenced by transcriptional, translational, and post-translational effects (**Feng et al., 2017**). Therefore, a strategy was devised to uncouple IRF1 protein abundance from its endogenous transcriptional program, thereby aiming to maximize the identification of regulators of protein degradation. To this end, a monoclonal cell line harboring Dox-inducible Cas9 was established, and a dual-color reporter construct under the control of a constitutively active promoter (**Figure 1C**), consisting of an mCherry-IRF1 cassette separated from BFP by a P2A ribosomal skip site. Although both proteins are translated from a single transcript in equimolar quantities, the unstable mCherry-IRF1 was predicted to accumulate at lower steady-state levels than the stable BFP internal control.

We reasoned that it would be important to screen in parallel with an unrelated protein that is degraded in a proteasome-dependent manner, in order to distinguish between general players in the ubiquitin proteasome system and factors specifically regulating IRF1 abundance. cMYC is constantly turned over by the proteasome with a 20–30 min half-life (**Gregory and Hann, 2000; Ramsay et al., 1986**). Therefore, a comparable cell line expressing mCherry-cMYC was established and characterized alongside the mCherry-IRF1 cell line.

To investigate whether the mCherry-tagged IRF1 reporter had comparable degradation characteristics as its endogenous counterpart, cells were subjected to a CHX chase to determine protein stability. In line with endogenous IRF1, mCherry-IRF1 levels rapidly diminished within 4 hr (**Figure 1D**). IRF1 degradation was prevented by simultaneous inhibition of the proteasome (**Figure 1D**), whereas inhibition of lysosomal degradation by Baf did neither stabilize endogenous IRF1 nor mCherry-IRF1 (**Figure 1E**). Similarly, mCherry-cMYC and endogenous cMYC were rapidly lost upon CHX treatment, which was prevented by simultaneous proteasome inhibition (**Figure 1—figure supplement 1C–D**). This indicated that mCherry-IRF1, mCherry-cMYC, and their endogenous equivalents are all predominantly degraded by the proteasome.

Next, we set out to determine whether our fluorescent reporters were compatible with fluorescence-activated cell sorting (FACS)-based genetic screening. In particular, we assessed by flow cytometry whether inhibition of protein synthesis or proteasomal degradation would specifically affect the mCherry-tagged unstable proteins, without affecting their stable BFP internal controls. Consistent

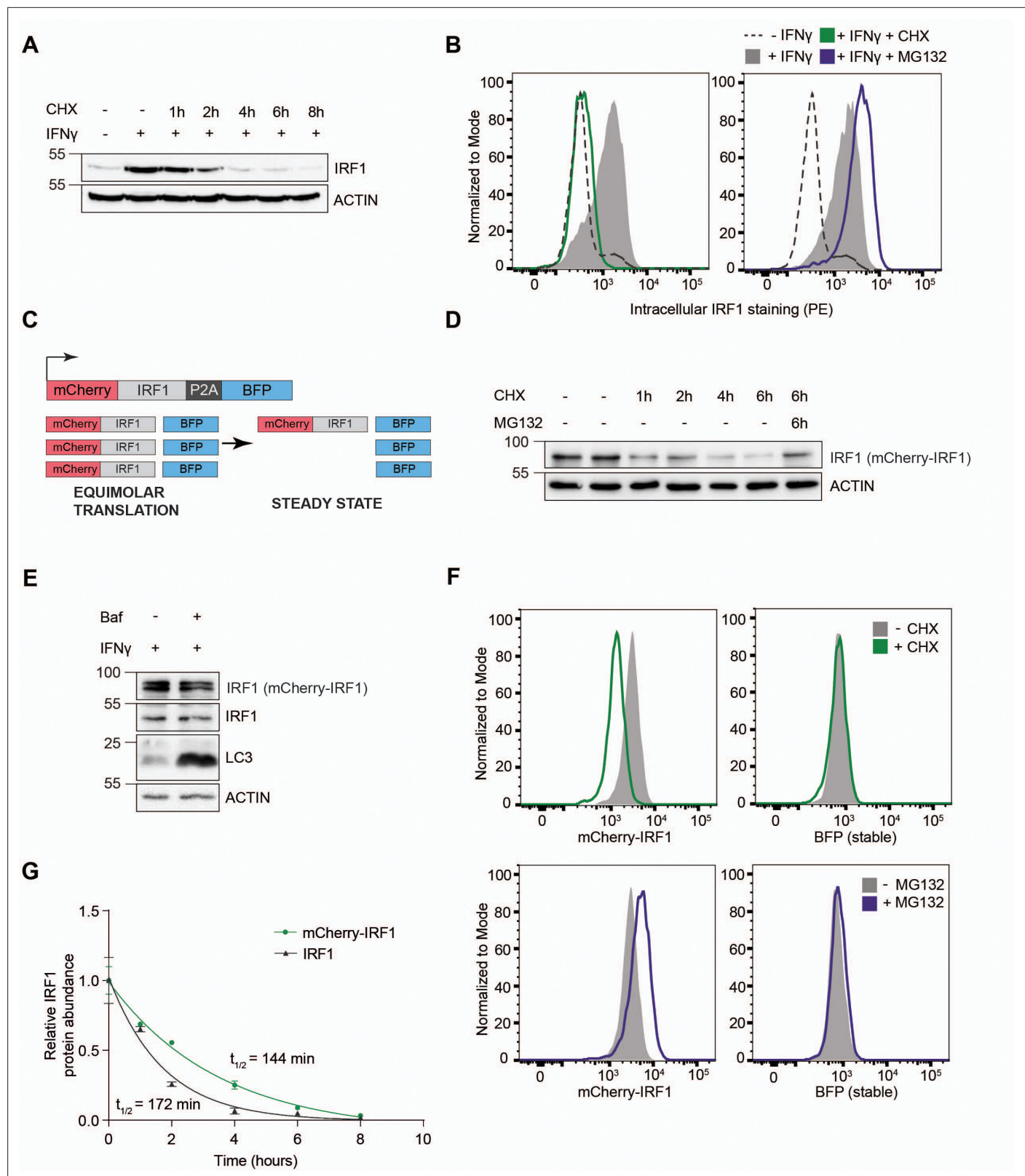


Figure 1. Establishment of an interferon regulatory factor 1 (IRF1) protein abundance reporter cell line for genetic screening. RKO cells were stimulated with IFN γ for 4 hr, and subsequently incubated with the translation inhibitor cycloheximide (CHX) in the absence or presence of proteasome inhibitor MG132, after which endogenous IRF1 levels were analyzed by (A) western blot or (B) intracellular staining and flow cytometry. (C) A dual-color reporter construct, from which unstable mCherry-IRF1 and stable BFP are synthesized in equimolar amounts, yet mCherry-IRF1 accumulates at low steady-state

Figure 1 continued on next page

Figure 1 continued

protein concentrations resulting from its proteasomal degradation. (D) RKO-mCherry-IRF1-P2A-BFP cells were incubated with CHX for the indicated times in the absence or presence of proteasome inhibitor MG132, after which mCherry-IRF1 levels were analyzed by western blot through detection of IRF1 protein. (E) RKO-mCherry-IRF1-P2A-BFP cells were stimulated with IFN γ for 4 hr, and subsequently incubated with lysosomal degradation inhibitor Bafilomycin A (Baf) for 4 hr, after which IRF1 levels were determined by western blot. (F) RKO-mCherry-IRF1-P2A-BFP cells were incubated with CHX or MG132 for 4 hr, after which mCherry-IRF1 and BFP protein levels were analyzed by flow cytometry. (G) RKO-mCherry-IRF1-P2A-BFP cells were incubated with CHX for the indicated times. Non-saturated western blot signals for mCherry-IRF1 and endogenous IRF1 protein were quantified, and normalized to ACTIN levels. Data represent means and s.d. (n=2 biological replicates). Half-life calculations were performed with GraphPad Prism (v9) by fitting the data to a one-phase decay nonlinear regression curve.

The online version of this article includes the following source data and figure supplement(s) for figure 1:

Source data 1. Western blots corresponding to **Figure 1A**.

Source data 2. Western blots corresponding to **Figure 1D**.

Source data 3. Western blots corresponding to **Figure 1E**.

Figure supplement 1. Establishment of an interferon regulatory factor 1 (IRF1) protein abundance reporter cell line for genetic screening.

Figure supplement 1—source data 1. Western blots corresponding to **Figure 1—figure supplement 1A**.

Figure supplement 1—source data 2. Western blots corresponding to **Figure 1—figure supplement 1C**.

with our findings by western blot readout, flow cytometry analysis also showed that both mCherry-IRF1 and mCherry-cMYC were degraded in the absence of de novo protein synthesis (**Figure 1F**, **Figure 1—figure supplement 1E**; top panels), and stabilized by proteasome inhibition (**Figure 1F**, **Figure 1—figure supplement 1E**; bottom panels), without affecting levels of the stable BFP internal control.

To determine IRF1 and cMYC half-lives, we quantified mCherry-IRF1 and IFN γ -induced endogenous IRF1 levels over time following inhibition of protein synthesis. Single exponential decay curve fitting indicated the half-life of endogenous IRF1 to be 72 min, whereas the half-life of mCherry-IRF1 was twice as long at 144 min (**Figure 1G**). We reasoned that the increased mCherry-IRF1 half-life would likely still allow identification of factors mediating degrading of endogenous IRF1, but could not rule out that some aspects of endogenous IRF1 degradation were differentially represented by mCherry-IRF1. Importantly, IFN γ treatment did not alter constitutively expressed mCherry-IRF1 levels (**Figure 1—figure supplement 1F**), indicating that its degradation machinery is likely already active under non-stimulated conditions. Similar measurements for mCherry-cMYC indicated a 24 min half-life for mCherry-cMYC and 19 min for endogenous cMYC in the same cells (**Figure 1—figure supplement 1C–D**), consistent with previously published work (Welcker et al., 2004; Yada et al., 2004). These combined data indicate that the N-terminal mCherry fusions of IRF1 and cMYC phenocopied the proteasomal degradation and instability of their endogenous counterparts.

Lastly, we assessed whether our selected monoclonal mCherry-IRF1 and mCherry-cMYC reporter cell lines had retained the ability to drive genome editing. To this end, cells were transduced with a lentiviral vector constitutively expressing an sgRNA targeting the cell-essential factor ribonucleotide reductase catalytic M1 (*RRM1*), while co-expressing iRFP. Subsequently, the ratio between these transduced cells and non-transduced cells in the same cell pool were measured over time by flow cytometry as a proxy for cell fitness.

As anticipated, the presence of Dox in the culture medium to induce Cas9 resulted in a near-complete loss of iRFP⁺ sgRRM1 cells from the cell pool in 6 days in both cell lines (**Figure 1—figure supplement 1G–H**), whereas parallel sgAAVS1 control samples remained at a constant ratio. In contrast, in the absence of Dox, sgRRM1 cells were not depleted over the 2-week course of the experiment. This indicated that the mCherry-IRF1 and mCherry-cMYC cell lines had minimal editing in the absence of Dox, yet efficient genome editing upon Dox-induced Cas9 expression.

Taken together, our data demonstrate that mCherry-IRF1 and mCherry-cMYC phenocopy the unstable nature of their endogenous counterparts and are likewise predominantly degraded by the proteasome. We concluded that the established reporter cell lines were suitable for subsequent genetic screening to identify IRF1 and cMYC stability regulators and that the established cell lines could be used for parallel screens to determine specificity of identified factors.

Identification of the Cullin substrate receptor SPOP as a regulator of IRF1 abundance

The characterized mCherry-IRF1 and mCherry-cMYC reporter cell lines were subsequently used for genome-wide genetic screening for regulators of their protein abundance. To this end, a genome-wide lentiviral sgRNA library was integrated in the cell lines at a low multiplicity of infection (MOI) to ensure the targeting of one gene per cell (**Figure 2A**, **Figure 2—figure supplement 2A**), followed by selection for G418 resistance to enrich for sgRNA-expressing cells (**Figure 2—figure supplement 2B–C**). At 3 and 6 days after Cas9 induction with Dox, the top 1–3% of cells with the highest and lowest mCherry levels were collected by flow cytometry (**Figure 2A**, **Figure 2—figure supplement 3**, **Figure 2—figure supplement 4**), to enrich for cells with decreased or increased levels of the unstable mCherry-IRF1 or mCherry-cMYC. In parallel, the top 1–3% of cells with the highest and lowest levels of the stable BFP control were collected to identify non-specific effects (**Figure 2A**, **Figure 2—figure supplement 3**, **Figure 2—figure supplement 4**).

The two harvesting time points were previously empirically determined to be optimal for capturing essential genes (day 3), and genes that encode proteins whose levels require longer times to deplete (day 6) (**Figure 1—figure supplement 1G–H**; *de Almeida et al., 2021*). Integrated sgRNA coding sequences from the selected cell pools were quantified by next-generation sequencing (NGS) and compared to relative frequencies determined in unsorted cells representing the initial library (**Figure 2—figure supplement 5A**). The robustness and uniformity of the screens in both cell lines is underpinned by the fact that all sgRNA coding sequences in unsorted samples were covered by 100–1000 reads (**Figure 2—figure supplement 5B–C**).

As anticipated, the mCherry-cMYC screen strongly identified its well-known degrader FBXW7 in mCherry-cMYC^{high} cells (**Figure 2B**, **Figure 2—figure supplement 1A–D**). Moreover, it identified other UPS components reported to mediate cMYC degradation (**Figure 2—figure supplement 1A–D**), such as proteasome components (PSMC5, PSMD4, PSMD11), the proteasome regulator AKIRIN2 (*de Almeida et al., 2021*), and the UPS-related ATPase VCP/p97 (*Heidelberger et al., 2018*). These factors were not enriched in the BFP^{high} stable control cells (**Figure 2—figure supplement 1C–D**), attesting to the specificity and identification power of the experimental system.

The mCherry-IRF1 screen identified 27 high confidence factors which specifically affected mCherry-IRF1 abundance, but neither mCherry-cMYC nor the stable BFP controls (**Figure 2C–E**). The only candidate strongly enriched at both 3 and 6 days after Cas9 induction was the Cullin E3 ligase receptor speckle type BTB/POZ protein (SPOP), which did not score in the mCherry-cMYC screen (**Figure 2B–E**, **Figure 2—figure supplement 1E–F**). In addition, seven nuclear transport-related factors (NUP35, NUP85, NUP93, NUP155, NUP160, TMEM48, NUTF2) were identified (**Figure 2E**, **Figure 2—figure supplement 1F**), suggesting that nuclear translocation of IRF1 or one of its degraders may be important for its degradation. Consistent with this notion, SPOP has been previously reported to localize to the nucleus and degrade nuclear substrates (*Marzahn et al., 2016*; *Usher et al., 2021*). Moreover, a chromatin reader component of the NuA4 histone acetyltransferase complex (YEATS4) and two factors with limited functional annotation (GPATCH1, C14ORF102) were identified (**Figure 2E**).

Previously described regulators of IRF1 proteasomal turnover such as FBXW7, MDM2, or STUB1/CHIP (*Garvin et al., 2019*; *Landré et al., 2013*; *Narayan et al., 2011b*) did not score as significant IRF1 regulators in our screen, indicating that they are unlikely to play a significant role in IRF1 degradation in this cellular context, although we cannot rule out that unknown technical aspects in the screen prevented their identification. Importantly, FBXW7 was identified as the strongest factor mediating cMYC degradation (**Figure 2B**, **Figure 2—figure supplement 1A–D**), underpinning the functionality and specificity of the screening approach. In summary, our genetic screen identified the E3 ligase substrate receptor SPOP as a specific candidate to regulate IRF1 protein abundance.

SPOP regulates IRF1 protein levels

To determine whether our genetic screen results were predictive, individual genes identified in cell pools with increased mCherry-cMYC or mCherry-IRF1 levels were targeted. Ablation of top-scoring putative regulators of cMYC (FBXW7, UBE3C, VCP) substantially increased mCherry-cMYC levels (**Figure 3—figure supplement 1A–C**), whereas targeting SPOP specifically increased the abundance of mCherry-IRF1 (**Figure 3A**).

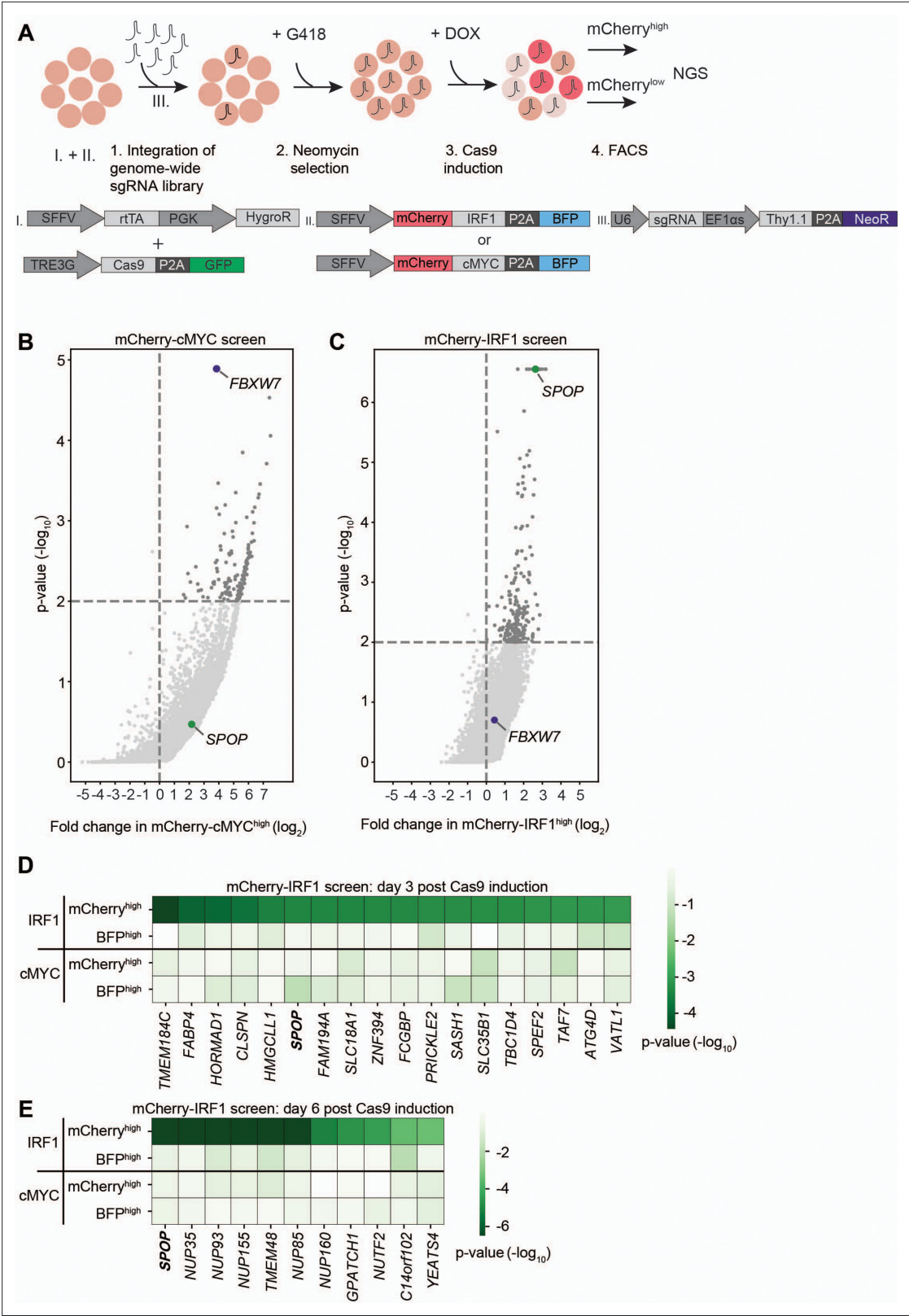


Figure 2. Identification of SPOP as a regulator of interferon regulatory factor 1 (IRF1) abundance. **(A)** Overview of fluorescence-activated cell sorting (FACS)-based CRISPR-Cas9 knockout screening procedure using the RKO-mCherry-IRF1-P2A-BFP or RKO-mCherry-cMYC-P2A-BFP cell lines. Cells expressing high and low levels of mCherry-cMYC or mCherry-IRF1 protein were sorted and their integrated sgRNA coding sequences determined by next-generation sequencing. **(B–C)** Read counts per million in the mCherry-cMYC^{high} and mCherry-IRF1^{high} cells at 6 days after Cas9 induction were

Figure 2 continued on next page

Figure 2 continued

compared to those in unsorted cells from the same day, sgRNA enrichment calculated by MAGeCK analysis, and log₂-fold change and adjusted p-value plotted. Specific top hits SPOP and FBXW7 are shown for the (B) mCherry-cMYC or (C) mCherry-IRF1 screen. (D–E) 20 genes enriched in the mCherry-IRF1^{high} sorted populations with lowest adjusted p-values were selected and any genes enriched in BFP^{high} or mCherry^{low} populations were excluded. Screen-specific adj. p-value heatmaps were generated for mCherry-IRF1^{high} cells at (D) 3 days or (E) 6 days post-Cas9 induction.

The online version of this article includes the following figure supplement(s) for figure 2:

Figure supplement 1. Identification of SPOP as a regulator of interferon regulatory factor 1 (IRF1) abundance.

Figure supplement 2. Identification of SPOP as a regulator of interferon regulatory factor 1 (IRF1) abundance.

Figure supplement 3. Identification of SPOP as a regulator of interferon regulatory factor 1 (IRF1) abundance.

Figure supplement 4. Identification of SPOP as a regulator of interferon regulatory factor 1 (IRF1) abundance.

Figure supplement 5. Identification of SPOP as a regulator of interferon regulatory factor 1 (IRF1) abundance.

Next, we set out to assess whether SPOP regulates endogenous IRF1 protein levels. To this end, cells were transduced with sgSPOP expression vectors, Cas9 was induced with Dox, and their endogenous intracellular IRF1 and cMYC protein levels determined by flow cytometry (**Figure 3B–D**). Consistent with our screen data (**Figure 2, Figure 2—figure supplement 1**), SPOP ablation increased endogenous IRF1 levels by 40%, whereas endogenous cMYC protein levels were not affected (**Figure 3C**).

The sgRNA library design with coverage of each gene by six sgRNAs made it unlikely that off-target effects of the SPOP-targeting sgRNAs were responsible for the selected phenotype of increased mCherry-IRF1. However, to measure whether individual sgRNAs would result in the same IRF1-stabilizing phenotype, we transduced cells individually with seven different, independent, sgSPOP sequences, and analyzed intracellular IRF1 levels by flow cytometry (**Figure 3D**). All seven different sgRNAs significantly increased IRF1 levels compared to the two independent controls, indicating that specific SPOP targeting underlies this phenotype, and not off-target effects (**Figure 3D**). Consistent with this notion, western blot analysis of three independent monoclonal SPOP knockout cell lines showed that IRF1 levels were increased by 50–70% (**Figure 3E–F**). This effect of SPOP ablation specifically affected IRF1 protein levels, as IRF1 mRNA concentrations were not significantly changed in SPOP knockout cells (**Figure 3—figure supplement 1D–E**).

Lastly, we assessed the importance of SPOP for IRF1 degradation in non-cancer-derived cells, and across species. In line with our results from human cell lines, SPOP ablation in mouse embryonic fibroblasts (MEFs) likewise increased endogenous IRF1 protein levels (**Figure 3G–H**), indicating a conserved mechanism across species and non-transformed cell types. In summary, these findings demonstrate that SPOP is important for regulating IRF1 protein levels, without affecting its mRNA concentrations.

SPOP targets S/T-rich degrons in IRF1

Having identified SPOP in a phenotypic genetic screen, it was possible that the effects of SPOP ablation on IRF1 were direct, or indirect through unknown other mediators. As a first step to investigate a possible direct targeting of IRF1 by SPOP, we first analyzed their subcellular distribution to determine whether they reside in the same compartment.

To this end, HeLa and RKO cells expressing exogenous IRF1 and SPOP were analyzed by confocal immunofluorescence microscopy. Consistent with previous reports, both IRF1 and SPOP localized in the nucleoplasm (**Figure 4A**), indicating that they are present in the same subcellular compartment, and thus could interact. While both proteins localized dispersedly in the nucleoplasm, in both tested cell types SPOP was also present at nuclear speckles, as previously described (**Marzahn et al., 2016**).

We reasoned that the steady-state levels of the SPOP-targeted IRF1 pool could be low under these conditions of ongoing IRF1 degradation. To stabilize the actively targeted IRF1 protein pool, cells were treated with proteasome inhibitor. Under these conditions, the diffuse nucleoplasmic IRF1 signal increased (**Figure 4—figure supplement 1A, Figure 4—figure supplement 2**), suggesting that this represents the unstable cellular IRF1 pool. Moreover, in the presence of proteasome inhibitor, the SPOP puncta increased in size and signal intensity, consistent with previous reports that these are sites of SPOP-dependent degradation of a subset of its targets (**Marzahn et al., 2016; Usher et al., 2021**). However, none of the IRF1 signal was detected in the SPOP puncta (**Figure 4—figure supplement**

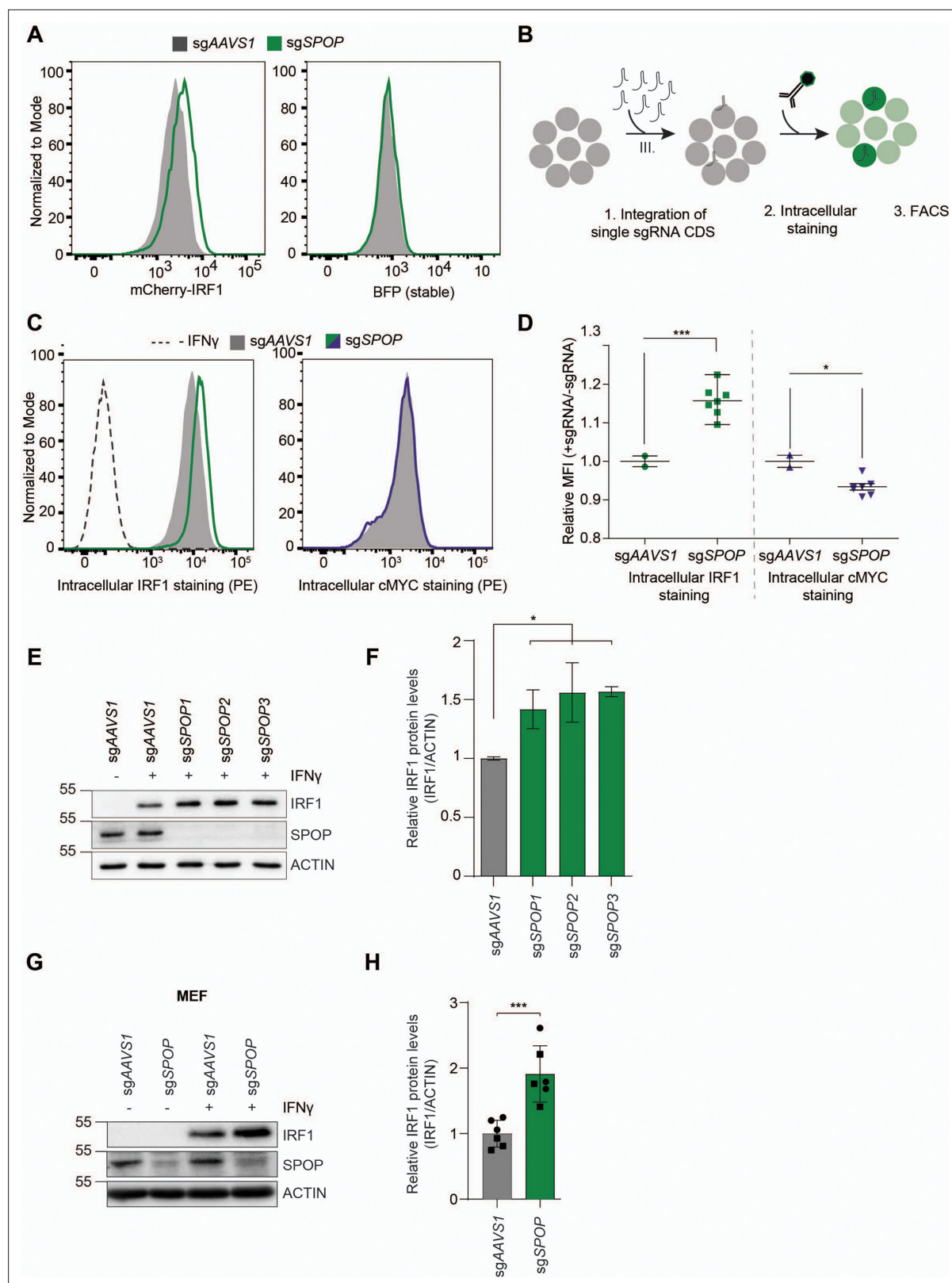


Figure 3. SPOP regulates interferon regulatory factor 1 (IRF1) protein levels. **(A)** RKO-mCherry-IRF1-P2A-BFP cells expressing sgAAVS1 or sgSPOP were treated with Dox to induce Cas9. Subsequently, mCherry-IRF1 and BFP protein levels were analyzed by flow cytometry. **(B)** RKO-Cas9-P2A-GFP cells expressing sgAAVS1 or sgSPOP were treated with Dox to induce Cas9, treated with IFN γ for 4 hr, after which endogenous IRF1 or cMYC levels were determined by **(C–D)** intracellular staining and flow cytometry. In **(D)**, each data point represents targeting with a different, independent sgSPOP

Figure 3 continued on next page

Figure 3 continued

sequence. Data were analyzed by unpaired two-tailed t-test; $n=2$ biological replicates. $*p<0.05$, $***p<0.001$. (E) Independent monoclonal RKO-Cas9-GFP cell lines with wt *IRF1* alleles derived from sgAAVS1 expressing cells, or homozygous SPOP disrupted alleles were stimulated with IFN γ for 4 hr, after which their IRF1 and SPOP protein levels were determined by western blot (WB), and (F) quantified (unpaired t-test, $n=2$). (G) Mouse embryonic fibroblast (MEF) cells were treated with IFN γ for 4 hr, after which endogenous IRF1 levels were analyzed by WB and (H) quantified (unpaired t-test, $n=6$ biological replicates from two independently established MEF cell lines indicated by circle and square symbols), $***p<0.001$.

The online version of this article includes the following source data and figure supplement(s) for figure 3:

Source data 1. Western blots corresponding to **Figure 3E**.

Source data 2. Western blots corresponding to **Figure 3G**.

Figure supplement 1. SPOP regulates interferon regulatory factor 1 (IRF1) protein levels.

1A). Together, these data suggest that IRF1 is likely targeted for degradation in the nucleoplasm, and that this could potentially be mediated by the diffuse nucleoplasmic SPOP pool.

To further extend these analyses, subcellular fractionation of AAVS1- and SPOP-targeted cells was performed. Consistent with our microscopy results (**Figure 4A**, **Figure 4—figure supplement 1A**), IFN γ treatment rapidly induced IRF1 in the nuclear fraction, which even further increased upon SPOP depletion (**Figure 4B**, **Figure 4—figure supplement 1B–C**). These data reinforce the conclusion that SPOP targets IRF1 in the nucleus, where it is subsequently degraded.

Previous studies had identified an S/T-rich SPOP binding consensus of $[\Phi]-[\Pi]-[S]-[S/T]-[S/T]$, in which Φ and Π are non-polar and polar residues, respectively (Zhuang et al., 2009). We reasoned that the presence of any sequences matching this SPOP degron in IRF1 could mediate SPOP binding to IRF1, thereby targeting it for ubiquitination and degradation.

A multiple sequence alignment of IRF1 across species (**Figure 4—figure supplement 3**) identified four sequences in IRF1 matching the SPOP degron consensus (**Figure 4C**), consistent with predictions (ScanProsite – Expasy, and Hou et al., 2022). One of the putative SPOP degrons was identified in the region between the DNA-binding domain and the IAD2 domain, whereas the remaining three were predicted to be inside the IAD2 domain, which is important for transactivation and dimerization (**Figure 4C**, **Figure 4—figure supplement 3**).

To test whether these predicted degron sites in IRF1 are important for SPOP-dependent degradation, an IRF1 mutant was generated in which the core S/T residues in each putative SPOP binding domain (SBD) were mutated to A residues (IRF1-SBD_{mut}). Subsequently, wild-type IRF1 or IRF1-SBD_{mut} were expressed in the presence of increasing amounts of a wild-type SPOP expression plasmid (**Figure 4D**). SPOP expression decreased wtIRF1 protein levels in a dose-dependent manner (**Figure 4D**), without affecting the stable EGFP internal control. In contrast, protein levels of the IRF1 degron mutant remained unaffected by exogenous SPOP expression, suggesting that one or more of the four predicted degrons are required for SPOP-dependent IRF1 degradation.

Next, we set out to investigate whether IRF1 and SPOP interact in cells and whether this interaction is mediated through the predicted IRF1 S/T degrons. To directly assess complex formation between SPOP and IRF1, SPOP was immunoprecipitated from cells expressing wtIRF1 or IRF1-SBD_{mut}. Subsequent western blot analysis for IRF1 identified wtIRF1, but not IRF1-SBD_{mut} (**Figure 4E**). The reciprocal approach in which wtIRF1 or IRF1-SBD_{mut} were immunoprecipitated, exclusively identified SPOP upon purification of wtIRF1, but not its degron mutant (**Figure 4F**).

SPOP interaction with S/T degrons has been previously mapped to its MATH domain (Zhuang et al., 2009). In line with this observation, wtIRF1 interacted with wtSPOP, yet failed to co-immunoprecipitate MATH-domain mutated SPOP (SPOP-MATH_{mut}) (**Figure 4G**). Consistently, the reciprocal pull-down of wtSPOP identified wtIRF1, whereas the SPOP mutant showed strongly reduced interaction with IRF1 (**Figure 4H**).

In line with one or more of the four identified degrons being important for SPOP-dependent IRF1 turnover, IRF1 ubiquitination was significantly increased upon co-expression of wtSPOP, but not with MATH-domain mutated SPOP (SPOP-MATH_{mut}). Upon mutation of the SPOP binding sequences, IRF1 ubiquitination was significantly decreased (**Figure 4I–J**). From this, we concluded that some or all of the SPOP degrons are required for IRF1 poly-ubiquitination, although the remaining ubiquitination in the IRF1 mutant indicates that additional E3 ligases may ubiquitinate IRF1 as well. It remains to be

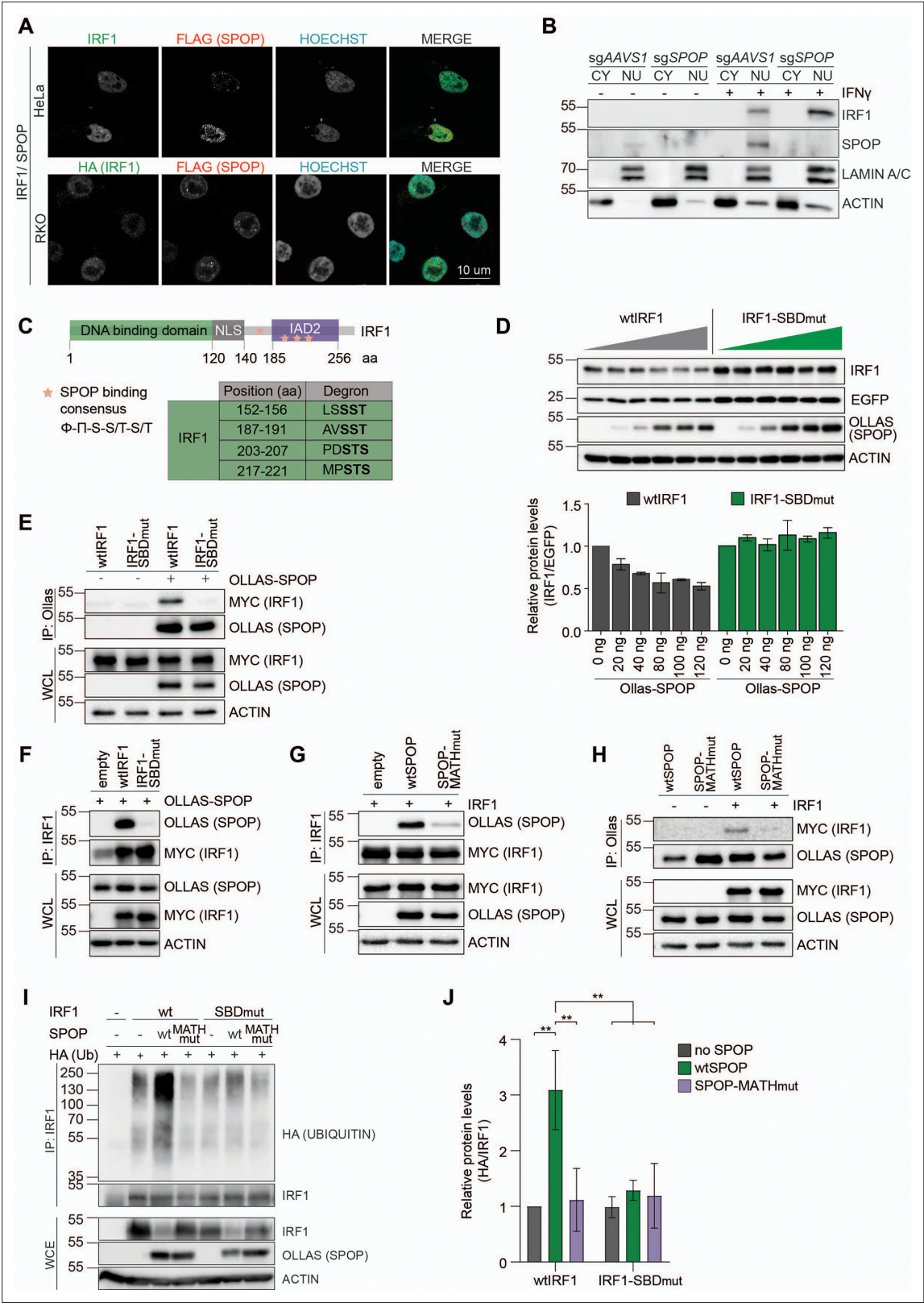


Figure 4. SPOP targets S/T-rich degrons in interferon regulatory factor 1 (IRF1). **(A)** Representative images of HeLa cells, transfected with expression plasmids for MYC-tagged IRF1 and FLAG-tagged SPOP, and RKO cells, stably expressing HA-tagged IRF1 and FLAG-tagged SPOP. At 48 hr post-transfection (HeLa) or 48 hr after seeding (RKO), cells were fixed, stained for the indicated factors, and their localization analyzed by confocal immunofluorescence microscopy. **(B)** Independent monoclonal RKO-Cas9-GFP cells lines with wt *IRF1* alleles derived from sgAAVS1 expressing cells, *Figure 4 continued on next page*

Figure 4 continued

or homozygous *SPOP* disrupted alleles were stimulated with IFN γ for 4 hr, after which whole cell (WCL), cytosolic (CY), and nuclear (NU) fractions were collected. IRF1 and *SPOP* protein levels were determined by western blot. **(C)** Schematic representation of human IRF1 and its conserved domains. The *SPOP* consensus binding sequence is displayed and matching degron sequences in human IRF1 indicated. **(D)** HEK-293T cells were co-transfected with expression plasmids for EGFP, Ollas-tagged wt*SPOP*, and either wtIRF1 or an IRF1 mutant in which the S/T residues in the four indicated *SPOP* binding domains (SBD) were mutated to A (IRF1-SBD_{mut}). At 36 hr post-transfection, cell lysates were analyzed by western blot and quantified. Data represent means and s.d. (n=3). **(E–H)** HEK-293T cells were transfected with the indicated expression plasmids for wtIRF1, IRF1-SBD_{mut}, Ollas-tagged wt*SPOP*, or an *SPOP* mutant in which its substrate interaction site in its MATH domain was mutated (*SPOP*-MATH_{mut}). At 36 hr post-transfection, cells were stimulated with epoxomicin (EPOX) for 4 hr, and subsequently interaction was analyzed by co-IP. **(I–J)** HEK-293T cells were transfected with the indicated plasmids encoding MYC-tagged wtIRF1 or IRF1-SBD_{mut}, Ollas-tagged wt*SPOP* and *SPOP*-MATH_{mut}, as well as HA-tagged ubiquitin. 36 hr post-transfection, cells were treated with EPOX for 4 hr, and subsequently MYC-tagged IRF1 was immunoprecipitated and **(I)** ubiquitination analyzed by western blot, and **(J)** quantified by densitometry. Data were analyzed by two-way ANOVA; n=3 biological replicates, data represent means and s.d., **p<0.01.

The online version of this article includes the following source data and figure supplement(s) for figure 4:

Source data 1. Western blots corresponding to **Figure 4A**.

Source data 2. Western blots corresponding to **Figure 4B**.

Source data 3. Western blots corresponding to **Figure 4D**.

Source data 4. Western blots corresponding to **Figure 4E**.

Source data 5. Western blots corresponding to **Figure 4F**.

Source data 6. Western blots corresponding to **Figure 4G**.

Source data 7. Western blots corresponding to **Figure 4H**.

Source data 8. Western blots corresponding to **Figure 4I**.

Figure supplement 1. *SPOP* targets S/T-rich degrons in interferon regulatory factor 1 (IRF1).

Figure supplement 1—source data 1. Western blots corresponding to **Figure 4—figure supplement 1A**.

Figure supplement 1—source data 2. Western blots corresponding to **Figure 4—figure supplement 1B**.

Figure supplement 1—source data 3. Western blots corresponding to **Figure 4—figure supplement 1I**.

Figure supplement 2. Identification of *SPOP* as a regulator of interferon regulatory factor 1 (IRF1) abundance.

Figure supplement 2—source data 1. Western blots corresponding to **Figure 4—figure supplement 2**.

Figure supplement 3. Identification of *SPOP* as a regulator of interferon regulatory factor 1 (IRF1) abundance.

determined whether these *SPOP*-independent poly-ubiquitin chains have a topology associated with proteasomal degradation.

Subsequently, we set out to identify the lysine residues required for IRF1 turnover. To this end we expressed *SPOP* together with full-length IRF1, in which all lysines in only the indicated protein domains were mutated to arginines to prevent ubiquitination (**Figure 4—figure supplement 1D–E**). Mutation of all lysines in the N-terminal half of the DNA-binding domain (DBD1), its C-terminal half (DBD2), the NLS region, or its C-terminal domain (C-term.) did not alter its sensitivity to treatment with proteasome inhibitor (**Figure 4—figure supplement 1D–E**). In contrast, an IRF1 mutant in which all lysines were mutated in the entire IRF1 protein (allKtoR) was no longer stabilized by proteasome inhibition, suggesting that *SPOP* targets lysines in multiple IRF1 domains.

Lastly, we tested whether wt*SPOP* or *SPOP*-MATH_{mut} could rescue IRF1 degradation in *SPOP* KO cells. To this end, expression plasmids for wt*SPOP* or its MATH domain mutant counterpart were delivered to *SPOP* KO cells, and endogenous IRF1 levels measured by flow cytometry (**Figure 4—figure supplement 1F**). In line with previous observations, knockout of *SPOP* increased IRF1 protein levels (**Figure 4—figure supplement 1G**). Expression of wt*SPOP* in AAVS1-targeted RKO cells did not alter endogenous IRF1 protein concentrations compared to an empty vector control (**Figure 4—figure supplement 1H**, left panel), indicating that *SPOP* levels in these cells were likely not limiting for IRF1 turnover. In contrast, the elevated IRF1 protein levels in *SPOP* KO cells were decreased by exogenous wt*SPOP* expression to levels measured in wild-type cells, whereas they were not affected by the empty vector control (**Figure 4—figure supplement 1H**; middle panel and **Figure 4—figure supplement 1I**). Consistent with our interaction data and requirement of the *SPOP* MATH domain for IRF1 targeting (**Figure 4G–H**), IRF1 levels were only rescued by wt*SPOP* expression, but not by its MATH domain mutant counterpart (**Figure 4—figure supplement 1H**, right panel).

Taken together, these data indicate that IRF1 is targeted by SPOP for ubiquitination through interaction of the SPOP MATH domain with S/T-rich degrons in IRF1, resulting in subsequent IRF1 degradation.

SPOP controls IRF1-dependent cellular output

IRF1 is required for efficient transcription of interferon-induced genes (ISGs). IRF1 is in particular important for transcriptional induction of a subset of ISGs, such as members of the guanylate binding protein (GBP) family in response to IFNs (Ramsauer et al., 2007). By querying the harmonizome project for IRF1 transcriptional targets (Rouillard et al., 2016), we identified the ISGs *GBP2*, *GBP3*, and *IFIT3* as putatively IRF1-dependent immune effector ISGs, and *PARP14* and *APOL2* as IRF1-dependent non-immune effector genes in RKO cells. *NLRC5* was included as an IRF1-independent control gene. We asked whether SPOP-dependent control of IRF1 protein abundance influences IRF1-dependent transcriptional output. To this end, mRNA levels of IRF1 target genes were measured by RT-qPCR in either control cells, SPOP-deficient, IRF1-deficient, or IRF1/SPOP double knockout cells (Figure 5A–B, Figure 5—figure supplement 1A).

As expected, SPOP ablation did not significantly change the mRNA concentration of IRF1 independently transcribed *NLRC5* (Figure 5A). In contrast, SPOP ablation significantly increased the mRNA levels of IRF1-dependent immune-related targets *GBP2*, *GBP3*, and *IFIT3* (Figure 5A) in the presence of IRF1. Expression was reduced to equal levels in both IRF1 and IRF1/SPOP knockout cells, indicating that differences in ISG expression in SPOP KO cells are dependent on IRF1-driven transcription. Moreover, non-immune transcripts *APOL2* and *PARP14* (Figure 5B) were likewise increased in the absence of SPOP in an IRF1-dependent manner.

To further investigate the effect of SPOP on IRF1-dependent transcriptional output, the effect of exogenous SPOP expression in a reporter assay was assessed. To this end, wtIRF1 or IRF1-SBD_{mut} were co-expressed with an IFN-stimulated response element (ISRE) luciferase reporter (Figure 5C). wtIRF1 expression increased reporter activation by 408-fold (Figure 5C). Consistent with our findings that the IRF1-SBD_{mut} is expressed at increased steady-state levels (Figure 4D), expression of the same amount of plasmid encoding this mutant resulted in an 85% increase in ISRE-reporter activity (Figure 5C). In line with SPOP degron-dependent degradation, wtIRF1-driven ISRE activation was reduced 11-fold by SPOP co-expression, whereas its degron-mutated IRF1-SBD_{mut} counterpart was unaffected (Figure 5C).

To exclude any confounding effects, we reduced the amount of IRF1-SBD_{mut} expression construct by half to match the steady-state protein levels of wtIRF1. Likewise, under these conditions wtSPOP expression specifically reduced wtIRF1-driven reporter expression, but not its IRF1-SBD_{mut} counterpart (Figure 5D). Moreover, IRF1-driven reporter activation was only reduced by wtSPOP, but not SPOP-MATH_{mut}, which cannot interact with IRF1 (Figure 5D). From these data, we concluded that one or more degrons in the IRF1 substrate as well as the substrate binding domain in SPOP are required for reducing IRF1 levels, thereby controlling its transcriptional output.

Unlike other IRFs, IRF1 also drives the expression of various cell cycle inhibiting factors (e.g. p21) (Armstrong et al., 2012; Tanaka et al., 1996), making it an important tumor suppressor (Nozawa et al., 1999; Xie et al., 2003). Therefore, we next determined whether SPOP loss would affect cell fitness in a competition assay. To avoid any confounding and desensitization effects of continuous IFN γ treatment to upregulate IRF1, we targeted SPOP in our mCherry-IRF1 screening cell line, and analyzed their relative cell fitness in a mixed culture with untransduced cells (Figure 5E). Over the course of 20 days, targeting SPOP in cells not exogenously expressing mCherry-IRF1 (wt) reduced fitness by 15%, whereas this was further reduced by nearly twofold in mCherry-IRF1-expressing cells, relative to their respective sgAAVS1 controls (Figure 5E). From these data we concluded that SPOP loss disproportionately reduces cell fitness in the presence of IRF1, consistent with increased IRF1 protein concentrations in the absence of SPOP.

Lastly, we addressed the biological relevance of SPOP loss on the cellular antiviral state. Pre-treatment of both sgAAVS1 and sgSPOP cells with IFN γ reduced vesicular stomatitis virus (VSV) progeny virus production by approximately fivefold in a multi-cycle infection experiment (Figure 5—figure supplement 1B). However, progeny virus titers were not significantly different from sgAAVS1 control cells (Figure 5—figure supplement 1B).

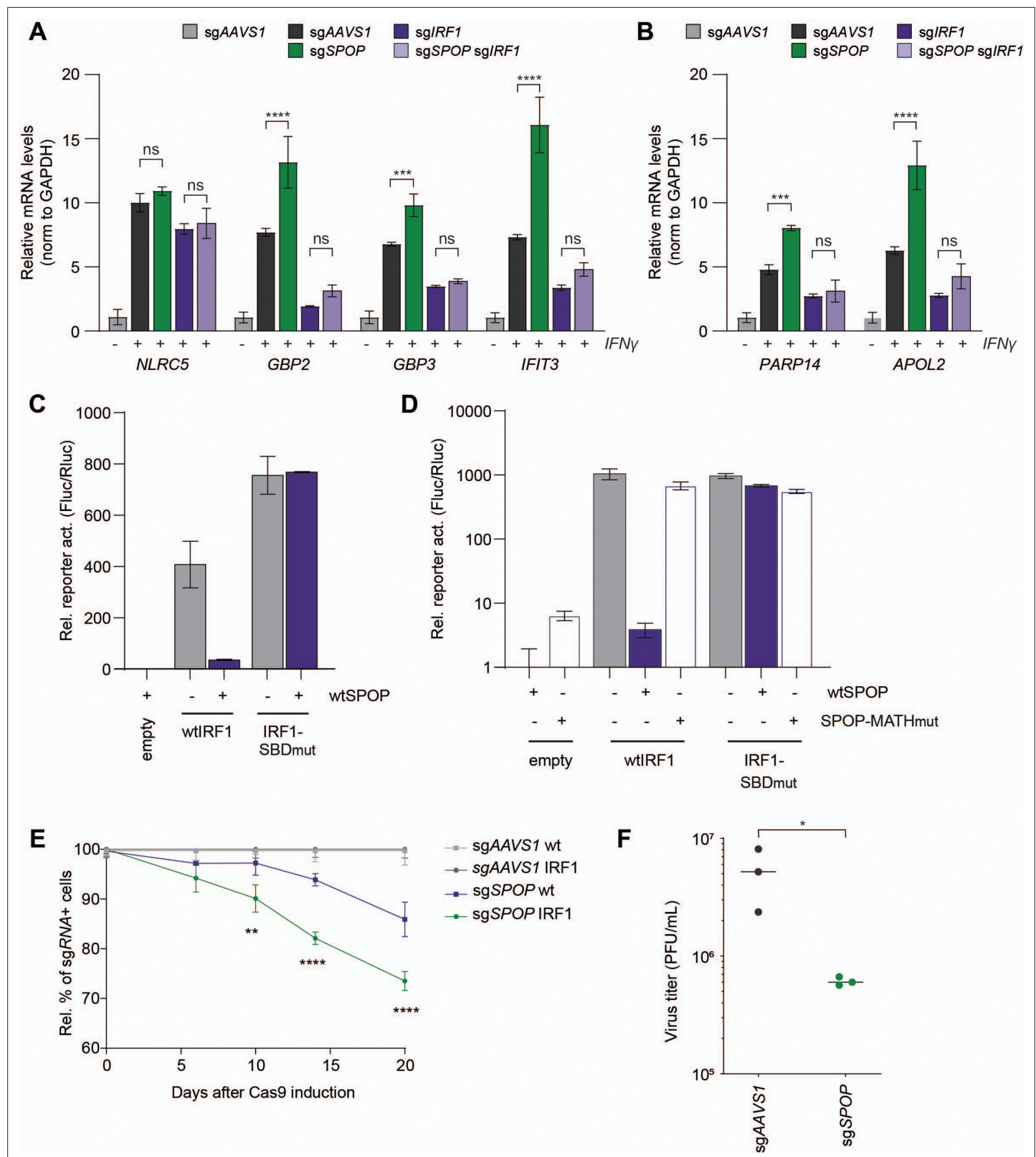


Figure 5. SPOP controls interferon regulatory factor 1 (IRF1)-dependent cellular output. RKO cells expressing either sgAAVS1, sgSPOP, sgIRF1, or sgSPOP and sgIRF1 together were stimulated with IFN γ for 4 hr and concentrations of the indicated (A) immune-related or (B) non-immune-related mRNAs analyzed by RT-qPCR (n=2 biological replicates). Data represent means and s.d. Data were analyzed by unpaired two-tailed t-test. *p<0.05, ***p<0.001. (C–D) HEK-293T cells were transfected with expression plasmids for an ISRE-Fluc reporter, and (C) equal amounts of the indicated IRF1

Figure 5 continued on next page

Figure 5 continued

and SPOP expression constructs, or (D) half of the amount of IRF1-SBD_{mut} plasmid to match IRF1 protein levels. After 24 hr cell lysates were analyzed by dual luciferase assay. n=3 biological replicates. Data represent means and s.d. (E) RKO-mCherry-IRF1-P2A-BFP cells or the parental cell line with only dox-inducible Cas9-GFP (wt) were transduced with iRFP vectors expressing sgAAVS1 or sgSPOP in 30–60% of all cells. Gene editing was induced with Dox and the fraction of transduced cells monitored for 20 days. At the indicated times, the fraction of sgRNA vector-positive cells was determined by flow cytometry. Graphs show relative abundance of sgSPOP-positive cells normalized to sgAAVS1 of the same cell line on day 0. Data were analyzed by two-way ANOVA; n=3 biological replicates; **p<0.01, ****p<0.0001. (F) RKO-mCherry-IRF1-P2A-BFP cells in which *IRF3* and *STAT1* were targeted by sgRNAs, were transduced with expression vectors for either sgAAVS1 or sgSPOP. sgRNA-positive cells were sorted by fluorescence-activated cell sorting (FACS), and subsequently infected with vesicular stomatitis virus (VSV) at a multiplicity of infection (MOI) of 0.0005. After 26 hr, supernatants were collected, and progeny virus titers determined by plaque assays.

The online version of this article includes the following source data and figure supplement(s) for figure 5:

Figure supplement 1. SPOP controls interferon regulatory factor 1 (IRF1)-dependent cellular output.

Figure supplement 1—source data 1. Western blots corresponding to **Figure 5—figure supplement 1A**.

Figure supplement 1—source data 2. Western blots corresponding to **Figure 5—figure supplement 1C**.

Since IRF1 drives only the transcription of a subset of ISGs in IFN γ -stimulated cells, we reasoned that most of the antiviral state in cells could be conferred by the STAT1 homo-dimeric transcription factor complex, in an IRF1-independent manner. Therefore, we next performed VSV infections in cells exogenously expressing mCherry-IRF1, in which most of the antiviral state is IRF1-dependent (Schoggins et al., 2011). To further limit IRF1-independent ISG induction during infection, sgRNAs targeting *IRF3* and *STAT1* were expressed (Figure 5—figure supplement 1C; Ramsauer et al., 2007; Schoggins et al., 2011), in combination with either sgAAVS1 or sgSPOP. sgAAVS1-expressing mCherry-IRF1 cells produced approximately 10-fold less progeny virus (Figure 5F), compared to its counterparts not expressing IRF1 (Figure 5—figure supplement 1B), indicating that mCherry-IRF1 expression conferred an antiviral effect. Consistent with increased IRF1 protein levels, loss of SPOP further reduced VSV progeny titers by approximately 10-fold (Figure 5F). These results indicate that SPOP plays an important role in limiting intracellular IRF1 protein concentrations, and that its loss increases IRF1-dependent cell fitness (Figure 5E) and antiviral immunity (Figure 5F).

Discussion

The importance of IRF1 in myeloid cells for driving CXCL9 and CXCL10 expression and thereby being broadly critical for their innate immune response has been described (Forero et al., 2019). Moreover, IRF1 is a critical tumor suppressor, which is underpinned by the observation that IRF1 is mutated or lost in many cancers (Nozawa et al., 1999; Xie et al., 2003). Some ubiquitin E3 ligases controlling IRF1 protein turnover have been described, such as FBXW7, MDM2, and STUB1 (Garvin et al., 2019; Landré et al., 2013; Narayan et al., 2011b). Their differential importance for regulating IRF1 turnover in different cell types and tissues draws an interesting functional parallel with cMYC, which is also targeted for degradation by cell-type-specific E3 ligases (Sun et al., 2021).

Here, we show that the Cullin E3 ligase receptor SPOP is important for IRF1 protein turnover. In contrast to previous findings in COS7 and MRC5 cells (Garvin et al., 2019), in our RKO cell-based experiments, FBXW7 did not contribute to IRF1 degradation, as its ablation did not alter IRF1 protein concentrations. Nevertheless, FBXW7 cellular functionality and efficient targeting was demonstrated, since it was key to cMYC degradation, consistent with previous reports (Welcker et al., 2004; Yada et al., 2004). These findings expand our knowledge on the pathways that can be employed by different cells to balance IRF1 levels. Moreover, it underpins the notion that, as for cMYC, the factors mediating IRF1 turnover can be cell-context-specific. This notion of cell-type-specific regulation of protein degradation is further underpinned by the fact that we show in this manuscript that SPOP is dispensable for cMYC degradation in our cell models, whereas it has been reported to mediate cMYC turnover in prostate and breast cancers (Geng et al., 2017; Luo et al., 2018).

SPOP has been found to be differentially expressed in many cancers (Cuneo and Mittag, 2019; Guo et al., 2016; Li et al., 2022; Patel et al., 2020; Song et al., 2020; Zhao et al., 2016). Moreover, mutations accumulate in SPOP target recognition domains in endometrial and prostate cancers, altering their sensitivity to BET inhibitor therapy (Dai et al., 2017; Janouskova et al., 2017; Zhang et al., 2017). Interestingly, these mutations enhanced BRD protein degradation in endometrial cancers,

whereas it did the opposite in prostate cancers (Dai et al., 2017; Janouskova et al., 2017; Zhang et al., 2017). Thus, SPOP substrate targeting, its importance as a tumor-promoting or -repressing factor, and therapy sensitivity may be cancer- or cell-type specific. This exemplifies the cell context-dependent similarities between IRF1 and SPOP.

Several SPOP substrates important in a cancer context have been previously identified, such as BRD proteins, DRAK1 and LATS1 (Pang et al., 2022; Wang et al., 2020). Our data presented here adds IRF1 to this growing list of SPOP substrates. Since IRF1 levels are low in many cells in the absence of cytokine stimulation, it may have previously been missed as a SPOP interactor and substrate in unbiased proteomics efforts (Dai et al., 2017; Janouskova et al., 2017; Zhang et al., 2017). Thus, while the contributions of SPOP to cancer establishment or progression are likely through deregulation of a manifold of its substrates, our work suggests that IRF1 may be one of them. In line with our finding that SPOP could be critical for lowering the tumor-suppressive levels of IRF1, SPOP is overexpressed in most clear-cell renal cell carcinomas (ccRCC) (Patel et al., 2020; Zhao et al., 2016). The observation that IFN γ signaling and IRF1 expression are important biomarkers in ccRCC (Kong et al., 2022), addressing the importance of the SPOP-IRF1 axis in this cancer type may be of particular interest in future studies.

The complexity of IRF1 turnover regulation in different cell types is further illustrated by the fact that SPOP may influence IRF1 stability in myeloid cells in an opposite manner as we identified here in stromal cell types (Tawaratsumida et al., 2022). Recent work identified SPOP in myeloid cells as a key bridging molecule, indirectly repressing IRF1 phosphorylation, and its subsequent degradation. In line with this model, loss of SPOP in plasmacytoid dendritic cells and a mouse macrophage cell line decreased IRF1 (Tawaratsumida et al., 2022). This opposite SPOP phenotype and function from what we describe here could be explained by the fact that many non-immune cells do not express TLRs and their associated adapter proteins, which were shown to be required for SPOP complex formation and repression of IRF1 phosphorylation and degradation. It should be noted that the RKO cells used in this study have been reported to express TLRs (Xu et al., 2020). This suggests that additional cellular factors, such as recruited adaptors or differential dependence on specific Src kinases for signaling, may determine SPOP-dependent regulation in myeloid cells.

Importantly, IRF1 degradation in myeloid cells is seemingly dependent on active TLR-dependent immune signaling, whereas in the stromal, non-myeloid cell models used in this study, IRF1 degradation was independent of immune signaling. Several other studies have indicated that SPOP may inhibit TLR signaling through several different proposed means (Hu et al., 2021; Jin et al., 2020; Li et al., 2020), suggesting that SPOP may balance biological output from the TLR-IRF1 axis through opposing effects. Together, these possible contrary effects of SPOP on IRF1 abundance in different cell types should thus be considered when addressing the impact of SPOP in the context of future whole-organism studies.

During the revision of this manuscript, Gao et al. also reported SPOP as an important IRF1 degrader in endometrial cancer cells (Gao et al., 2023). They found that cancer-associated SPOP mutations result in diminished IRF1 degradation, and consequentially increased expression of the IRF1-driven immune-suppressive PD-L1. Most of the cell-mechanistic data of how SPOP targets IRF1 for ubiquitination and degradation are in agreement between both studies. However, a notable difference between both studies is that in our experiments, we showed that in RKO and HeLa cells, SPOP is localized in the nucleoplasm and nuclear speckles, whereas IRF1 is exclusively diffusely distributed in the nucleoplasm. Since proteasome inhibition increased only the diffuse nucleoplasmic IRF1 pool, we concluded that in our cell models SPOP most likely targets IRF1 in the nucleoplasm, and not in nuclear speckles. In contrast, Gao et al. found that IRF1 strongly associates with SPOP in nuclear speckles in KLE endometrial cancer cells (Gao et al., 2023). It remains unclear where this difference stems from. It could be that SPOP-IRF1 subcellular interaction sites differ in different cell types, or that technical differences – such as SPOP/IRF1 expression levels – influence subcellular interaction sites.

Previous work has indicated that SPOP recognizes nuclear targets with intrinsically disordered regions through multivalent interactions with S/T-rich degrons (Usher et al., 2021). In addition, its related paralog SPOP-like (SPOPL) has been proposed to regulate the oligomeric state of the SPOP E3 ligase complex (Errington et al., 2012). While our genetic screens indicate that SPOPL does likely not play an important role in IRF1 targeting, our data from this study are fully consistent with the reported substrate profile: IRF1 has IDRs and localizes almost exclusively to the nucleoplasm. Moreover, IRF1 is

likely targeted by SPOP through recognition of one or more S/T-rich degrons, although we have not determined the contribution of the four individual S/T-rich degrons to IRF1 degradation.

The S/T residues in these SBD are theoretical sites of phosphorylation, which as such could influence SPOP-IRF1 interaction. Indeed, previous work has indicated that SPOP preferentially binds some substrate S/T-rich degrons in their non-phosphorylated form, whereas in other substrates, phosphorylation of these sites seems a prerequisite for interaction and degradation (Jiang *et al.*, 2021; Ostertag *et al.*, 2019; Zhuang *et al.*, 2009). Whether any of the identified IRF1 degrons are phosphorylated, and how this would affect its SPOP-dependent degradation, remains to be determined. IRF1 phosphorylation on S219 and S221 in the fourth degron was identified with low coverage in available IRF1 proteomics data (Garvin *et al.*, 2019) and independently in a region that includes this site (Lin and Hiscott, 1999), suggesting that this particular identified SBD could be post-translationally modified. The same sites were identified to be phosphorylated in breast cancer cells by the immune-activating kinase IKK ϵ (Remoli *et al.*, 2020). Phosphorylation under these conditions increased IRF1 proteasomal degradation. Since IKK ϵ is activated under physiological conditions during infection, this could suggest that in some cellular contexts, ongoing immune signaling could thus provide a regulatory circuit driving transcription of *IRF1*, as well as providing its subsequent signal for degradation of the encoded IRF1 protein by SPOP.

Three of the identified S/T-rich degrons in IRF1 are located in its IAD2 transactivation domain. This domain is key for heterodimerization with other transcriptional activators or repressors (Antoniczyk *et al.*, 2019). Therefore, an additional regulatory implication of our findings could be that IRF1 heterodimerization through its IAD2 domain could shield three of the S/T-rich degrons from recognition by SPOP. As such, SPOP could thus preferentially target the inactive nucleoplasmic IRF1 pool that is not engaged in transcription.

Taken together, our study identified and characterized SPOP as a cellular factor targeting IRF1 for proteasomal degradation. It extends the growing set of E3 ligases which collectively mediate proper intracellular IRF1 protein levels in various different cellular contexts. Our findings lay the foundation for future studies to address how transcriptionally inactive and active immune transcription factor pools are properly spatiotemporally targeted in diverse cell types.

Materials and methods

Vectors

The lentiviral human genome-wide sgRNA library consists of six sgRNAs per gene for a whole coding human genome and was described elsewhere (Michlits *et al.*, 2020). Lentiviral vectors expressing sgRNA from a U6 promoter and eBFP2 or iRFP from a PGK promoter have been described elsewhere (de Almeida *et al.*, 2021). sgRNA CDSs were cloned in pLentiv2-U6-PGK-iRFP670-P2A-Neo (de Almeida *et al.*, 2021) to perform knockouts in RKO cell lines. The IRF1 and cMYC stability reporters (pLX-SFFV-mCherry-IRF1-P2A-eBFP2 and pLX-SFFV-mCherry-cMYC-P2A-eBFP2) were constructed by cloning the open reading frame (ORF) of human IRF1 or cMYC into a modified pLX303 vector (Addgene plasmid 25897). cDNAs encoding IRF1-SBD_{mutr}, wtSPOP, and SPOP-MATH_{mut} were purchased from Twist Bioscience. The IRF1-SBD_{mut} degron mutant (S154A, S155A, T156A, S189A, S190A, T191A, S205A, T206A, S207A, S219A, T220A, S221A) and the SPOP-MATH_{mut} binding mutant (Y87C, F102C, W131G) were cloned into a modified pLX303 vector. The plasmids and sgRNAs used in this study are listed in **Supplementary file 3** and **Supplementary file 4**.

Cell culture

All experiments in this study have been reproduced at least twice in independent experiments. RKO-mCherry-IRF1-P2A-eBFP2 or RKO-mCherry-cMYC-P2A-eBFP2 cells were generated by transducing RKO-Dox-Cas9 cells (de Almeida *et al.*, 2021) with SFFV-MYC-mCherry-IRF1-P2A-eBFP2 or SFFV-MYC-mCherry-cMYC-P2A-eBFP2 lentiviral vectors. Cas9 was induced with 100 ng/ml of doxycycline hyclate (Dox, Sigma-Aldrich, D9891) and mCherry-, eBFP2-, and GFP-positive single cells were sorted by FACS into 96-well plates using a FACSaria III cell sorter (BD Biosciences) to obtain single cell-derived clones. Cas9 genome editing and expression in the absence of Dox from the TRE3G promoter was tested in competitive proliferation assays. The cell lines and culture conditions used in this study are listed in **Supplementary file 5**. Cell lines used in this study were authenticated by STR analysis.

Transfections

Transfections for analysis by western blot were performed by mixing DNA and PEI (Polysciences, 23966) in a 1:3 ratio (wt/wt) in DMEM (Sigma-Aldrich, D6429) without supplements. Transfection was performed using 500 ng of total DNA. The day before transfection, 1.5×10^5 HEK-293T cells were seeded in six-well clusters in fully supplemented DMEM media. Cells were harvested 36 hr after transfection, washed with ice-cold PBS, and stored at -80°C until further processing. For dual luciferase assays, HEK-293T cells in M24 well clusters were transfected with 170 ng ISRE-Fluc reporter, 30 ng pRL-TK (Rluc; Promega), 320 ng of wtIRF1 or 160 ng of IRF1-SBD_{mut} expression plasmid, and 640 ng of wtSPOP or SPOP-MATH_{mut} expression plasmid. Twenty-four hr after transfection, cells were lysed with Passive Lysis Buffer (Promega, E194A) and Firefly- and Renilla luciferase activities measured in a dual-luciferase assay using a Synergy H1 plate reader (BioTek). Substrates were prepared as previously reported (Hampf and Gossen, 2006).

FACS-based CRISPR-Cas9 screens

Lentivirus-like particles were used to transduce RKO-mCherry-IRF1-P2A-eBFP2 or RKO-mCherry-cMYC-P2A-eBFP2 cells at an MOI of less than 0.2 TU/cell, and 500- to 1000-fold library representation. The percentage of library-positive cells was determined after 3 days of transduction by immunostaining of the Thy1.1 surface marker and subsequent flow cytometric analysis. RKO cells with integrated lentiviral vectors were selected with G418 (1 mg/ml, Sigma-Aldrich, A1720) for 7 days. After G418 selection, 120 million cells of unsorted reference sample corresponding to 1000-fold library representation were collected, and stored at -80°C until further processing. Cas9 genome editing was induced with Dox (100 ng/ml, Sigma-Aldrich, D9891) and after 3 days and 6 days, cells were sorted by FACS. Cells were harvested, washed with PBS, and stained with Fixable Viability Dye eFluor (1:1000, eBioscience, 65-0865-14) for 30 min. Subsequently, cells were washed three times with PBS and sorted in supplemented RPMI-1640 using the FACSaria III cell sorter operated by BD FACSDiva software (v8.0). RKO cells were gated for non-debris, singlets, negative for Viability Dye, GFP-positive, mCherry-positive, and BFP-positive. Two to 3% of cells with the lowest and 1–2% of cells with the highest mCherry or BFP signals were sorted into PBS. For both mCherry-IRF1 and mCherry-cMYC genome-wide screens, at least 1×10^6 (mCherry^{low} and BFP^{low}) and 2×10^6 (mCherry^{high} and BFP^{high}) cells were collected for each time point. Sorted samples were re-analyzed for purity, pelleted and stored at -80°C until further processing. The gating strategy for flow cytometric cell sorting is shown in **Figure 2—figure supplement 3** and **Figure 2—figure supplement 4**.

Protein half-life determination

To estimate endogenous IRF1 and cMYC as well mCherry-IRF1 and mCherry-cMYC protein half-lives, RKO mCherry-IRF1 or mCherry-cMYC-expressing cells were treated with 30 $\mu\text{g}/\text{ml}$ of CHX (Sigma-Aldrich, C1988). At indicated time points, total protein extracts were generated using RIPA buffer, analyzed by western blot, quantified, and normalized to ACTIN levels and to time point 0 as indicated. Single exponential decay curves were plotted using GraphPad Prism (v9), from which protein half-lives were calculated.

Immunofluorescence confocal microscopy

200,000 HeLa cells/35 mm well were seeded onto coverslips (Marienfeld, 630-2190) and transfected using polyethylenimine (PEI) transfection reagent (Polysciences, 23966) in a 1:6 (wt/wt) DNA/PEI ratio in non-supplemented DMEM with lentiviral plasmids expressing MYC-tagged wtIRF1 and FLAG-tagged wtSPOP. 48 hr after transfection, cells were fixed with 4% paraformaldehyde (PFA) for 15 min. Similarly, 250,000 RKO cells stably expressing HA-tagged wtIRF1 and FLAG-tagged wtSPOP were seeded onto coverslips and after 48 hr stimulated with epoxomicin (10 μM , 4 hr) followed by fixation. Cells were permeabilized with 0.25% Triton X-100 in PBS for 5 min, followed by blocking of non-specific sites by incubation with 1% BSA for 30 min at RT. Coverslips were incubated for 1 hr at RT with primary anti-IRF1 antibody (Cell Signaling Technology, 1:100), anti-HA (Cell Signaling Technology, 1:1000), or anti-FLAG (M2, 1:500) antibody in 1% BSA, followed by incubation with anti-rabbit IgG Alexa Fluor 488 (Abcam, 1:800), anti-mouse Alexa Fluor 647 (Invitrogen, 1:1000), or anti-mouse IgG DyLight 550 (Invitrogen, 1:500) secondary antibodies, and incubation for 5 min with 0.4X Hoechst (Thermo Fisher Scientific, H3569) in PBS. The coverslips were mounted using ProLong Gold Antifade

Mountant (Invitrogen, P36934). Images were collected using a Zeiss LSM 980 confocal microscope at $\times 40$ magnification.

Subcellular fractionation

Subcellular fractionation was performed as previously described (Suzuki *et al.*, 2010). In brief, 2 million cells were washed in 1 ml PBS and lysed in 500 μ l ice-cold REAP buffer (0.1% NP-40 in 1x PBS) supplemented with 1 mM PMSF, 1X protease inhibitor cocktail (Sigma-Aldrich, 11836145001), and 25 U/ml benzonase. 240 μ l of the lysates were collected as whole-cell fractions, the remaining lysate was centrifuged at $3000 \times g$ for 60 s at 4°C. 240 μ l of supernatants were collected as cytosolic fractions, after which pellets were washed with 500 μ l of REAP buffer by centrifugation at $3000 \times g$ for 60 s at 4°C, then resuspended in 240 μ l of REAP buffer (nuclear fraction). All fractions were subsequently supplemented with 6x Laemmli sample buffer (62.5 mM Tris-HCl [pH 6.8], 5.8% glycerol, 2% SDS, and 1.7% β -mercaptoethanol), and boiled for 10 min. Equal volumes of fractions were loaded on a 10% SDS polyacrylamide gel.

Western blot

Cells were lysed in RIPA lysis buffer (50 mM Tris HCl [pH 7.4], 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA, 0.1% SDS, 0.1 mM PMSF, and 1X protease inhibitor cocktail). Cells were rotated for 20 min at 4°C and then centrifuged at $18,500 \times g$ for 10 min at 4°C. Supernatants were transferred to new tubes and protein concentrations were determined by Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, 23225). Between 20 and 50 μ g of protein per sample was mixed with Laemmli sample buffer (62.5 mM Tris-HCl [pH 6.8], 5.8% glycerol, 2% SDS, and 1.7% β -mercaptoethanol), and boiled for 5 min. Proteins were loaded on SDS polyacrylamide gels, the percentage of which was adjusted based on the molecular weight of the proteins of interest. Proteins were blotted on PVDF or nitrocellulose membranes at 4°C for 1 hr at 300 mA in Towbin buffer (25 mM Tris pH 8.3, 192 mM glycine, and 20% ethanol). Membranes were blocked in 5% BSA in PBS-T for 1 hr at RT, and subsequently incubated with primary antibodies overnight at 4°C. The next day, the membranes were washed three times for 5 min each with PBS-T and incubated with HRP-coupled secondary antibodies for 1 hr at RT and imaged with the ChemiDoc Imaging System from Bio-Rad. Relative protein levels were quantified using Image Lab (Bio-Rad). Antibodies used in this study are listed in **Supplementary file 1**.

Co-immunoprecipitation assays

Cells were lysed in 100 μ l of Frackelton lysis buffer (10 mM Tris [pH 7.4], 50 mM NaCl, 30 mM $\text{Na}_4\text{P}_2\text{O}_7$, 50 mM NaF, 2 mM EDTA, 1% Triton X-100, 1 mM DTT, 1 mM PMSF, and 1X protease inhibitor cocktail). Cells were incubated on a rotating wheel at 4°C for 30 min and subsequently centrifuged at $20,000 \times g$ at 4°C for 30 min. The supernatant was transferred to a new tube and 10 μ l (10% of the lysate used for the immunoprecipitations [IPs]) was collected as input. 300 μ g of lysates were incubated overnight at 4°C on a rotating wheel with an IgG Isotype Control (Cell Signaling Technology, 1:300), anti-Ollas (Novus, 1:100), or anti-IRF1 antibody (Cell Signaling Technology, 1:100). The next day, magnetic beads (Pierce Protein A/G Magnetic Beads, Thermo Fisher Scientific, 88803) used for anti-IRF1 antibody IPs, or Protein G Sepharose beads (Protein G Sepharose 4 Fast Flow, Sigma-Aldrich, GE17-0618-01) used for anti-Ollas antibody IPs, were blocked by rotation in 3% BSA in Frackelton Buffer for 1 hr at 4°C. 25 μ l of beads were added to 300 μ g of lysates and rotated for 2 hr at 4°C. Then, the beads were washed five times with 1 ml of RIPA buffer, supplemented with 300 mM NaCl. Proteins were eluted by boiling in 2X disruption buffer (2.1 M urea, 667 mM β -mercaptoethanol, and 1.4% SDS) for 5 min at 95°C.

IPs for ubiquitination

Cells were lysed in 1 ml of RIPA lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100), supplemented with 40 mM *N*-ethylmaleimide, 40 mM iodoacetamide, 25 U/ml benzonase, 1 mM PMSF, and 1X protease inhibitor cocktail. Cells were incubated on a rotating wheel at 4°C for 30 min, and centrifuged at $20,000 \times g$ at 4°C for 15 min. Supernatants were transferred to new tubes and 50 μ l (20% of the lysates used for the IPs) were collected as input. 500 μ g of lysates were incubated overnight at 4°C on a rotating wheel with an anti-IRF1 antibody (Cell

Signaling Technology, 1:100). The next day, magnetic beads (Pierce Protein A/G Magnetic Beads, Thermo Fisher Scientific, 88803) were blocked by rotation in 3% BSA in RIPA Buffer for 1 hr at 4°C. 25 µl of beads were added to 500 µg of lysates and rotated for 2 hr at 4°C. Subsequently, beads were washed five times with 1 ml of RIPA buffer, supplemented with 300 mM NaCl. Proteins were eluted by boiling in 2X disruption buffer (2.1 M urea, 667 mM β-mercaptoethanol, and 1.4% SDS) for 5 min at 95°C. Volumes loaded for the IP were adjusted based on the relative IRF1 amount in those samples, quantified by Image Lab.

Lentivirus production and transduction

HEK-293T cells were transfected with DNA mixes containing lentiviral transfer plasmids, pCRV1-Gag-Pol (*Hatzioannou et al., 2004*) and pHCMV-VSV-G (*Yee et al., 1994*) using PEI (Polysciences, 23966) in a 1:3 µg DNA/PEI ratio in non-supplemented DMEM. Virus containing supernatants were clarified of cellular debris by filtration through a 0.45 µm filter. Virus-like particles were directly used after harvesting, or stored at –80°C. Target cells were transduced in the presence of 5 µg/ml polybrene (Sigma-Aldrich, TR1003G).

Intracellular staining for flow cytometry

For staining of intracellular proteins, cells were collected, washed twice with PBS, and subsequently fixed with 2% PFA for 15 min at RT. After one wash with FACS buffer (PBS supplemented with 1% FCS), cells were permeabilized in ice-cold MeOH for 10 min and pelleted three times for 10 min at 900 × g. Subsequently, cells were incubated for 10 min at RT in FACS buffer to inhibit non-specific antibody binding. Cells were then incubated with primary antibodies, or left unstained for 1 hr at RT. Following two FACS buffer washes, cells were incubated with secondary antibodies for 15 min at 4°C., washed two times with FACS buffer and resuspended in FACS buffer for flow cytometric analysis on an LSRFortessa (BD Biosciences) operated by BD FACSDiva software (v8.0). FACS data were analyzed in FlowJo (v10.8).

NGS library preparation

NGS libraries of sorted and unsorted control samples were processed as previously described (*de Almeida et al., 2021*). In brief, isolated genomic DNA was subjected to two-step PCR. The first PCR allowed the amplification of the integrated sgRNA cassettes, the second PCR introduced the Illumina adapters. Purified PCR products' size distribution and concentrations were measured using a fragment analyzer (Advanced Analytical Technologies). Equimolar ratios of the obtained libraries were pooled and sequenced on a HiSeq 2500 platform (Illumina). Primers used for library amplification are listed in *Supplementary file 7*.

Analysis of pooled CRISPR screens

The analysis of the CRISPR-Cas9 screens was carried out as previously described (*de Almeida et al., 2021*). In brief, sgRNAs enriched in day 3 and day 6 post-Cas9 induction-sorted samples were compared against the matching unsorted control populations harvested on the same days using MAGeCK (*Li et al., 2014*). In addition, depletion of sgRNAs over time was calculated by comparing the unsorted populations to the initial day 0 population.

Cell competition assays

Competitive cell fitness assays were performed as described previously (*Lee et al., 2012*). In brief, parental RKO cells harboring Dox-inducible Cas9 or the derived monoclonal RKO-mCherry-IRF1-P2A-BFP cell line were transduced with iRFP lentiviral sgRNA plasmids targeting either AAVS1 or SPOP. The multiplicity of transduction was such that 30–60% of cells were iRFP-positive before the start of fitness measurements. Gene editing was induced with Dox and the percentage of iRFP-positive cells monitored for 20 days by flow cytometry at the indicated days. The relative fraction of sgSPOP-positive cells was normalized to sgAAVS1 of the same cell line on day 0. Data were analyzed by two-way ANOVA.

VSV infections and titration

RKO-mCherry-IRF1-P2A-eBFP2 cells harboring STAT1 and IRF3 sgRNA expression vectors were additionally transduced with vectors expressing either sgAAVS1 or sgSPOP. Subsequently, sgRNA-positive

cells were collected by FACS. After Cas9 induction by Dox, cells were seeded in M24 clusters, and infected with VSV Indiana at an MOI of 0.0005 PFU/cell as previously described (Mata et al., 2011). After 26 hr, supernatants were collected, clarified, and viral titers determined by plaque assays as previously described (Matrosovich et al., 2006).

RNA isolation, cDNA synthesis, and qPCR

Total RNA was extracted from RKO-Dox-Cas9-P2A-GFP cells harboring either AAVS1/CCR5, AAVS1/IRF1, SPOP/CCR5, SPOP/IRF1-targeting sgRNAs. 1×10⁶ cells were lysed using Trizol reagent (Thermo Fisher Scientific, 5596-018) and treated with Turbo DNase (Thermo Fisher Scientific, 10792877). cDNA was prepared using random hexamer primer and RevertAid Reverse Transcriptase (Thermo Fisher Scientific, EP0441). Real-time PCR experiments were run on a Mastercycler (Bio-Rad), using SYBR Green (Thermo Fisher Scientific). Primers for qPCR are listed in **Supplementary file 6**.

Materials availability statement

All data generated or analyzed during this study are included in the manuscript and supporting files.

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Additional information

Competing interests

Johannes Zuber: JZ is a founder, shareholder and scientific advisor of Quantro Therapeutics GmbH. The Zuber lab receives research support and funding from Boehringer Ingelheim. The other authors declare that no competing interests exist.

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Additional files

Supplementary files

- Supplementary file 1. Antibodies used in this study.
- Supplementary file 2. Reagents used in this study.
- Supplementary file 3. Plasmids used in this study.
- Supplementary file 4. sgRNA sequences used in this study.
- Supplementary file 5. Cell lines used in this study.
- Supplementary file 6. qPCR primers used in this study.
- Supplementary file 7. Next-generation sequencing (NGS) library PCR primers used in this study.
- Supplementary file 8. Results from CRISPR-Cas9 genetic screens of mCherry-IRF1 and mCherry-cMYC.
- MDAR checklist

Data availability

All data generated or analysed during this study are included in the manuscript and supporting files.

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5.3 Results 3

Publication “HUWE1 controls tristetraprolin proteasomal degradation by regulating its phosphorylation”

5.3.1 Preamble

The mechanism of timely TTP protein activity and degradation remains unclear, with reports suggesting a potential ubiquitin independent process. In this study, we show that TTP on the contrary is significantly ubiquitinated, which is required for its proteasomal turnover. Furthermore, through a genetic screen we identified the E3 ligase HUWE1 as a critical regulator of TTP phosphorylation, and in this way, indirectly of its degradation. Previously, only two out of more than thirty phospho-sites of TTP have been functionally described, and a model was proposed in which MK2-mediated phosphorylation is negatively correlated with TTP activity and degradation. Here, we expand upon this model and show that it might be somewhat overly simplified. Through various TTP mutants and pharmacological assays, we show that multiple kinases and phosphatases are involved in HUWE1-regulated TTP phosphorylation, with its C-terminal part being an important hotspot of phosphorylation and potential binding sites for E3 ligases and phosphatases. Moreover, we show that this state of phosphorylated TTP remains functionally active as it reduces the half-life of TTP-targeted mRNAs like *Tnf*. Overall, this study exemplifies how the intricate interplay between multiple regulatory mechanisms can control the timely activity of key innate immune RNA processing factors like TTP.

5.3.2 Authors and status

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5.3.3 Contributing statement

My contributions to this study were formal analysis, investigation, methodology, review and editing.

5.3.4 Publication

HUWE1 controls tristetraprolin proteasomal degradation by regulating its phosphorylation

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Abstract Tristetraprolin (TTP) is a critical negative immune regulator. It binds AU-rich elements in the untranslated-regions of many mRNAs encoding pro-inflammatory mediators, thereby accelerating their decay. A key but poorly understood mechanism of TTP regulation is its timely proteolytic removal: TTP is degraded by the proteasome through yet unidentified phosphorylation-controlled drivers. In this study, we set out to identify factors controlling TTP stability. Cellular assays showed that TTP is strongly lysine-ubiquitinated, which is required for its turnover. A genetic screen identified the ubiquitin E3 ligase HUWE1 as a strong regulator of TTP proteasomal degradation, which we found to control TTP stability indirectly by regulating its phosphorylation. Pharmacological assessment of multiple kinases revealed that HUWE1-regulated TTP phosphorylation and stability was independent of the previously characterized effects of MAPK-mediated S52/S178 phosphorylation. HUWE1 function was dependent on phosphatase and E3 ligase binding sites identified in the TTP C-terminus. Our findings indicate that while phosphorylation of S52/S178 is critical for TTP stabilization at earlier times after pro-inflammatory stimulation, phosphorylation of the TTP C-terminus controls its stability at later stages.

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Editor's evaluation

The study by Scinicariello et al. set out to identify novel factors that controlled TTP stability and identified HUWE1 by CRISPR screening in macrophages. HUWE1 limited TTP phosphorylation at later times post inflammatory stimulation on residues distinct from those functionally modified by MAPKs in early stages. Overall, the biochemical and cellular signaling experiments were thoughtfully designed and well executed, leading to the discovery of HUWE1 as a TTP regulator.

Introduction

Dynamic regulation of the immune system is essential to mount a defense against pathogens upon infection, yet shut-off the response at the appropriate time during resolution. Since most cytokines and other pro-inflammatory mediators are transcriptionally induced during infection, an essential aspect of returning to homeostatic conditions is the timely removal of their mRNAs during resolution.

Tristetraprolin (TTP; also known as ZFP36 or TIS11A) is an RNA-binding protein that interacts with AU-rich elements (ARE) present in the 3'-untranslated-regions (UTR) of many mRNAs encoding pro-inflammatory mediators (Galloway et al., 2016; Lai et al., 1999; Sedlyarov et al., 2016). Subsequently, TTP recruits the CCR4-NOT decapping and deadenylation complex to target mRNAs, resulting in their destabilization and removal from the cell (Fabian et al., 2013; Lai et al., 2003; Lykke-Andersen and Wagner, 2005; Sandler et al., 2011; Tiedje et al., 2016). TTP binds to the AREs of a multitude of mRNAs encoding cytokines and other immune-related factors, yet not all of them are destabilized (Moore et al., 2018; Sedlyarov et al., 2016; Tiedje et al., 2016; Zhang et al., 2017). This has suggested that additional -hitherto unknown- regulatory mechanisms are at play controlling TTP-dependent mRNA degradation, which may differ in various cell types.

The biological importance of TTP for proper dampening of the inflammatory response is underpinned by the observation that *Zfp36* (the gene encoding TTP)-deficient mice develop systemic inflammation characterized by arthritis, dermatitis, conjunctivitis, and cachexia, which has been coined TTP deficiency syndrome (Taylor et al., 1996). One of the main deregulated ARE-containing mRNAs driving the inflammatory phenotype in *Zfp36*-deficient mice is *Tnf* (Carballo et al., 1998; Taylor et al., 1996), although additionally *Il1a/b*, *Il23*, and *Ccl3* have been implicated as well (Kang et al., 2011; Molle et al., 2013; Sneezum et al., 2020).

TTP itself is regulated at the transcriptional, post-transcriptional, and post-translational levels. Most cell types express low levels of *Zfp36* mRNA in unstimulated conditions, the transcription of which is robustly induced by proinflammatory stimuli including the Toll-like receptor 4 (TLR4) agonist lipopolysaccharide (LPS) in myeloid cells such as macrophages (Carballo et al., 1998; Lai et al., 1995; Sauer et al., 2006; Schaljo et al., 2009; Suzuki et al., 2003). At the post-translational level, TTP is phosphorylated at over 30 residues by inflammation-activated stress kinases (Brook et al., 2006; Clark and Dean, 2016; Hitti et al., 2006; Ronkina et al., 2019).

The biological relevance of most TTP phospho-sites and the identity of the involved kinases remain unknown (Clark and Dean, 2016; Ronkina et al., 2019). Most characterized are phosphorylation events at residues S52 and S178 in murine TTP that are mediated by the inflammation-activated kinase MK2, which acts down-stream of p38 mitogen-activated protein kinase (MAPK; Brook et al., 2006; Hitti et al., 2006). In mice, TTP mutants lacking these phosphorylation sites are highly unstable and rapidly degraded, yet highly biologically active (Ross et al., 2015).

This has given rise to a model in which TTP is predominantly unphosphorylated and rapidly degraded in unstimulated cells, whereas pro-inflammatory cell signaling not only increases *Zfp36* transcription, but also TTP S52/S178 phosphorylation and stabilization through interaction with 14-3-3 proteins (Kratochvill et al., 2011; Sedlyarov et al., 2016). However, in this S52/S178 phosphorylated state, TTP is thought to be inactive, whereas during dephosphorylation of these residues at later times in the inflammatory response, TTP actively mediates mRNA degradation (Kratochvill et al., 2011; Sedlyarov et al., 2016). Nevertheless, the impact of the other 30+ phosphorylated residues on TTP stability and activity has remained largely elusive.

Proteasomes are the main degradation machines of cells for homeostatic protein turn-over (Bard et al., 2018). 20S core particles contain catalytic activity, yet lack receptors for ubiquitin and ATPase activity for unfolding and translocation of proteins into the catalytic chamber. Association of 19S regulatory particles containing ubiquitin receptors and AAA+ ATPase activity assembles 26S proteasomes, which are considered the main degradative entities for poly-ubiquitinated proteins (Bard et al., 2018).

TTP protein is degraded through the proteasome, as previous studies showed that 20S proteasome inhibition stabilizes TTP. Moreover, a previous study suggested that TTP may be directly degraded by 20S proteasomes in a ubiquitin-independent manner. In this context, important destabilizing intrinsically disordered regions in the N and C termini of TTP were identified and have been suggested to putatively allow direct degradation by 20S proteasomes (Brook et al., 2006; Ngoc et al., 2014). Yet, other regulators of intracellular TTP protein abundance have remained elusive. In this study, we set out to identify and characterize novel factors that control TTP turn-over, thereby affecting pro-inflammatory output.

Through genetic loss-of-function screening, we identified several novel determinants of TTP abundance, including the giant ubiquitin E3 ligase HUWE1. Our data indicate that TTP is strongly poly-ubiquitinated on lysines in its zinc finger domain, and degraded by the proteasome in a ubiquitin-dependent manner. Moreover, we identified a novel role for the E3 ligase HUWE1 in indirectly

controlling TTP turn-over through mediating its phosphorylation via multiple stress kinases, and reduced dephosphorylation.

Results

TTP is degraded in a ubiquitin-dependent manner

Pro-inflammatory stimuli such as LPS drive both transcription of *Zfp36* (the gene encoding TTP), and phosphorylation of the TTP protein. To study how TTP protein levels are regulated, we established a macrophage cell line expressing exogenous TTP from a constitutively active promoter, uncoupling *Zfp36* transcription from regulatory effects on TTP protein stability in the absence or presence of pro-inflammatory signals.

Consistent with previous studies, endogenous TTP protein was rapidly induced by LPS (Carballo *et al.*, 1998; Lai *et al.*, 1995; Sauer *et al.*, 2006; Schaljo *et al.*, 2009; Suzuki *et al.*, 2003), and in the absence of de novo protein synthesis, rapidly degraded (Figure 1A; Brook *et al.*, 2006; Ngoc *et al.*, 2014). Treatment with proteasome inhibitor MG132 almost completely prevented TTP degradation, indicating that its degradation is predominantly through the proteasome. Under these conditions, a high-MW form of TTP accumulated, suggesting that phosphorylation is important for regulation of its stability.

To investigate whether pro-inflammatory stimuli are exclusively stabilizing TTP, or also provide degradation signals, a macrophage cell line stably expressing HA-tagged TTP was established (Figure 1B). Under non-stimulated conditions, HA-TTP was detected as medium-range MW species migrating at and above its predicted MW of 36.7 kDa, consistent with it being partially phosphorylated under non-stimulated conditions. Stimulation with LPS resulted in rapid TTP stabilization after 30 min, followed by a reduction of its protein levels at 3 hr and 7 hr post-treatment (Figure 1B; lanes 2–3). This suggested that pro-inflammatory stimuli may also provide the signaling required for TTP turn-over at longer stimulation times, possibly through regulating its phosphorylation.

To determine whether TTP proteasomal degradation was mediated by ubiquitination, cells were treated with the ubiquitin E1 inhibitor TAK-243, which inhibits de novo ubiquitination (Figure 1B and Figure 1—figure supplement 1A–B). This stabilized endogenous and exogenously expressed TTP under baseline and LPS-stimulated conditions (Figure 1B, and Figure 1—figure supplement 1A–B), demonstrating that TTP is degraded in a ubiquitination-dependent manner. Consistent with this notion, exogenous HA-TTP and endogenous TTP was detected to be ubiquitinated in denaturing lysates from these cells (Figure 1C, and Figure 1—figure supplement 1C–D).

Moreover, a TTP mutant in which all of its five lysine residues in the TTP zinc finger domain were mutated to arginines (KtoR), accumulated at high steady-state levels, and was substantially less ubiquitinated (Figure 1—figure supplement 1C). Consistent with its strongly reduced ubiquitination, the KtoR TTP mutant was stabilized (Figure 1—figure supplement 1E). Mutation of individual lysines had no significant effects on TTP accumulation (Figure 1—figure supplement 1F), suggesting that multiple lysine residues in TTP may be functionally redundant for its ubiquitination and degradation. A TTP mutant with simultaneous mutation of four residues (K97/115/133/135 R) did significantly accumulate, albeit to a lesser extent than a mutant in which all five lysines were mutated (Figure 1—figure supplement 1F). In line with lysine poly-ubiquitination playing an important role in TTP degradation, degradative K48-linked poly-ubiquitin chains were detected on TTP, whereas non-degradative K63-linked chains were not (Figure 1—figure supplement 1G). Collectively, these results indicate that TTP is covalently poly-ubiquitinated in its TTP zinc finger domain, and that all five lysines are functionally important for TTP degradation.

To enable identification of TTP abundance regulators by genetic screening, a macrophage cell line stably expressing unstable mCherry-TTP and stable BFP was established (Figure 1D). The stable BFP served as an internal control, as it is translated in equimolar amounts from the same transcript through a P2A ribosomal skip site. mCherry-TTP accumulated in cells as a stable protein under non-stimulated conditions (Figure 1E; top panel). In contrast, LPS stimulation initially further stabilized mCherry-TTP, yet subsequently facilitated its degradation, phenocopying its endogenous TTP counterpart (Figure 1E; bottom panel, and Figure 1—figure supplement 1H). Treatment of lysates from these cells with alkaline phosphatase collapsed higher migrating endogenous and exogenous TTP species

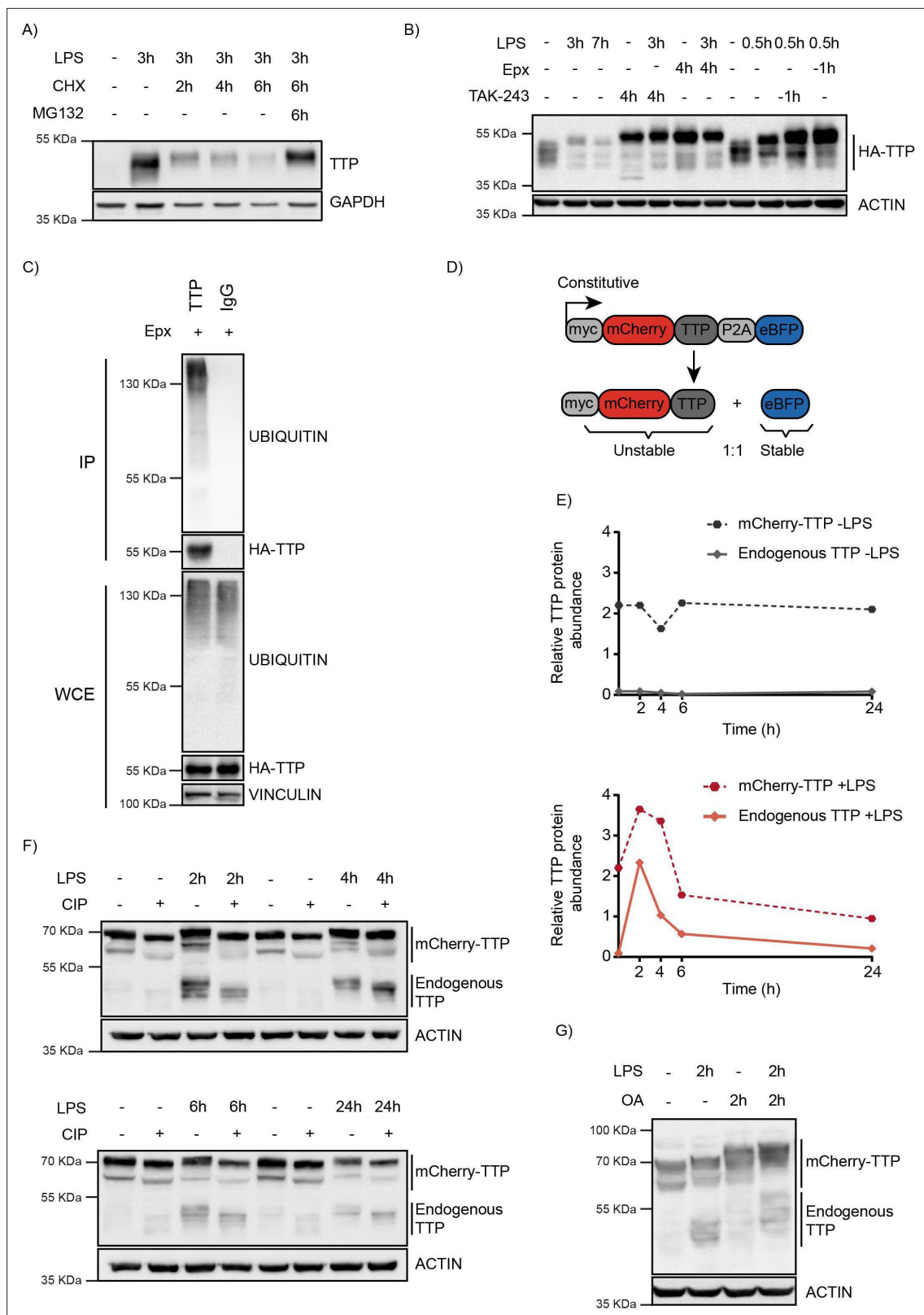


Figure 1. TTP is degraded by the proteasome in a ubiquitin-dependent manner. **(A)** RAW264.7 murine macrophages were stimulated with LPS and incubated with the translation inhibitor cycloheximide (CHX) and the proteasome inhibitor MG132 for the indicated times (h), after which TTP levels were analyzed by western blot. **(B)** 3xHA-TTP-expressing RAW264.7 cells were incubated with LPS or left unstimulated. Cells were then treated with E1 enzyme inhibitor (TAK-234) or the proteasome inhibitor Epoxomicin (Epx). Protein levels were assessed by western blot. **(C)** RAW264.7 cells stably

Figure 1 continued on next page

Figure 1 continued

expressing 3xHA-tagged TTP protein were treated with Epx for 5 hr, after which TTP was immunoprecipitated, and its ubiquitination analyzed by western blot. (D) Schematic representation of the TTP stability reporter construct. Constitutively expressed myc-tagged mCherry-TTP fusion protein and enhanced blue fluorescent protein (eBFP2) are translated at equimolar levels through a P2A site. (E–F) RAW264.7-Dox-Cas9-mCherry-TTP cells were stimulated with LPS for the indicated times. Subsequently, cell lysates were treated with Calf Intestinal Phosphatase (CIP) for 2 hr at 37 °C, and TTP levels analyzed by western blot. Non-saturated western blot signals for mCherry-TTP and endogenous TTP protein were quantified, normalized to ACTIN levels, and plotted. (G) RAW264.7-Dox-Cas9-mCherry-TTP cells were treated with LPS for 2 hr, after which PP1/2 inhibitor okadaic acid (OA) was added to the culture medium for 2 hr. TTP electrophoretic mobility was assessed by western blot.

The online version of this article includes the following source data and figure supplement(s) for figure 1:

Source data 1. Blots corresponding to **Figure 1A**.

Source data 2. Blots corresponding to **Figure 1B** and **Figure 1—figure supplement 1A**.

Source data 3. Blots corresponding to **Figure 1C**.

Source data 4. Blots corresponding to **Figure 1F**.

Source data 5. Blots corresponding to **Figure 1G**.

Figure supplement 1. TTP is degraded by the proteasome in a ubiquitin-dependent manner.

Figure supplement 1—source data 1. Blots corresponding to **Figure 1—figure supplement 1B**.

Figure supplement 1—source data 2. Blots corresponding to **Figure 1—figure supplement 1C**.

Figure supplement 1—source data 3. Blots corresponding to **Figure 1—figure supplement 1D**.

Figure supplement 1—source data 4. Blots corresponding to **Figure 1—figure supplement 1E**.

Figure supplement 1—source data 5. Blots corresponding to **Figure 1—figure supplement 1F**.

Figure supplement 1—source data 6. Blots corresponding to **Figure 1—figure supplement 1G**.

Figure supplement 1—source data 7. Blots corresponding to **Figure 1—figure supplement 1I**.

Figure supplement 1—source data 8. TurboID-TTP proximity labelling in RAW264.7 cells.

(**Figure 1F**), whereas inhibition of the phosphatases PP1 and PP2 by okadaic acid (OA) increased them (**Figure 1G**), indicating that mCherry-TTP is phosphorylated in a similar fashion as endogenous TTP.

Together, these data show that LPS-stimulation initially stabilizes TTP, whereas at later time points its induced cell signaling events direct TTP degradation.

The E3 ligase HUWE1 is a major determinant of cellular TTP protein abundance

Next, we set out to identify cellular factors regulating TTP protein abundance. To this end, a RAW264.7 mouse macrophage cell line with Dox-inducible Cas9 was established, which in addition expresses mCherry-TTP (**Figure 1D** and **Figure 2A**). To enable identification of essential genes, a cell line was established which only functionally edits in the presence of doxycycline (Dox), but not in its absence (**Figure 2—source data 4**).

A genome-wide lentiviral sgRNA library was transduced into these cells, ensuring one integration per cell. Knock-outs were induced by treatment with Dox for three and six days to identify regulators irrespective of essential gene functions and different protein half-lives. Subsequently, TTP was destabilized by LPS treatment, cells with high and low mCherry-TTP content were sorted, and their integrated sgRNA coding sequences quantified by next-generation sequencing (**Figure 2A**, and **Figure 2—source data 5**). In parallel, sorted cells with high or low levels of the stable BFP control (**Figure 1D**, and **Figure 2—source data 5**) were likewise processed, and used for identifying non-specific factors.

As anticipated, factors previously reported to be important for stabilizing TTP (e.g. *Mapkapk2* (*Mk2*), *Ywhag* (*14-3-3*), *Mapk14* (*p38*)) were significantly enriched in the mCherry-TTP^{low} cell pool (**Figure 2—source data 6**, **Figure 2B** and **Figure 2—figure supplement 1A**; left and top panels, respectively). Consistent with mCherry-TTP proteasomal degradation being LPS-dependent (**Figure 1E**), key factors for TLR4-signaling (*Tlr4*, *Ly96* (*Md2*)), and components of the proteasome (*Psma5*, *Psmb7*) were significantly enriched in mCherry-TTP^{high} sorted cells (**Figure 2B** and **Figure 2—figure supplement 1A**; right and bottom panels, respectively). Moreover, various additional new candidates controlling cellular TTP abundance were identified, including the giant ubiquitin E3 ligase *Huwe1* (**Figure 2B** and **Figure 2—figure supplement 1A**, right and top panels, respectively). Individual targeting of these

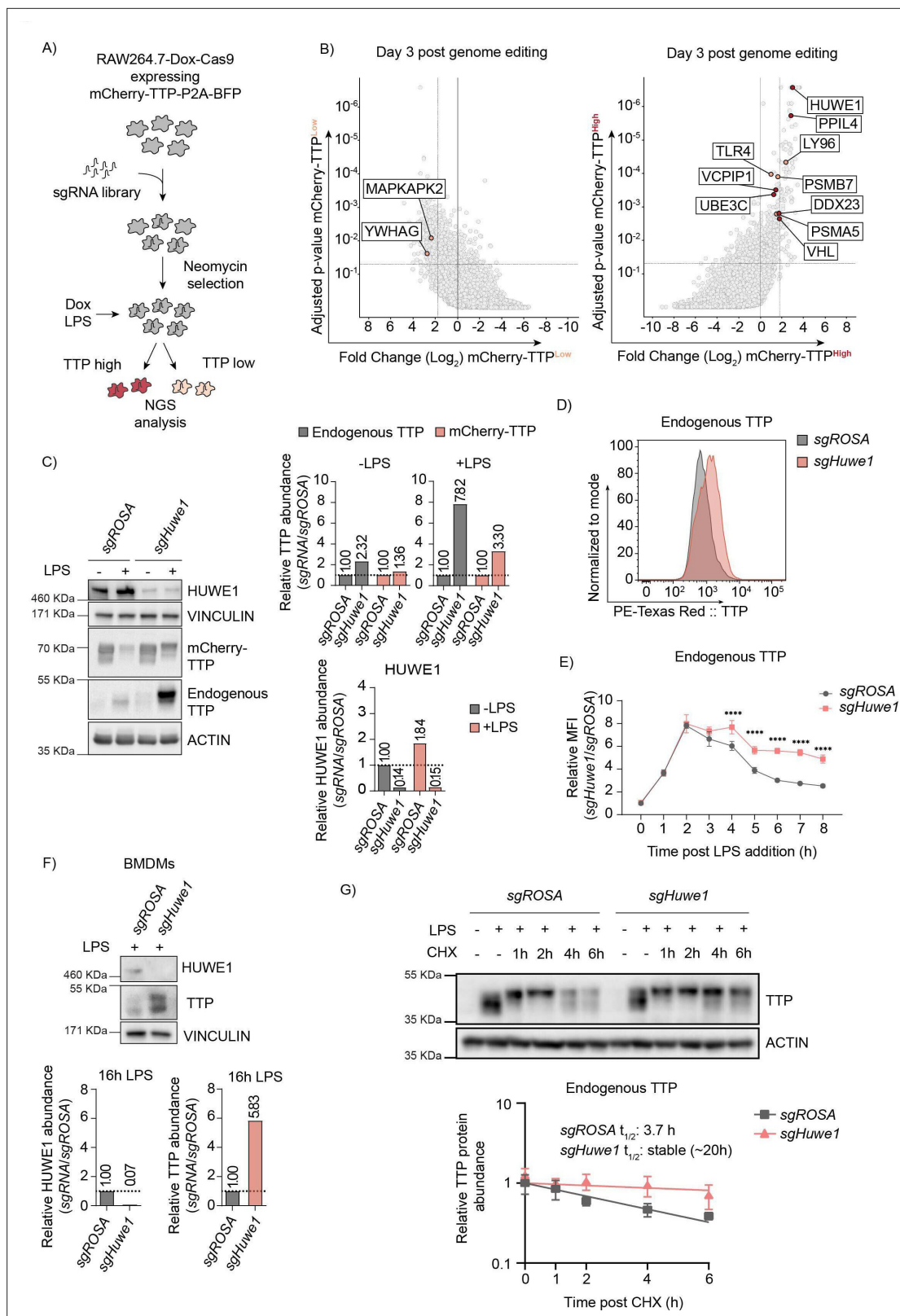


Figure 2. Genome-wide CRISPR-Cas9 knockout screen identified the E3 ligase HUWE1 as a regulator of TTP stability. **(A)** Overview of FACS-based CRISPR-Cas9 knockout screening procedure using the RAW264.7-Dox-Cas9-mCherry-TTP cell line. Cells expressing high and low levels of mCherry-TTP protein were sorted, and their integrated sgRNA coding sequences determined by next generation sequencing. **(B)** Read counts per million in the mCherry-TTP^{high} cells at 3 days after Cas9 induction were compared to those in unsorted cells from the same day, sgRNA enrichment calculated by

Figure 2 continued on next page

Figure 2 continued

MAGeCK analysis, and log2-fold change and adjusted p-value plotted. Genes enriched in the sorted populations that met the following criteria are indicated in red: a log2 fold-change of <1.8 (mCherry-TTP^{low}) or >1.8 (mCherry-TTP^{high}), adjusted p-value <0.05 , not enriched in the matching eBFP2^{low} or eBFP2^{high} sorted cells. (C) Cas9 was induced with Dox for 5 days in RAW264.7-Dox-Cas9-mCherry-TTP cells expressing either sgROSA or sgHuwe1. Subsequently, cells were treated with LPS for 16 hr, and TTP protein levels were assessed by western blot. HUWE1, mCherry-TTP and endogenous TTP abundance was quantified and plotted. The TTP and ACTIN panels are the left four lanes from the blot presented in **Figure 2—figure supplement 1B**. (D) RAW264.7-Dox-Cas9 cells expressing sgROSA or sgHuwe1 were treated with Dox for 5 days to induce Cas9. Then, cells were incubated with LPS for 16 hr or left unstimulated, and endogenous TTP protein levels analyzed by intracellular staining, followed by flow cytometry. (E) sgROSA- or sgHuwe1-targeted RAW264.7-Dox-Cas9 cells were treated with LPS for the indicated times (h), and TTP levels were analyzed by flow cytometry. Normalized mean fluorescence intensity (MFI) was plotted. Data represent the mean and s.d.; n=3 biological replicates. ****p $\leq$ 0.0001. (F) Bone marrow-derived macrophages (BMDMs) isolated from Cas9-expressing knock-in mice were stably transduced with sgROSA or sgHuwe1. Cells were incubated with LPS for 16 hr or left unstimulated. Endogenous TTP protein levels were determined by western blot. Quantified TTP levels normalized to VINCULIN are plotted. (G) sgROSA- or sgHuwe1-RAW264.7-Dox-Cas9 cells were treated for 2 hr with LPS, followed by CHX chase in the continued presence of LPS. Protein lysates were harvested at the indicated time points (h). Endogenous TTP levels were measured by WB, quantified, plotted, and TTP half-life calculated. Data represent means and s.d.; n=3 biological replicates.

The online version of this article includes the following source data and figure supplement(s) for figure 2:

Source data 1. Blots corresponding to **Figure 2C** and to **Figure 2—figure supplement 1B**.

Source data 2. Blots corresponding to **Figure 2F**.

Source data 3. Blots corresponding to **Figure 2G**.

Source data 4. Cas9 functionality and leakiness evaluation.

Source data 5. Adopted gating strategy of FACS-based mCherry-TTP stability regulator screen.

Source data 6. MAGeCK analysis of D3 and D6 CRISPR screen in RAW-Dox-Cas9.

Figure supplement 1. Genome-wide CRISPR-Cas9 knockout screen identified the E3 ligase HUWE1 as a regulator of TTP stability.

Figure supplement 1—source data 1. Blots corresponding to **Figure 2—figure supplement 1F**.

Figure supplement 1—source data 2. Blots corresponding to **Figure 2—figure supplement 1G**.

Figure supplement 1—source data 3. Blots corresponding to **Figure 2—figure supplement 1H**.

candidates increased endogenous TTP and exogenous mCherry-TTP protein levels by western blot (**Figure 2—figure supplement 1B**), attesting to the validity and predictive quality of our screen.

In particular, HUWE1 was identified as a strong determinant of endogenous and exogenous TTP protein abundance by western blot (**Figure 2C** and **Figure 2—figure supplement 1B**; compare LPS-treated samples), and flow cytometry (**Figure 2D**), without affecting *Zfp36* mRNA levels (**Figure 2—figure supplement 1C**). Consistent with an increase in protein stability, inducible *Huwe1* knock-out significantly increased endogenous TTP protein levels at later time points post-LPS stimulation (**Figure 2E**). Given that after initial stabilization, LPS mediates TTP degradation (**Figure 1E**), we tested whether *Huwe1* knock-out affected TLR4 signaling. To this end, the effect of *Huwe1* loss on I κ B α , which is degraded in a proteasome-dependent manner upon LPS stimulation (**Figure 2—figure supplement 1D**), was measured. *Huwe1* knock-out increased TTP protein levels (**Figure 2—figure supplement 1E**; top panel), but did neither affect LPS-induced degradation of mCherry-I κ B α by flow cytometry (**Figure 2—figure supplement 1E**; bottom panel), nor endogenous I κ B α by western blot (**Figure 2—figure supplement 1F**). This shows that the *Huwe1* knock-out does not affect cell signaling between TLR4 and I κ B α , and this does not contribute to TTP stabilization in *Huwe1*-deficient cells.

Next, we determined whether the HUWE1-dependent control of TTP abundance in the RAW264.7 mouse macrophage cell line was conserved across species and cell types. To this end, *HUWE1* was targeted in the human colon carcinoma cell line RKO, which -unlike most myeloid cells- have low detectable levels of TTP in the absence of any stimulation (**Figure 2—figure supplement 1G**). Similar to the phenotype in RAW264.7 cells, *HUWE1* knock-out in RKO cells strongly increased TTP protein levels (**Figure 2—figure supplement 1G**), indicating that HUWE1 has a similar role in human, non-myeloid cells independent of the TLR4 axis. Moreover, targeting of *Huwe1* in mouse bone marrow derived macrophages (BMDMs), likewise strongly increased high and low molecular weight species of endogenous TTP (**Figure 2F**), indicating that the biological importance of *Huwe1* for TTP abundance is relevant in primary cells.

Lastly, we measured whether *Huwe1* ablation affected TTP protein half-life. To this end, sgROSA and sgHuwe1 RAW264.7-Dox-Cas9 cells were continuously stimulated with LPS, chased in the

presence of translation inhibitor cycloheximide (CHX), analyzed by western blot, and single-step exponential decay curves plotted. Endogenous TTP was stabilized ≥ 5 -fold in the absence of *Huwe1* (estimated half-life of ~ 20 hr), compared to sgROSA cells in which TTP half-life was measured to be 3.7 hr (**Figure 2G**). In similar stability assessments with exogenously expressed TTP in the absence of LPS, *Huwe1* knock-out increased HA-TTP protein half-life by 83% from 35 min to 55 min (**Figure 2—figure supplement 1H**). Together, these data demonstrate that loss of *Huwe1* increases TTP protein half-life, and positioned HUWE1 as a strong, conserved regulator of TTP protein stability.

Loss of *Huwe1* decreases the half-life of pro-inflammatory mRNAs controlled by TTP

TTP is essential for the degradation of transcripts with AU-rich elements in their 3'-UTR, encoding pro-inflammatory cytokines such as TNF and IL6. Phosphorylation of S52 and S178 stabilizes TTP, yet reduces its degradation of mRNAs. In contrast, the effect of phosphorylation on other sites has remained elusive. Therefore, we reasoned that increased TTP protein levels upon *Huwe1* ablation could (i) either result in increased intracellular TTP protein concentrations, and consequently diminished levels of transcripts encoding pro-inflammatory cytokines, or -as a consequence of increased TTP phosphorylation- (ii) decrease the bio-active pool of TTP, resulting in equal or increased mRNA levels in *Huwe1* knock-out cells.

To investigate whether increased TTP levels upon *Huwe1* loss are biologically relevant, we measured *Tnf* and *Il6* mRNA levels in *Huwe1*-targeted BMDMs and RAW264.7 cells. Consistent with the fact that non-stimulated cells have very low levels of TTP, *Huwe1* knock-out did not alter baseline *Tnf* (**Figure 3A**, and **Figure 3—figure supplement 1A**), or *Il6* (**Figure 3—figure supplement 1B/C**) mRNA levels.

LPS stimulation transcriptionally induces *Tnf* and *Il6*, and in parallel influences TTP protein stability. Loss of *Huwe1* resulted in significantly decreased concentrations of *Tnf* and *Il6* transcripts at 3–16 hr post-stimulation (**Figure 3A**, and **Figure 3—figure supplement 1A–C**), consistent with increased TTP protein levels in *Huwe1* knock-out cells (**Figure 2C**). In line with TTP-dependent post-transcriptional effects, *Huwe1*-loss only altered mature *Tnf* mRNA concentrations, whereas its pre-mRNA levels remained unaffected (**Figure 3B**), indicating that *Tnf* transcription was likely unaffected. Neither did any differences stem from altered macrophage differentiation from bone marrow, as no differences in F4/80 surface expression were measured between sgROSA and sg*Huwe1* BMDMs (**Figure 3—figure supplement 1D**). Instead, Actinomycin D mRNA chase experiments indicated that the decreased levels of pro-inflammatory mRNAs are consistent with a 57% decrease in *Tnf* mRNA stability (**Figure 3C**). Moreover, targeting of *Zfp36* in *Huwe1*-deficient cells partially rescued *Tnf* mRNA concentrations (**Figure 3—figure supplement 1E**), indicating that the effects of *Huwe1* loss on *Tnf* mRNA levels are at least in part TTP-dependent. Lastly, measurements of intracellular cytokines by flow-cytometry showed that the decreased *Tnf* mRNA levels in *Huwe1* KO cells, were matched by significantly decreased intracellular TNF protein (**Figure 3—figure supplement 1F**).

Taken together, these data show that loss of *Huwe1* increases the bio-active pool of cellular TTP, resulting in enhanced turn-over of TTP target mRNAs encoding pro-inflammatory mediators.

HUWE1 regulates TTP phosphorylation and its increase is responsible for increased TTP stability

Since HUWE1 is a ubiquitin E3 ligase and was identified as a regulator of TTP protein stability by genetic means, we reasoned that the effects from its ablation on TTP could be direct through complex formation and ubiquitination of TTP, or indirect by influencing the activity or abundance of proteins that regulate TTP. Neither co-IP, nor TurboID proximity labeling assays identified complex formation between TTP and HUWE1 in cells (**Figure 1—figure supplement 1I/J**).

This suggested that the effects of HUWE1 on TTP may be indirect, although direct ubiquitination of TTP by HUWE1 cannot be ruled out as their interaction may have been too transient to detect in our assays. Attempts to address direct TTP ubiquitination by HUWE1, or any of the other E3 ligases identified in the genetic screen (**Figure 2B** and **Figure 2—figure supplement 1A–B**; VHL, UBE3C, and the Cullin adapters Elongin B/C) were hindered by the inability to purify sufficient amounts of recombinant TTP protein.

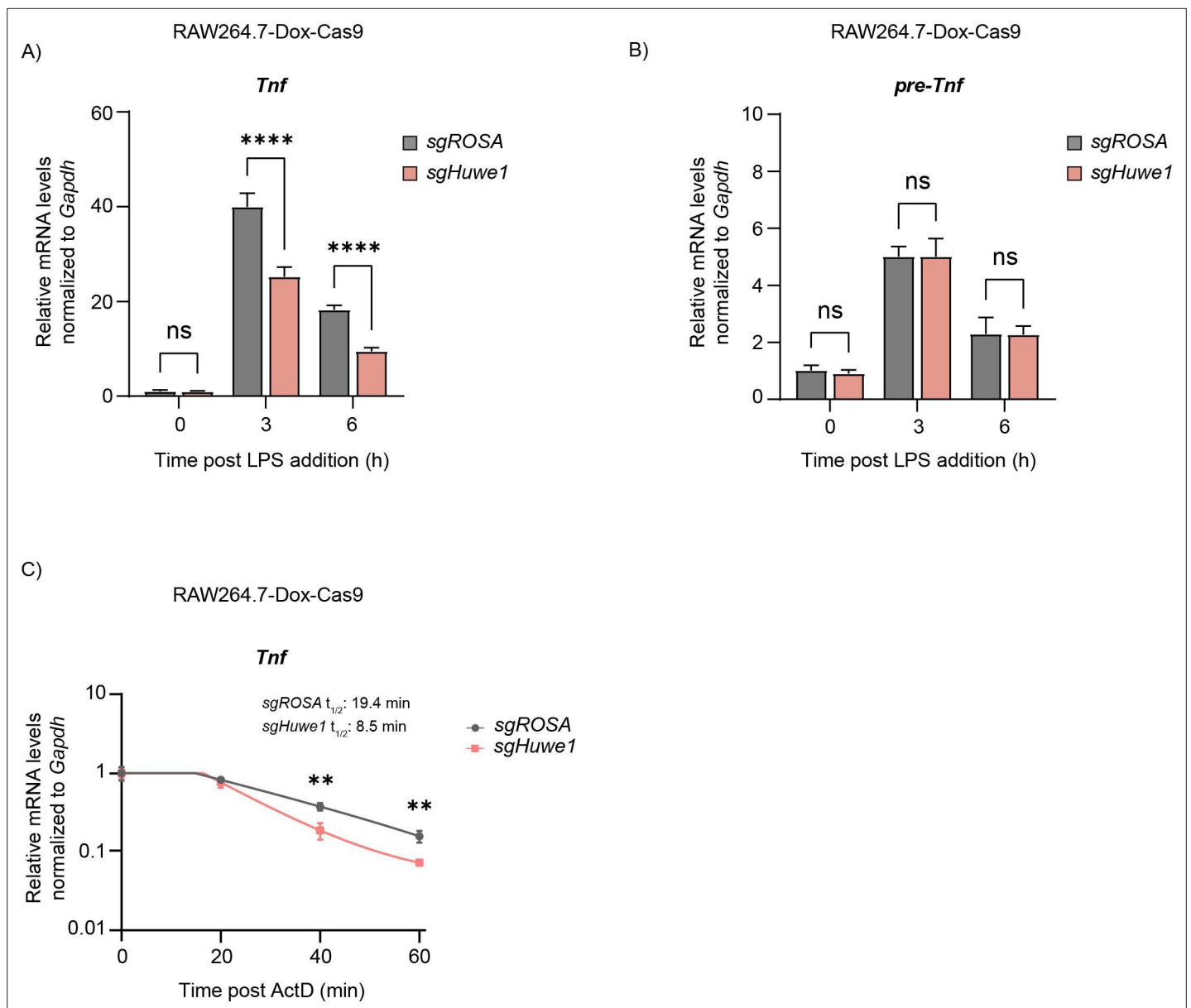


Figure 3. TTP mRNA targets are dysregulated upon *Huwe1* depletion. RAW264.7-Dox-Cas9 cells expressing *sgROSA* or *sgHuwe1* were treated with Dox for 5 days to induce Cas9. Cells were incubated with LPS for the indicated time points (h). (A) Mature *Tnf* mRNA levels, and (B) *Tnf* pre-mRNA levels were measured by RT-qPCR and normalized to *Gapdh*. Data represent the mean and s.d.; n=3 biological replicates. ****p<0.0001. Two-way ANOVA was performed. (C) RAW264.7-Dox-Cas9 cells expressing *sgROSA* or *sgHuwe1* were treated with Dox for 5 days to induce Cas9. Cells were incubated with LPS for 3 hr, after which Actinomycin D (ActD) was added for the indicated times (min), and *Tnf* mRNA levels were determined by RT-qPCR. Data represent the mean and s.d.; n=3 biological replicates. **p<0.01. Unpaired t-tests were performed for the 40 min and 60 min time points.

The online version of this article includes the following figure supplement(s) for figure 3:

Figure supplement 1. TTP mRNA targets are dysregulated upon *Huwe1* depletion.

Since TTP stability is regulated for an important part through phosphorylation by the stress kinase p38-MK2 axis and to a lesser extent ERK (Brook et al., 2006; Deleault et al., 2008; Ronkina et al., 2019), we set out to determine whether *Huwe1* ablation would alter TTP levels indirectly by affecting the cellular concentrations or activity of these kinases. Data from *Huwe1* knock-out cells indicated that the effect of *Huwe1* loss on TTP stability was predominantly at time points after the initial two hours of LPS stimulation (Figure 2E) during which TTP dephosphorylation of S52/S178 happens, resulting in its degradation (Kratochvill et al., 2011; Sedlyarov et al., 2016). Consistent with this finding, *Huwe1*

ablation did not significantly alter the total protein levels or change the early phosphorylation/activation kinetics of stress kinases p38, MK2, ERK, and JNK between 0 and 60 min post-LPS treatment (**Figure 4—figure supplement 1A–D**).

In contrast, ablation of *Huwe1* strongly increased endogenous TTP levels upon its induction by LPS at all measured later time points from 2 to 16 hr post-stimulation (**Figures 2E and 4A**). In the same lysates, total and activated/phosphorylated levels of p38, MK2, ERK, and JNK were determined.

The total levels of all four kinases varied slightly between the different time points post-LPS stimulation, yet these differences were independent of the targeted locus (*ROSA* or *Huwe1*). In contrast, *Huwe1*-targeting consistently increased the activated phosphorylated forms of all kinases at 2 hr post-stimulation (**Figure 4A–D**). While activated p38 and ERK levels in *Huwe1*-targeted cells were comparable to the *ROSA*-targeted control, or even lower, at later time points (**Figure 4A–C**), MK2 and JNK activation was increased for prolonged times, up to 6 hr post-stimulation (**Figure 4B–D**). Importantly, in the absence of LPS stimulation, *Huwe1* knock-out did not affect, or even decreased, baseline phosphorylated levels of all kinases (**Figure 4A–D**). Moreover, *Huwe1* ablation in the presence of LPS did not induce unrelated stress responses, such as p53 activation (**Figure 4—figure supplement 1E**), indicating that loss of *Huwe1* does not induce a general stress response in the cell, but is specific for pro-inflammatory cellular conditions mediated by LPS. Together, these data indicated that in the absence of *Huwe1*, multiple stress kinases may be activated more, and for prolonged times.

Based on the increased levels of phosphorylated TTP (**Figure 2C**) and stress kinase activation in *Huwe1* knock-out cells, we hypothesized that increased phosphorylation of TTP by some or all of the four deregulated kinases could be responsible for the elevated TTP protein stability. In particular, p38 and its downstream target MK2 were prime candidates, given their importance in LPS-induced TTP stabilization through phosphorylation of S52 and S178.

We reasoned that if HUWE1-dependent stability effects on TTP occurred through altering TTP phosphorylation, that either preventing TTP phosphorylation by kinase inhibition, or saturating TTP phosphorylation by phosphatase inhibition would negate TTP stabilization in *Huwe1* KO cells. To investigate whether the increased activity of the four individual kinases in *Huwe1*-targeted cells was causative for the increased TTP stability, a mixed genetic/inhibitor epistasis experiment was performed. To this end, endogenous TTP levels were assessed by intra-cellular staining in *ROSA*- or *Huwe1*-targeted cells, which were additionally treated with individual inhibitors of p38, MK2, ERK, JNK, or a combination of all four inhibitors (**Figure 4E**).

Consistent with our other results, *Huwe1* knock-out increased TTP protein levels in 6 hr LPS-treated DMSO control cells (**Figure 4E**; sample set 3). In line with previous reports that the p38-MK2 axis is an important determinant of TTP stability, treatment of sg*ROSA*-targeted cells with p38 or MK2 inhibitors significantly decreased TTP levels (**Figure 4E**; sample sets 4 and 5). However, simultaneous *Huwe1* knock-out still elevated TTP levels in the presence of these individual inhibitors, indicating that either HUWE1 does not affect TTP through the p38-MK2 axis, or that there are compensatory mechanisms affecting TTP stability in the absence of p38-MK2 kinase activity.

Consistent with previous findings of a minor effect of ERK activity, and no effect of JNK on TTP stabilization (**Deleault et al., 2008**), ERK or JNK inhibition did not influence TTP levels (**Figure 4E**; sample sets 6 and 7). Moreover, the levels of TTP in the presence of either ERK or JNK inhibitors were still increased upon *Huwe1* knock-out, indicating that the activity of neither of these individual kinases alone is required for the HUWE1-dependent effect on TTP.

We hypothesized that the deregulated increase in activity of multiple of these four stress kinases could in a partially functionally compensatory manner contribute to elevated TTP phosphorylation (**Figure 4A–D**) and stability. Indeed, inhibition of all four kinases (4i) simultaneously rendered TTP highly unstable as expected, yet in contrast to the single kinase inhibitors, this was no longer affected by *Huwe1* knock-out (**Figure 4E**; sample set 8, and **Figure 2F**). From these data, we concluded that the HUWE1 effect on TTP stability is dependent on the activity of multiple stress kinases. Together, these results indicate that HUWE1 is important for curtailing TTP phosphorylation, thereby indirectly influencing TTP stability. We reasoned that the increased TTP phosphorylation in *Huwe1* knock-out cells could stem from either increased phosphorylation by the stress kinases, and/or decreased dephosphorylation.

Since MK2/p38, ERK, and JNK are activated/phosphorylated through independent cellular pathways, yet inactivated/dephosphorylated by the same phosphatases as TTP itself (PP1/2) (**Kruse et al.,**

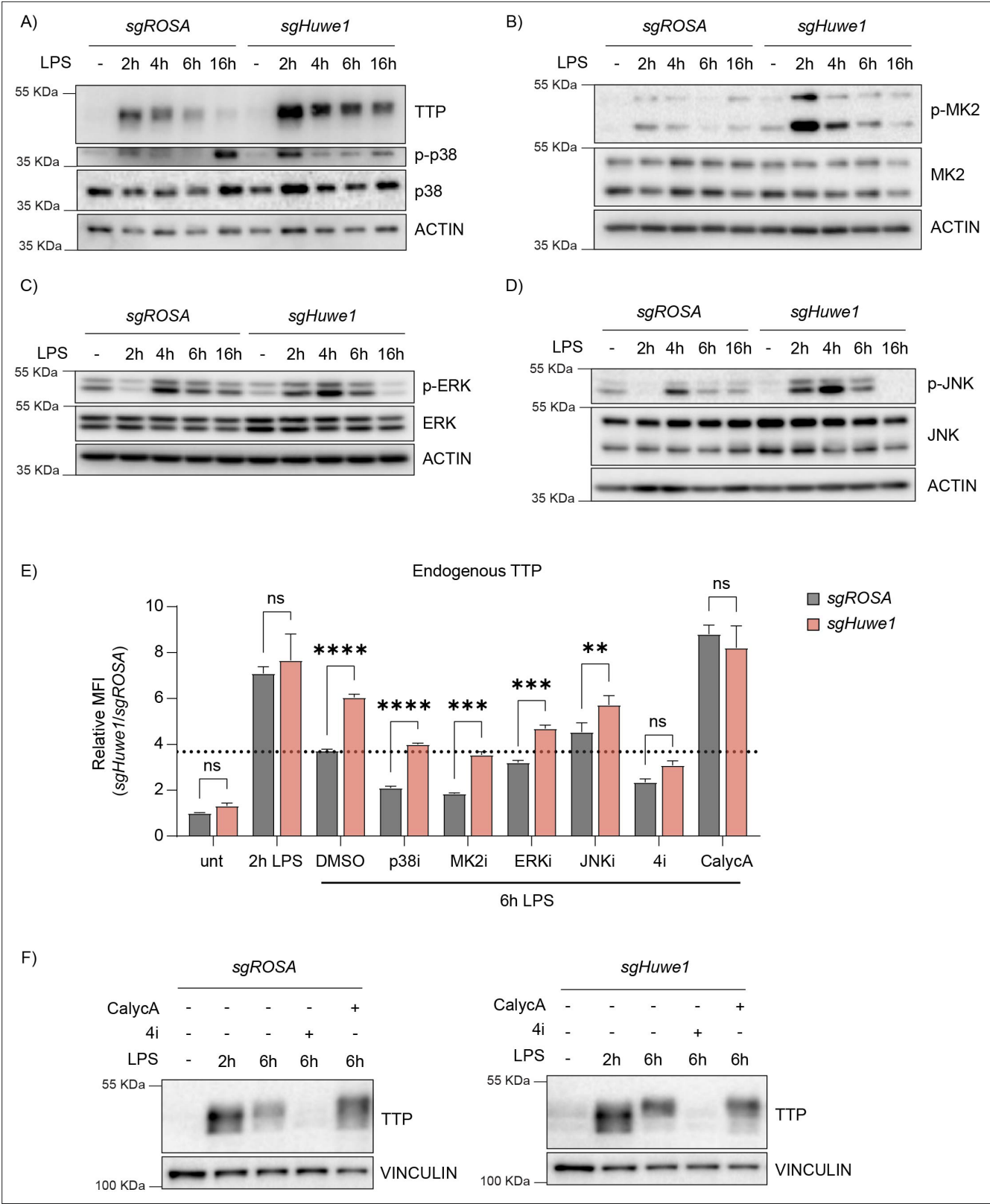


Figure 4. HUWE1 regulates TTP phosphorylation status, and thereby TTP stability. **(A–D)** RAW264.7-Dox-Cas9 cells expressing *sgROSA* or *sgHuwe1* were treated with Dox for 5 days to induce Cas9. Cells were incubated with LPS for the indicated time points (h). Phosphorylation of **(A)** p38, **(B)** MK2, **(C)** ERK, and **(D)** JNK was determined by western blot. **(E)** *sgROSA*- or *sgHuwe1*-RAW264.7-Dox-Cas9 cells were treated with LPS or left untreated. After 2 h of LPS treatment, cells were incubated with p38i, MK2i, ERKi, JNKi, or PP1/2 inhibitor Calyculin A (CalycA). TTP levels were analyzed by flow cytometry

Figure 4 continued on next page

Figure 4 continued

and normalized MFI plotted. Data represent the mean and s.d.; n=3 biological replicates. **p≤0.01; ***p≤0.001; ****p≤0.0001. Two-way ANOVA was performed. Dotted horizontal line indicates TTP abundance in the DMSO control at 6 hr post-LPS treatment. (F) sgROSA- or sgHuwe1-RAW264.7-Dox-Cas9 cells were treated with LPS for the indicated times. During the last 4 hr of LPS stimulation, the indicated inhibitors were added, after which endogenous TTP levels were analyzed by WB.

The online version of this article includes the following source data and figure supplement(s) for figure 4:

Source data 1. Blots corresponding to **Figure 4A**.

Source data 2. Blots corresponding to **Figure 4B**.

Source data 3. Blots corresponding to **Figure 4C**.

Source data 4. Blots corresponding to **Figure 4D**.

Source data 5. Blots corresponding to **Figure 4F**.

Figure supplement 1. HUWE1 regulates TTP phosphorylation status, and thereby its TTP stability.

Figure supplement 1—source data 1. Blots corresponding to **Figure 4—figure supplement 1A**.

Figure supplement 1—source data 2. Blots corresponding to **Figure 4—figure supplement 1B**.

Figure supplement 1—source data 3. Blots corresponding to **Figure 4—figure supplement 1C**.

Figure supplement 1—source data 4. Blots corresponding to **Figure 4—figure supplement 1D**.

Figure supplement 1—source data 5. Blots corresponding to **Figure 4—figure supplement 1E**.

2020; Nguyen and Shiozaki, 1999; Takekawa et al., 1998; Takekawa et al., 2000; Warmka et al., 2001), we reasoned that it was most likely that HUWE1 may be important to regulate PP1/2 activity or its cellular concentrations. Therefore, we hypothesized that decreased PP1/2 output in *Huwe1* knock-out cells could prolong TTP phosphorylation by: (i) diminishing direct TTP dephosphorylation by PP1/2, and (ii) indirectly prolonging stress kinases activation as a consequence of their diminished dephosphorylation by PP1/2.

To test this hypothesis, sgROSA or sgHuwe1 cells were treated with LPS for 6 hr, and from 2 hr onward, co-incubated with PP1/2 inhibitor Calyculin A (**Figure 4E–F**). As expected, preventing dephosphorylation by this inhibitor stabilized TTP, and prevented TTP degradation by 6 hr of LPS treatment (**Figure 4E**; compare sgROSA 6 hr LPS with sgROSA 6 hr LPS +CalycA; sample set 9, and **Figure 2F**). In contrast to sgHuwe1 samples treated for 6 hr with LPS (in which TTP protein levels were increased), *Huwe1* knock-out no longer increased TTP protein concentrations in the presence of Calyculin A (**Figure 4E**; compare sgHuwe1 6 hr LPS with sgHuwe1 6 hr LPS +CalycA, and **Figure 2F**).

From these results, we conclude that in the absence of HUWE1, decreased cellular output of PP1/2 may prolong stress kinase activation. Increased kinase activity and decreased dephosphorylation of TTP by PP1/2 consequently increases TTP phosphorylation, thereby stabilizing it.

HUWE1 controls only a small fraction of proteasome targets, and regulates the abundance of TTP paralog ZFP36L1

HUWE1 has been shown to associate with proteasomes (Besche et al., 2009), the biological significance of which has remained elusive. We reasoned that HUWE1 might be important for proteasome activity, and its ablation could cause a general impaired degradation of proteasome targets such as TTP. To investigate whether this was the case, we compared the proteomes of LPS-stimulated RAW264.7-Dox-Cas9 cells in which we targeted either *Huwe1* or proteasome core particle component *Psmb7* by label-free mass-spectrometry.

As expected, *Psmb7* targeting altered the abundance of a large number of proteins, many of which are known targets of proteasomal degradation (**Figure 5—figure supplement 1A** and **Figure 5—source data 2**). In contrast, *Huwe1* ablation significantly changed the concentrations of only a select number of proteins (**Figure 5A** and **Figure 5—figure supplement 1B**). In line with expectations of an E3 ligase, HUWE1 targets showed a trend of also being increased in *Psmb7* knock-out cells, and vice versa (**Figure 5A** and **Figure 5—figure supplement 1A/B**). However, there was no clear correlation between the most affected proteins in the two genotypes, indicating that HUWE1 is likely not essential for proteasome function in cells, and that the increase of TTP in *Huwe1* knock-out cells is unlikely to have resulted from diminished overall proteasome activity. Among the differentially regulated

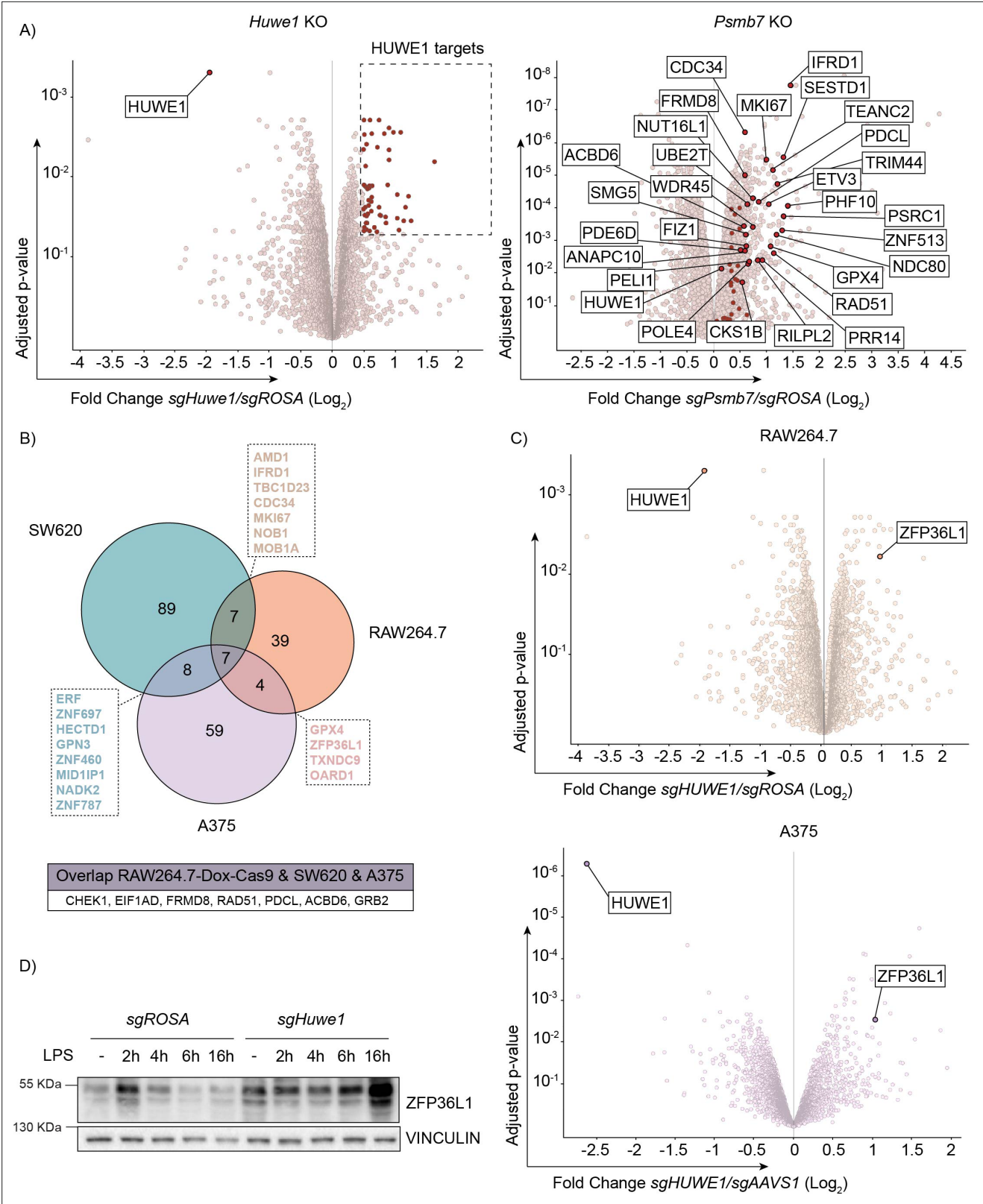


Figure 5. TTP family member ZFP36L1 abundance is increased upon *HUWE1* knockout in human and mouse cells. **(A)** RAW264.7-Dox-Cas9 expressing *sgROSA*, *sgHuwe1* or *sgPsmb7* were treated with Dox for 3 days to induce Cas9. Proteome changes were assessed by quantitative mass spectrometry. Proteins classified as HUWE1 targets are highlighted in red. Shared HUWE1 and proteasome targets are labelled in the *Psmb7* knock-out volcano plot. (adjusted p-value ≤0.05 and Fold Change (Log₂) ≥0.5; n=3 biological replicates). **(B)** Venn diagram showing the overlap between proteome changes of

Figure 5 continued on next page

Figure 5 continued

Huwe1-targeted RAW264.7, A375, and SW620 cell lines. Shared targets are listed (adjusted p-value ≤ 0.05 and Fold Change (Log_2) ≥ 0.5 ; $n=3$ biological replicates). (C) Volcano plots representing proteome changes of *Huwe1*- and AAVS1/ROSA-targeted A375 human melanoma cells and RAW264.7-Dox-Cas9 cells (adjusted p-value ≤ 0.05 and Fold Change (Log_2) ≥ 0.5 ; $n=3$ biological replicates). The shared HUWE1 target ZFP36L1 is highlighted. (D) sgROSA or sg*Huwe1* knockout RAW264.7-Dox-Cas9 cells were treated with Dox for 5 days to induce Cas9, followed by LPS treatment for the indicated times (h). Endogenous ZFP36L1 protein levels were determined by western blot.

The online version of this article includes the following source data and figure supplement(s) for figure 5:

Source data 1. Blots corresponding to **Figure 5D**.

Source data 2. Quantitative proteomics to systematically assess protein changes after HUWE1 knockout, in RAW264.7-Dox-Cas9, A375, and SW620 cell lines.

Figure supplement 1. Abundance of the TTP family member ZFP36L1 is increased upon *HUWE1* knockout in human and mouse.

proteins were factors previously identified as HUWE1 targets (Cassidy et al., 2020; Thompson et al., 2014; Xu et al., 2016), including GRB2, CHEK1, and CDC34 (Figure 5—figure supplement 1C).

Our data so far indicated that HUWE1 is important for proper regulation of TTP phosphorylation, and that in its absence the equilibrium shifted to a hyper-phosphorylated state (Figure 4), dependent on a decrease in phosphatase activity, and an increase in stress kinase activity, without major effects on their protein levels.

To further assess in an unbiased manner whether *Huwe1* deficiency would affect MAPK or PP1/2 protein levels, we extended our proteome mass-spectrometry for two additional human cell lines (A375 and SW620). Consistent with our previous findings (Figure 4), *Huwe1* ablation did not substantially or consistently affect the protein levels of detected MAPK or PP1/2 subunits in the three cell lines (Figure 5—figure supplement 1D/E). In line with the data presented above (Figure 4), this suggests that the hyper-phosphorylated TTP state in *Huwe1*-targeted cells does not result from changes in MAPK or PP1/A protein levels.

Previous studies have indicated that HUWE1 can target broader classes of cellular substrates (Grabarczyk et al., 2021; Hunkeler et al., 2021), but that the targeted proteins may be cell type specific to some degree. Analysis of proteome changes in the three different cell lines identified seven proteins that were consistently increased in all *Huwe1*-targeted cell lines (Figure 5B). Moreover, the protein concentration of other proteins was changed in only two of the three cell lines, whereas it was not detected in the third (Figure 5B).

We reasoned that any of these common deregulated proteins in *Huwe1* knock-out cells could contribute to the TTP hyper-phosphorylation/stabilization phenotype. However, analysis of overlap between factors that regulate TTP abundance identified in the genetic screen (Figure 2B and Figure 2—figure supplement 1A), and proteins deregulated by *Huwe1*-ablation did not identify any overlap, suggesting that these *Huwe1*-regulated proteins are unlikely to drive the effect on TTP protein stability.

Importantly, proteome measurements by mass-spectrometry are limited to detection of only reasonably abundant proteins. Even after LPS stimulation, no TTP peptides were identified in any of the three analyzed cell lines (Figure 5B), indicating that its absolute intra-cellular concentrations in these cells are too low to be detected by this method. In contrast, peptides of its paralog ZFP36L1 were readily identified in RAW264.7 and A375 cells and among the most increased proteins identified in *Huwe1*-targeted cells (Figure 5C). In line with this observation, independent western blot analysis of ZFP36L1 in cell lysates from *Huwe1*-deficient RAW264.7 cells showed that ZFP36L1 protein levels were increased in *Huwe1* knock-out cells (Figure 5D).

Collectively, these findings indicate that *Huwe1* ablation does not alter MAPK or PP1/2 protein levels, but that rather their differential activation alters TTP phosphorylation and stability. Moreover, ZFP36L1 abundance is regulated by HUWE1, akin to its closest related family member -TTP-, indicating that they could be regulated by HUWE1 in a conserved manner.

Residues in the TTP 234-278 region are important for its stability

Lysines are exclusively located in the TTP zinc finger domain, and our data indicate that this is the site of poly-ubiquitination (Figure 1—figure supplement 1C–G). In line with this notion, upon mutation of the five lysine residues in its zinc finger domain (KtoR), TTP accumulated as a stable, phosphorylated

species (**Figure 6A–B** and **Figure 6—figure supplement 1A–B**). Moreover, this mutant was no longer affected by *HUWE1* loss, indicating that the effects of *HUWE1* on TTP stability are dependent on ubiquitination in the zinc finger domain.

We reasoned that the E3 ligase ubiquitinating TTP at that site could bind TTP in its folded zinc finger domain, or that the mRNA-engaged TTP pool could be the predominant *HUWE1*-dependent target. Therefore, we addressed whether a TTP mutant with a disrupted zinc finger domain (C116R, C139R; *Lai et al., 2018; Ming et al., 2001*) would be stabilized. Zinc finger domain disruption did not accumulate at higher steady-state levels than its wtTTP counter-part, and was still increased upon *HUWE1* loss (**Figure 6B** and **Figure 6—figure supplement 1B**), demonstrating that neither recognition of the folded zinc finger domain structure by an E3 ligase, nor TTP functionality are required for the *HUWE1* effects.

Our data support a role of *HUWE1* in determining TTP phosphorylation, and thereby its stability. Therefore, we next analyzed whether phosphorylation of the two best-characterized TTP residues in this context (S52, S178) are important for *HUWE1* effects. As for the zinc finger domain mutant, a S52A/S178A TTP mutant was still stabilized by *HUWE1* loss (**Figure 6B** and **Figure 6—figure supplement 1B/C**). This indicated that while phosphorylation of these residues importantly controls TTP stability, *HUWE1* effects are likely independent of these phospho-residues.

Together, these data from full-length TTP point mutants suggest that an unknown E3 ligase likely binds TTP outside of its zinc finger domain, but ubiquitinates it on lysines inside the zinc finger domain. Moreover, we concluded that *HUWE1* regulation of TTP stability and phosphorylation is independent of the MK2-stabilized S52/S178 residues. This is consistent with the finding that TTP levels were still increased in *Huwe1* knock-out cells treated with inhibitors of the p38 and MK2 kinases phosphorylating these two sites (**Figure 4E**; subsets 4–5). Lastly, these data indicate that TTP does not require an intact zinc finger domain for stability, suggesting that TTP engagement with target mRNAs is likely not a prerequisite for *HUWE1*-dependent stability regulation.

Next, we set out to determine which part of TTP regulates its *HUWE1*-dependent phosphorylation and stability. To this end, progressive N- and C-terminal TTP deletion mutants (**Figure 6A**) were analyzed in cells for their steady-state concentrations, phosphorylation, and sensitivity to *HUWE1* ablation. Since TTP is predicted to be mostly disordered outside of its zinc finger domain (<https://alphafold.ebi.ac.uk/entry/P22893>), we reasoned that the effects of the truncations on overall protein structure would be limited.

N-terminal deletions did neither affect TTP protein levels, its phosphorylation, nor the effect of *HUWE1* loss (**Figure 6C** and **Figure 6—figure supplement 1D–E**; deletions Δ N1-4), indicating that *HUWE1* does not influence TTP stability through residues N-terminal of the zinc finger domain. Likewise, the two most C-terminal deletions did also not affect TTP stability (**Figure 6D** and **Figure 6—figure supplement 1F**; Δ C1-2). In contrast, further truncation of the C-terminus rendered mutant Δ C3 (259–278 region) less sensitive to *HUWE1* loss, yet retained its heterogeneous size distribution for phosphorylated species. Further deletion of the 234–258 region in the Δ C4 mutant strongly stabilized TTP at a homogeneously phosphorylated size, and rendered it insensitive to *HUWE1* knock-out (**Figure 6D** and **Figure 6—figure supplement 1F**). Likewise, the Δ C5 and Δ C6 mutants were insensitive to *HUWE1* knock-out, but accumulated as unphosphorylated TTP species. Together, these data indicate that the 234–278 region (**Figure 6D** and **Figure 6—figure supplement 1F**) is important for *HUWE1*-dependent regulation of TTP stability, and its phosphorylation status.

Since the TTP- Δ C3 mutant was stabilized in a *HUWE1*-insensitive manner (**Figure 6D** and **Figure 6—figure supplement 1F**), we reasoned that this region (259–278) could be important for proteasomal targeting (e.g. an E3 ligase binding site). In contrast, the TTP- Δ C4 mutant accumulated as a lower MW homogeneously phosphorylated TTP species (**Figure 6D** and **Figure 6—figure supplement 1F**), suggesting that the 234–258 region regulates TTP stability by affecting its phosphorylation status (e.g. a phosphatase binding site).

To test these possibilities, we analyzed mutants in which either only the 259–278 or 234–258 regions were deleted, while retaining the rest of the protein (**Figure 6A**). Consistent with the data from **Figure 6D**, a TTP mutant only lacking the 259–278 region was strongly stabilized, accumulated predominantly as a relatively homogeneous phosphorylated species, and was insensitive to *HUWE1* knock-out (**Figure 6E** and **Figure 6—figure supplement 1G**). Moreover, deletion of the 234–258

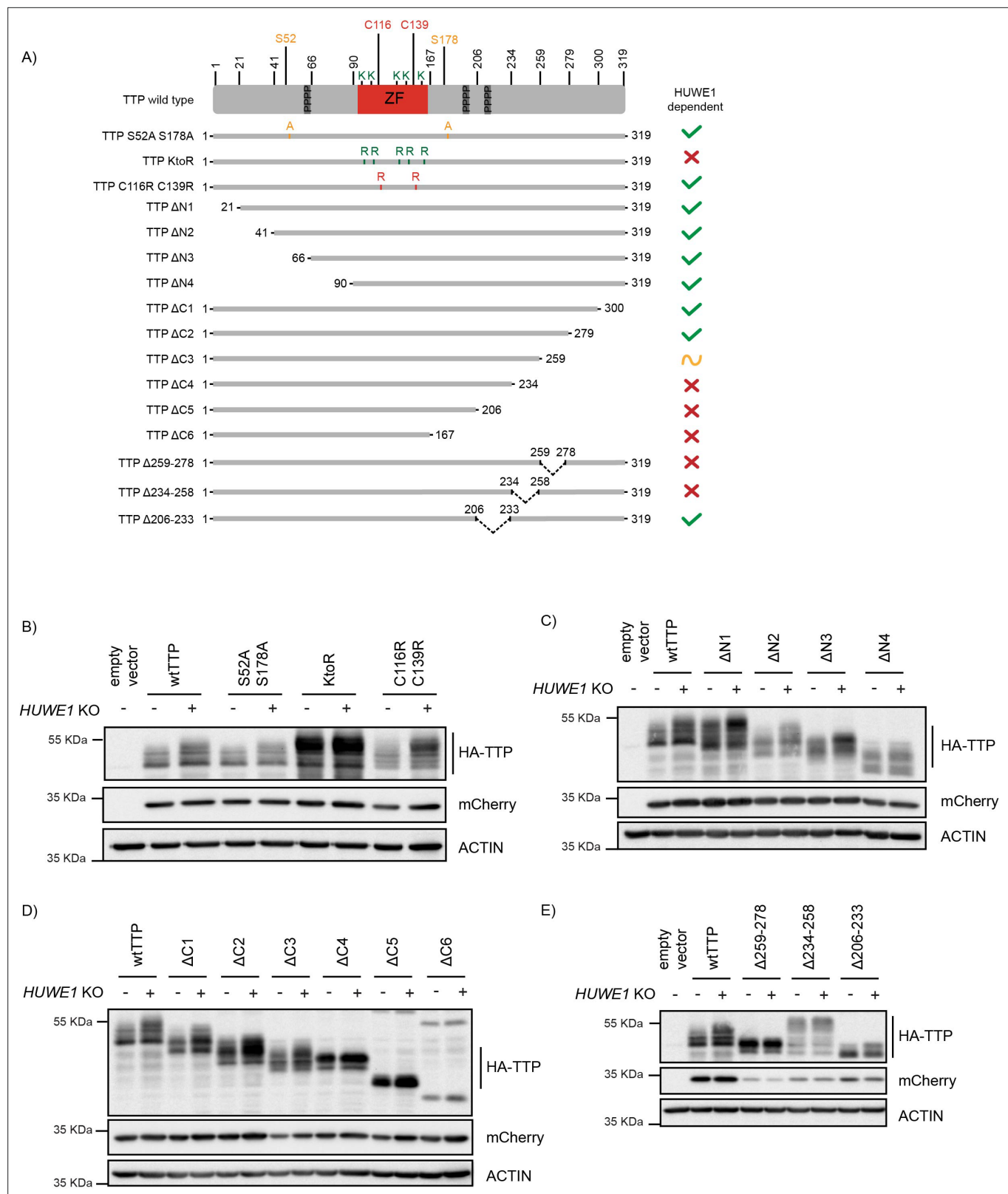


Figure 6. Residues in the TTP 234–278 region are important for its stability. (A) Schematic representation of 3xHA-TTP mutants. Colors denote amino acid substitutions. ZF indicates the zinc finger domain, and the three tetraprolin motifs are presented as dark grey boxes. (B–E) sgAAVS1- and sgHUWE1-depleted HEK-293T-Cas9 cells were transfected with the indicated mutants, and 3xHA-TTP stability was determined by western blot. mCherry is expressed as a stable internal control through a P2A site.

Figure 6 continued on next page

Figure 6 continued

The online version of this article includes the following source data and figure supplement(s) for figure 6:

Source data 1. Blots corresponding to **Figure 6B**.

Source data 2. Blots corresponding to **Figure 6C**.

Source data 3. Blots corresponding to **Figure 6D**.

Source data 4. Blots corresponding to **Figure 6E**.

Figure supplement 1. Residues in the TTP 234–278 region are important for its stability.

Figure supplement 1—source data 1. Blots corresponding to **Figure 6—figure supplement 1C**.

Figure supplement 1—source data 2. Blots corresponding to **Figure 6—figure supplement 1E**.

region resulted in TTP hyper-phosphorylation (**Figure 6E** and **Figure 6—figure supplement 1G**), consistent with the idea of it being important for phosphatase binding.

Together, these data indicate that the TTP Δ C3-specific region (259–278) is consistent with a possible binding site for an E3 ligase, whereas the Δ C4-specific region (234–258) is a likely interaction site of a phosphatase (**Figure 7A**). Importantly, the Δ C5- and Δ C6-mutants were stabilized, yet not hyperphosphorylated (**Figure 6B**). This suggests that the phosphorylated residues contributing to TTP stabilization in *HUWE1* knock-out cells are likely in the possible E3 ligase binding site in the TTP Δ C3-specific region (259–278) (**Figure 7A**).

In summary, we provide evidence for ubiquitin-dependent proteasomal degradation as a key regulatory mechanism for TTP protein abundance in cells. A genetic screen identified *HUWE1* as a strong regulator of TTP proteasomal turn-over. In the absence of *Huwe1*, TTP is heavily phosphorylated and stabilized, which is dependent on multiple ubiquitination sites in the TTP zinc finger domain, and phosphorylation in the 259–278 region. We propose that this region in its unphosphorylated form is also a likely binding site for an E3 ligase directing TTP ubiquitination and degradation (**Figure 7A**). Moreover, the adjacent 234–258 region is consistent with an interaction site for the main TTP phosphatases (PP1/2) (**Figure 7A**).

We propose a model in which *HUWE1* under physiological conditions curtails stress kinase activation, thereby limiting their stabilizing effects on TTP (**Figure 7B**). However, in the absence of *Huwe1*, the collective activity increase of these stress kinases results in TTP hyper-phosphorylation in the 259–278 region, increased TTP stability, and decreased pro-inflammatory output. Since we found that

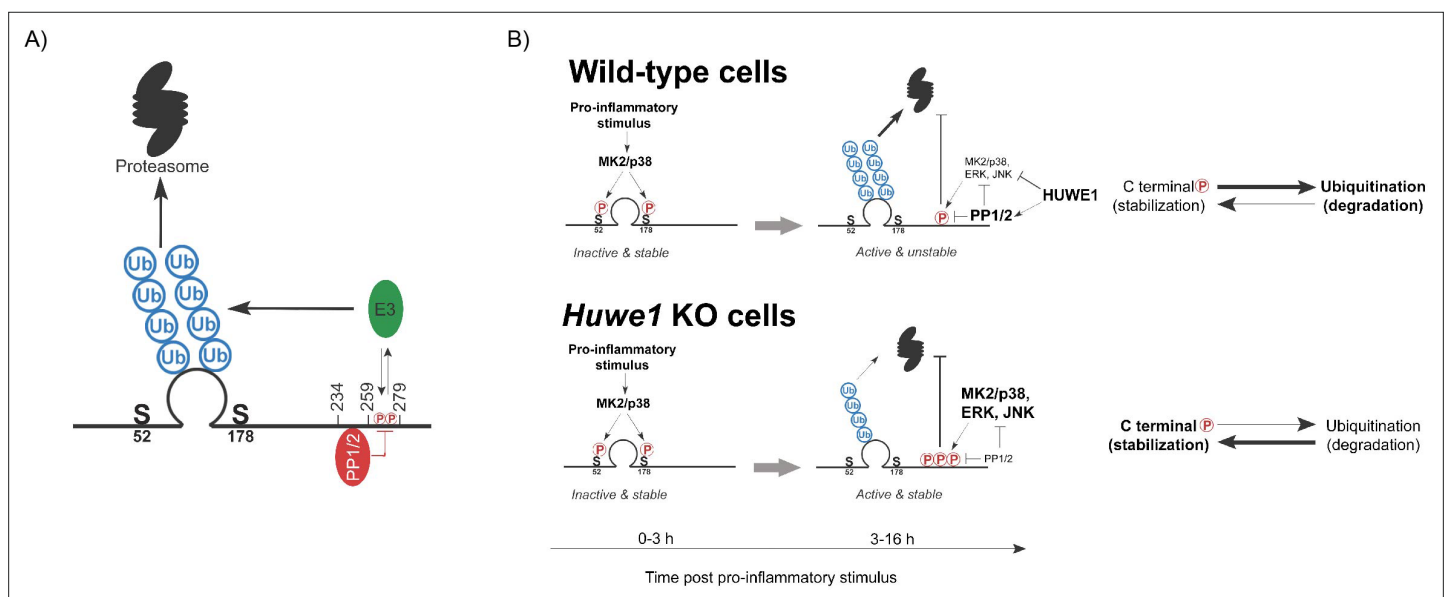


Figure 7. Model of *HUWE1*-dependent TTP regulation. **(A)** Model indicating the TTP regions in its C-terminus speculated to recruit PP1/2 and an unknown E3 ligase that ubiquitinates the zinc finger domain. **(B)** Model of TTP stability regulation through phosphorylation in wild-type cells and *Huwe1*-deficient cells.

TTP phosphorylation is inversely correlated with ubiquitination and degradation, we speculate that phosphorylation in this region could prevent E3 ligase binding (**Figure 7A**).

Discussion

Previous studies have predicted that TTP is disordered outside of its zinc finger domain, and showed that these unstructured regions contribute to its rapid proteasomal turn-over (**Ngoc et al., 2014; Ross et al., 2015**). Protein disorder is often associated with proteasomal turn-over, as these regions often contain degrons, accessible ubiquitination sites, or provide an initiation side for threading into the proteasome and initiating unfolding and translocation into the catalytic chamber (**Aufderheide et al., 2015; van der Lee et al., 2014**). However, previous work did not identify TTP poly-ubiquitination, and showed that incubation of TTP with purified 20S proteasomes -which lack the Ub-receptor containing 19 S regulatory particle-, were sufficient to degrade TTP (**Ngoc et al., 2014**).

Work from Ngoc et al. showed that fusion to GFP of either the N-terminal TTP part, or the TTP C-terminal part (aa 214–436), destabilized GFP in cells (**Ngoc et al., 2014**). Thus, the GFP destabilization was seemingly indiscriminate, and possibly caused by the disordered nature of the fusion construct per se. Since the C-terminal TTP part fused to GFP included aa 214–436, we cannot rule out that part of this effect was HUWE1-dependent. However, the discrepancy with our finding that the TTP N-terminus does not contribute to HUWE1-dependent TTP regulation, may suggest that the GFP fusions by Ngoc et al. were destabilized by more general protein principles, rather than HUWE1-specific effects.

It has been reported that oxidized, unfolded proteins could be directly degraded by 20S proteasomes (**Davies, 2001; Inai and Nishikimi, 2002**). However, the prevailing notion is that association of a regulatory particle is critical to open access to the catalytic chamber (**Coux et al., 1996; Davies, 2001; Driscoll and Goldberg, 1990; Eytan et al., 1989**), and efficient substrate degradation in cells. Here, we demonstrate robust poly-ubiquitination of TTP in denaturing RAW264.7 lysates, indicating that these poly-ubiquitin chains are covalently attached to TTP, and do not interact through a putative ubiquitin-interaction domain. Moreover, we show that mutation of lysines in the zinc finger domain stabilized TTP, and that an inhibitor preventing de novo ubiquitination in cells, stabilized TTP. These data demonstrate that poly-ubiquitination of TTP is essential for its degradation, which is likely mediated by 26S proteasomes.

In contrast to other published work (**Ngoc et al., 2014**), non-degradative TRAF2-driven K63-linked poly-ubiquitination of TTP has been previously reported to mediate the balance between NF κ B and JNK-dependent signaling using transfected HEK-293T cells (**Schichl et al., 2011**). Consistent with TTP being rapidly proteasomally turned-over, we readily detected K48-linked poly-ubiquitination of TTP in the same cell system, yet failed to detect substantial K63-linked poly-ubiquitination. This dissimilarity could stem from differences in expression levels, lack of co-expression of TRAF2, or insufficient sensitivity of the K63-specific antibody in our assays. As TNF and TLR activation activate NF κ B and stress kinases -including JNK- in parallel, it will be of interest to further dissect in future studies the interplay between HUWE1-dependent phospho-regulation of TTP, and its K63-ubiquitination in stimulated myeloid cells.

Most proteins with disordered regions will eventually be degraded in *in vitro* reactions containing high concentrations of 20S proteasomes (**Liu et al., 2003; Lu et al., 2015**). This could explain the previous finding of ubiquitin-independent TTP degradation *in vitro* (**Ngoc et al., 2014**). Future comparisons of TTP degradation kinetics of ubiquitinated and non-ubiquitinated forms in the presence of 26S proteasomes will be important to further address this issue.

Several E3 ligases to putatively poly-ubiquitinate TTP were identified in our genetic screen (**Figure 2**): HUWE1, VHL, UBE3C, and the Cullin adapters Elongin B/C. *Huwe1* ablation most robustly stabilized TTP, based on which we hypothesized that HUWE1 may directly poly-ubiquitinate TTP. However, multiple independent techniques, including TurboID proximity labeling and co-IPs, failed to identify an interaction between TTP and HUWE1, which suggested that it may instead indirectly influence TTP stability.

At this point, we cannot rule out that HUWE1 directly poly-ubiquitinates TTP, resulting in its proteasomal degradation. Alternatively, one or more of the other identified E3 ligases could contribute to direct TTP ubiquitination. If indeed the other E3 ligases contribute to TTP ubiquitination, the fact that their knock-out phenotype is substantially less than that of *Huwe1* loss (**Figure 2—figure supplement**

1B) may suggest that multiple of them could be functionally redundant. Irrespective of potential direct TTP ubiquitination by HUWE1, multiple lines of evidence point towards a strong contribution of HUWE1-dependent differential TTP phosphorylation as an indirect means to control TTP stability and functional activity. This is consistent with our finding that TTP phosphorylation and ubiquitination appear to be inversely correlated, as the TTP KtoR mutant accumulates as a hyper-phosphorylated species (**Figure 6B**).

Published data support a model in which TTP upon translation is initially phosphorylated by the p38/MK2 kinase axis on residues S52 and S178 (mTTP numbering), resulting in its stabilization, yet repressing TTP function (**Deleault et al., 2008; Hitti et al., 2006; Kratochvill et al., 2011; Ross et al., 2015**). At later stages, diminishing p38/MK2 kinase activity is thought to shift the equilibrium to dephosphorylation of TTP at these residues, rendering it active, but unstable. Under these conditions, TTP is rapidly turned over by the proteasome (**Deleault et al., 2008; Ross et al., 2015**).

Consistent with these data, we also found that an S52A/S178A mutant is unstable in the absence and presence of LPS stimulation (**Figure 6B**, and **Figure 6—figure supplement 1C**). Importantly, this mutant was still stabilized in the absence of *Huwe1*, indicating that the stabilizing effects of phosphorylation on S52/S178 are independent of HUWE1. Moreover, it indicates that HUWE1-dependent effects on TTP stability target other sites in TTP. Thus, S52/S178 phosphorylation seems predominantly relevant for TTP stabilization at the early (2–3 hr) time points post-LPS-stimulation (**Ross et al., 2015**), whereas HUWE1-dependent effects occur later (between 3–16 hr) post-LPS stimulation. In contrast to S52/178, TTP phosphorylation in its C-terminal 259–278 region and its associated stabilization in the absence of *Huwe1*, is paralleled by decreased concentrations of known TTP mRNA targets, which suggests that phosphorylation in this region may not inhibit TTP functional output.

To uncouple LPS-induced *Zfp36* transcription from PTMs influencing TTP protein stability, we complemented experiments using endogenous TTP with exogenously expressed counterparts. In the absence of LPS stimulation, *Huwe1* loss did increase exogenous TTP, albeit rather mildly (**Figure 2C** and **Figure 2—figure supplement 1B**). In contrast, upon LPS stimulation and stress kinase activation, the effect of *Huwe1* knock-out was much stronger, resulting in strong TTP protein accumulation, which included a substantial fraction of phosphorylated forms (**Figure 2C and F**, and **Figure 2—figure supplement 1B**). These results suggest that there may be low baseline levels of activated stress kinases in the cell that affect TTP stability in the absence of LPS. However, in contrast to these mild phenotypes, the predominant effects of *Huwe1* loss on TTP protein stability and phosphorylation occur after LPS stimulation, in line with the notion that HUWE1 regulates TTP stability through influencing stress kinase-dependent TTP phosphorylation (**Figures 5 and 6**).

Huwe1 loss increased the activity of multiple stress kinases (**Figure 4A–D**) without affecting their total protein levels. The previous findings that (i) the phosphorylation and activation of these kinases is controlled by PP2A (**Kruse et al., 2020; Nguyen and Shiozaki, 1999; Takekawa et al., 1998**), and (ii) the observation that combined inhibition of the stress kinases, or inhibition of PP1/2 activity with Calyculin A, rescued *Huwe1* knock-out effects on TTP (**Figure 4E–F**), suggest that HUWE1 may be important for PP1/2 activity. In line with this notion, decreased PP1/2 activity in *Huwe1* knock-out cells could affect TTP phosphorylation and stability by directly affecting its dephosphorylation, and in parallel maintain high stress kinase activity, which increases TTP phosphorylation even more.

This functional interaction between HUWE1 and the activity of PP1/2 and stress kinases has not been described previously, although it should be noted that HUWE1 has been reported to control the abundance and activity of other kinases and phosphatases (**Cassidy et al., 2020; Jang et al., 2014; Su et al., 2021**). Our findings broaden the understanding of how HUWE1 may indirectly influence numerous cellular proteins beyond direct recognition as ubiquitination substrates.

Taken together, our data support a model in which HUWE1 is important to maintain PP1/2 cellular output, and curtail stress kinase activation. This in turn limits phosphorylation in the TTP region spanning residues 259–278, allowing for recruitment of HUWE1 itself or another -yet unidentified- E3 ligase to that same region, subsequent poly-ubiquitination on lysine residues in the zinc finger domain, and ultimately proteasomal degradation. Phosphorylation in this region could prevent E3 ligase binding. Although the HUWE1-dependent phosphorylation effect appears to be dependent on the putative E3 ligase binding site (259–278), phosphatase recruitment to the 234–258 region in TTP seemingly controls dephosphorylation of most or all phospho-sites on TTP (**Figure 6C**). Since ZFP36L1 abundance is also strongly regulated by HUWE1, this suggests that its C-terminal region is orthologous to

TTP 234–278 and how it controls HUWE1-dependent degradation could be conserved across these family members.

Materials and methods

Vectors

The lentiviral mouse genome-wide sgRNA library (six sgRNAs/gene) has been described previously (*Michlits et al., 2020*). Lentiviral vectors driving the expression of a single sgRNA or a dual sgRNA from a U6 promoter, and either eBFP2 or iRFP from a PGK promoter have been described previously (*de Almeida et al., 2021*). Single sgRNA CDSs were cloned in pLentiCRISPRv2 (Addgene plasmid 52961) to perform stable knock-outs in HEK293T cells. A Dox-inducible Cas9 lentiviral vector was modified from LT3GEPiR (Addgene plasmid 111177): T3G-GFP-(miR-E)-PGK-Puro-IRES-rtTA3, in which the GFP-miR-E cassette was replaced by Cas9-P2A-GFP from pLentiCRISPRv2. The TTP stability reporter (pLX-SFFV-mCherry-TTP-P2A-BFP) was constructed by cloning the open-reading frame (ORF) of murine TTP into a modified pLX303 vector (Addgene plasmid 25897). Lentiviral N-terminally HA-tagged-TTP deletions or point mutant variants were obtained by cloning the indicated variants of murine TTP ORF into a modified pLX303 vector. For this purpose, cDNAs encoding mTTP mutants were purchased from Twist Bioscience. All 3xHA-TTP constructs co-expressed mCherry through a P2A site to monitor protein expression and protein stability. All plasmids and sgRNAs used in this study are listed in **Table 1** and **Table 2**.

Cell culture and reagents

All experiments in this study have been reproduced at least twice in independent experiments. Cell lines were tested negative for mycoplasma contamination. All cell lines used in this study and their applications are listed in **Table 3**. None of the used cell lines are on the current list of commonly misidentified cell lines (v12). Parental cell lines were obtained from ATCC: A375 (CRL-1619), RKO (CRL-2577), SW620 (CCL-227), and authenticated by short tandem repeat analysis. These cell lines were used to generate dox-inducible Cas9 derivatives as indicated in **Table 3**. RAW264.7 Dox-Cas9 cells were generated by transducing RAW264.7 cells with pRRL-TRE3G-Cas9-P2A-GFP-PGK-IRES-rtTA3 lentiviral vector. Cas9 expression was induced with 500 ng/ml of Doxycycline hyclate (Dox, Sigma-Aldrich, D9891) and single cells were sorted by FACS into 96-well plates using a FACSAria III cell sorter (BD Biosciences) to obtain single-cell-derived clones. Cas9 function and leakiness of the TRE3G promoter in the absence of Dox was tested in competitive proliferation assays. For mCherry-TTP reporter cells, pLX303-SFFV-mCherry-TTP-P2A-BFP was transduced into RAW264.7-Dox-Cas9 cells, and cells co-expressing mCherry and BFP were sorted by FACSAria III cell sorter into 96-well plates to obtain single-cell-derived clones. To obtain 3xHA-TTP expressing cells, pLX303-SFFV-mCherry-P2A-3xHA-TTP was transduced into RAW264.7-Dox-Cas9 cells, and cells expressing mCherry were bulk sorted using a FACSAria III. Bone marrow-derived macrophages (BMDMs) were differentiated from bone marrow isolated from femurs and tibias of 8-to-12-week-old mice from Cas9 knock-in mice of both sexes (*Platt et al., 2014*). Femur and tibia marrow was centrifuged and cells were resuspended in DMEM. Cells were differentiated in DMEM (Sigma-Aldrich, D6429) containing recombinant M-CSF for 10 days. All cells were cultured at 37 °C and 5% CO₂ in a humidified incubator. All animals were maintained in the pathogen-free animal facility of the Research Institute of Molecular Pathology, and all procedures were carried out according to an ethical animal license that is approved and regularly controlled by the Austrian Veterinary Authorities (License Number: GZ: 516079/2017/14). All reagents used in this study are listed in the **Table 4**. All antibodies used in this study are listed in **Table 5**.

Transfections

All transfections were performed by mixing DNA and polyethylenimine (PEI, Polysciences, 23966) in a 1:3 ratio (μg DNA/μg PEI) in DMEM (Sigma-Aldrich, D6429) without supplements. Transfection was performed using 500 ng of total DNA. The day before transfection, 2 × 10⁵ HEK293T cells were seeded in six-well clusters in fully supplemented media. Cells were harvested 48 hr after transfection, washed with ice cold PBS and stored at –80 °C until further processing.

Western blot

Cells were lysed in Frackelton lysis buffer (10 mM Tris-HCl pH 7.4, 50 mM NaCl, 30 mM Na₂P₂O₇, 50 mM NaF, 2 mM EDTA, 1% Triton X-100, 1 mM DTT, 0.1 mM PMSF, and 1 X protease inhibitor

Table 1. Vectors.

Plasmid	Purpose	Reference or source
pRRL-TRE3G-Cas9-P2A-GFP-PGK-IRES-rtTA3	Dox inducible Cas9	Johannes Zuber, IMP
pLX303-mCherry.TTP-P2A-BFP	TTP reporter	This study
pLX303-MYC-mCherry-P2A-3xHA.TTP	TTP reporter	This study
pLX303-MYC-mCherry-P2A-3xHA.TTP ΔC1	TTP reporter	This study
pLX303-MYC-mCherry-P2A-3xHA.TTP ΔC2	TTP reporter	This study
pLX303-MYC-mCherry-P2A-3xHA.TTP ΔC3	TTP reporter	This study
pLX303-MYC-mCherry-P2A-3xHA.TTP ΔC4	TTP reporter	This study
pLX303-MYC-mCherry-P2A-3xHA.TTP ΔC5	TTP reporter	This study
pLX303-MYC-mCherry-P2A-3xHA.TTP ΔC6	TTP reporter	This study
pLX303-MYC-mCherry-P2A-3xHA.TTP ΔN1	TTP reporter	This study
pLX303-MYC-mCherry-P2A-3xHA.TTP ΔN2	TTP reporter	This study
pLX303-MYC-mCherry-P2A-3xHA.TTP ΔN3	TTP reporter	This study
pLX303-MYC-mCherry-P2A-3xHA.TTP ΔN4	TTP reporter	This study
pLX303-MYC-mCherry-P2A-3xHA.TTP Δ259–278	TTP reporter	This study
pLX303-MYC-mCherry-P2A-3xHA.TTP ΔC234-258	TTP reporter	This study
pLX303-MYC-mCherry-P2A-3xHA.TTP Δ206–233	TTP reporter	This study
pLX303-MYC-mCherry-P2A-3xHA.TTP KtoR	TTP reporter	This study
pLX303-MYC-mCherry-P2A-3xHA.TTP S52A S178A	TTP reporter	This study
pLX303-MYC-mCherry-P2A-3xHA.TTP ZNF C116R C139R	TTP reporter	This study
pLX303-MYC-mCherry-P2A-3xHA.TTP K97R	TTP reporter	This study
pLX303-MYC-mCherry-P2A-3xHA.TTP K115R	TTP reporter	This study
pLX303-MYC-mCherry-P2A-3xHA.TTP K133R	TTP reporter	This study
pLX303-MYC-mCherry-P2A-3xHA.TTP K135R	TTP reporter	This study
pLX303-MYC-mCherry-P2A-3xHA.TTP K141R	TTP reporter	This study
pLX303-MYC-mCherry-P2A-3xHA.TTP K97R/K115R	TTP reporter	This study
pLX303-MYC-mCherry-P2A-3xHA.TTP K97R/K115R/K133R	TTP reporter	This study
pLX303-MYC-mCherry-P2A-3xHA.TTP K97R/K115R/K133R/K135R	TTP reporter	This study
pLX303-MYC-mCherry-P2A-3xHA.TTP K135R/K141R	TTP reporter	This study
pLX303-MYC-mCherry-P2A-3xHA.TTP K133R/K135R/K141R	TTP reporter	This study
pLX303-MYC-mCherry-P2A-3xHA.TTP K115R/K133R/K135R/K141R	TTP reporter	This study
CMV-Flag-TTP	TTP reporter	Pavel Kovarik, Max Perutz Labs
DualCRISPR-hU6-sgRNA-mU6-sgRNA-EF1as-BFP	Dual sgRNA	de Almeida M, Hinterndorfer M et al, 2021
pLentiv2-U6-PGK-iRFP670-P2A-Neo	Single sgRNA	de Almeida M, Hinterndorfer M et al, 2022
pLentiv2-U6-PGK-BFP-P2A-Neo	Single sgRNA	de Almeida M, Hinterndorfer M et al, 2023
PRRL-PBS-U6-sgRNA-EF1as-Thy1-P2A-NeoR (sgETN)	Library sgRNA	Johannes Zuber, IMP

cocktail). Cells were incubated for 5 min on ice and then centrifuged at 18,500 x g for 10 min at 4 °C. The supernatant was transferred to a new tube and protein concentration was determined using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, 23225). Between 20 and 40 micrograms of protein were mixed with Laemmli sample buffer supplemented with β-mercaptoethanol and boiled

Table 2. sgRNA coding sequences.

Gene	Species	Sequence (5' to 3')
<i>ROSA_1</i> (RAW264.7 & BMDMs)	mouse	AGATGGGCGGGAGTCTTC
<i>ROSA_2</i> (RAW264.7 & BMDMs)	mouse	TTTAGATGGGCGGGAGTCTTCGTTTA
<i>Huwe1_1</i> (RAW264.7 & BMDMs)	mouse	GATTTGCTGCAGTTCCAAG
<i>Huwe1_2</i> (RAW264.7 & BMDMs)	mouse	ATAAAATTCAAAGTGTAGTG
<i>Psmb7_1</i> (RAW264.7 & BMDMs)	mouse	GCTGTAACAACCTCTCGGG
<i>Psmb7_2</i> (RAW264.7 & BMDMs)	mouse	GAAAACTGGCACTACCATCG
<i>Vcpip1</i> (RAW264.7 & BMDMs)	mouse	GACGTGCTCTGGTTCGATG
<i>Ppil4</i> (RAW264.7 & BMDMs)	mouse	GTGTTTGGTGAAAGTGACAGA
<i>Tceb1</i> (RAW264.7 & BMDMs)	mouse	GCTGAGAATGAAACCAACG
<i>Ube3c</i> (RAW264.7 & BMDMs)	mouse	GAGAGTCAAAGTTCAAAA
<i>Ddx23</i> (RAW264.7 & BMDMs)	mouse	GGATGGAGCGGGAGACCAA
<i>Cnot10</i> (RAW264.7 & BMDMs)	mouse	GATTTACAGGGTAGCGG
<i>Ttp</i> (RAW264.7 & BMDMs)	mouse	GAAGCGGGCGTTGTCGCTACG
<i>AAVS1_1</i> (RKO & HEK-293T)	human	CTGTGCCCCGATGCACAC
<i>AAVS1_2</i> (RKO & HEK-293T)	human	GCTGTGCCCCGATGCACAC
<i>HUWE1_1</i> (RKO & HEK-293T)	human	GTGCGAGTTATATCACTGGG
<i>HUWE1_2</i> (RKO & HEK-293T)	human	GTGCGAGTTATATCACTGGGTGG
<i>AAVS1_3</i> (A375 & SW620)	human	GCTGTGCCCCGATGCACAC
<i>AAVS1_4</i> (A375 & SW620)	human	GCTTGGCAAACCTCACTCTT
<i>HUWE1_3</i> (A375 & SW620)	human	GTGCGAGTTATATCACTGGG
<i>HUWE1_4</i> (A375 & SW620)	human	GACAGTGGAATATGTCA

for 10 min. Proteins were loaded on SDS polyacrylamide gels. The percentage of the gel was chosen based on the MW of the proteins of interest. Proteins were blotted on a PVDF or on a nitrocellulose membrane at 4 °C for 16 hr at 200mA and then for 2 hr at 400mA in Towbin buffer (25 mM Tris-HCl pH 8.3, 192 mM glycine, and 20% ethanol) or in carbonate transfer buffer (3 mM Na₂CO₃, 10 mM NaHCO₃, and 20% ethanol). The membrane was blocked in 5% BSA in PBS-T for 1 hr at room temperature and then incubated with the primary antibody overnight at 4 °C while shaking. The next day, the membrane was washed three times with PBS-T and incubated with HRP-coupled secondary antibody for 1 hr at room temperature and imaged with the ChemiDoc Imaging System from Bio-Rad. Relative protein levels were quantified using Image Lab (BioRad).

Immunoprecipitation

Cells were lysed in 1 ml of RIPA lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% SDS, 0.5% Sodium deoxycholate, 1% Triton X-100) supplemented with 40 mM NEM, 40 mM iodoacetamide, 25 U/ml Benzonase, 0.1 mM PMSF, and 1 X protease inhibitor. Cells were incubated on a rotating wheel at 4 °C for 30 min, and centrifuged at 20,000 x g at 4 °C for 30 min. The supernatant was transferred to a new tube and 50 µl (20% of the lysate used for the IP) were collected as input. A total of 500 µg of lysates were incubated overnight at 4 °C on a rotating wheel with an IgG Isotype control (Cell Signaling Technology, 1:300), anti-HA antibody (Cell Signaling Technology, 1:100), or anti-TTP antibody (Cell Signaling Technology, 1:100). The next day, magnetic beads (Pierce Protein A/G Magnetic Beads, Thermo Fisher Scientific, 88803) were blocked by rotation in 3% BSA in RIPA Buffer for 1 hr at 4 °C. Twenty-five µl of beads were added to 500 micrograms of lysates and rotated for 2 hr at 4 °C. Then, the beads were washed five times with 1 ml of RIPA buffer supplemented with

Table 3. Cells and culture conditions.

Cell lines and primary cells	Type	Reference or source	Purpose	Media	Supplements
RAW264.7	Murine macrophages	ATCC TIB-71	parental cell line	Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, D6429)	10% FBS (Sigma-Aldrich, F7524) and 1% penicillin/streptomycin (Sigma-Aldrich, P4333)
RAW264.7-Dox-Cas9	Murine macrophages	This study	Dox-inducible Cas9	Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, D6429)	10% FBS (Sigma-Aldrich, F7524) and 1% penicillin/streptomycin (Sigma-Aldrich, P4333)
RAW264.7-Dox-Cas9 mCherry-TTP-P2A-BFP	Murine macrophages	This study	mCherry-TTP reporter	Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, D6429)	10% FBS (Sigma-Aldrich, F7524) and 1% penicillin/streptomycin (Sigma-Aldrich, P4333)
RAW264.7-Dox-Cas9 mCherry-IkBα-P2A-BFP	Murine macrophages	This study	mCherry-IkBα reporter	Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, D6429)	10% FBS (Sigma-Aldrich, F7524) and 1% penicillin/streptomycin (Sigma-Aldrich, P4333)
RAW264.7-Dox-Cas9 3xHA-TTP	Murine macrophages	This study	3xHA-TTP reporter	Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, D6429)	10% FBS (Sigma-Aldrich, F7524) and 1% penicillin/streptomycin (Sigma-Aldrich, P4333)
Bone Marrow Derived Macrophages, BMDMs	Murine macrophages	This study	constitutive Cas9 expression	Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, D6429)	10% FBS (Sigma-Aldrich, F7524) and 1% penicillin/streptomycin (Sigma-Aldrich, P4333)
HEK293T	Human kidney neural tissue	CRL-3216	3xHA-TTP mutants	Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, D6429)	10% FBS (Sigma-Aldrich, F7524) and 1% penicillin/streptomycin (Sigma-Aldrich, P4333)
Lenti-X 293T	Human kidney neural tissue	Takara, Cat# 632180	VLP production	Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, D6429)	10% FBS (Sigma-Aldrich, F7524) and 1% penicillin/streptomycin (Sigma-Aldrich, P4333)
RKO	human colon carcinoma	de Almeida M, Hinterndorfer M et al, 2021	Dox-inducible Cas9	RPMI 1640 (Thermo Fisher Scientific, 21875)	10% FBS (Sigma-Aldrich, F7524), L-glutamine (4 mM, Gibco), sodium pyruvate (1 mM, Sigma-Aldrich), and 1% penicillin/streptomycin (Sigma-Aldrich, P4333)

Table 3 continued on next page

Table 3 continued

Cell lines and primary cells	Type	Reference or source	Purpose	Media	Supplements
A375	human melanoma	This study	Dox-inducible Cas9	Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, D6429)	10% FBS (Sigma-Aldrich, F7524), L-glutamine (4 mM, Gibco) and 1% penicillin/streptomycin (Sigma-Aldrich, P4333)
SW620	human colon carcinoma	This study	Dox-inducible Cas9	Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, D6429)	10% FBS (Sigma-Aldrich, F7524), L-glutamine (4 mM, Gibco) and 1% penicillin/streptomycin (Sigma-Aldrich, P4333)

Table 4. reagents.

Description	Abbreviation	Application	Dilution/ concentration	Manufacturer	Catalogue number
Lipopolysaccharides from <i>Escherichia coli</i> O55:B5	LPS	Cell culture	10 ng/ml	Sigma-Aldrich	L2637
Cycloheximide	CHX	Cell culture	40 µg/m	Sigma-Aldrich	C1988
MG132	MG132	Cell culture	10 µM	Sigma-Aldrich	M7449
Epoxomicin	EPX	Cell culture	10 µM	Gentaur Molecular Products	607-A2606
TAK-243		Cell culture	0.5 µM	ChemScence	CS-0019384
Doxycycline hyclate	DOX	Cell culture	500 ng/ml	Sigma-Aldrich	D9891
G418 disulfate salt	G418	Cell culture	0.5–1 mg/ml	Sigma-Aldrich	A1720
PH-797804, p38 inhibitor	p38i	Cell culture	1 µM	Selleckchem	S2726
JNK Inhibitor II, JNK inhibitor	JNKi	Cell culture	20 µM	Sigma-Aldrich	420119
PF-3644022, MK2 inhibitor	MK2i	Cell culture	10 µM	Sigma-Aldrich	PZ0188
U0126, MEKi inhibitor	ERKi	Cell culture	250 nM	Cell Signaling Technology	9903
Okadaic Acid	OA	Cell culture	1 µM	Cell Signaling Technology	5934
Calyculin A	CalycA	Cell culture	50 nM	Cell Signaling Technology	9902
Etoposide		Cell culture	5 µM	Sigma-Aldrich	E1383
Brefeldin A		Cell culture	10 ug/ml	Sigma-Aldrich	B7651

Table 5. antibodies.

Target	Application	Dilution	Conjugate	Manufacturer	Catalogue number	Name	Type
TTP	Western blot	1:1000		Cell Signaling Technology	71632	D113T	Primary
Myc Tag	Western blot	1:5000		Sigma-Aldrich	05–724	4A6	Primary
HA tag	Western blot	1:1000		Cell Signaling Technology	3724	C29F4	Primary
HECTH9	Western blot	1:1000		Cell Signaling Technology	5695	AX8D1	Primary
Lasu1/Ureb1	Western blot	1:1000		Bethyl	A300-486A		Primary
Vinculin	Western blot	1:1000		Sigma-Aldrich	V9131	V9131	Primary
phospho-p38 MAPK, Thr180/Tyr182	Western blot	1:1000		Cell Signaling Technology	9211		Primary
p38 MAPK	Western blot	1:1000		Cell Signaling Technology	9212		Primary
phospho-SAPK/JNK, Thr183/Tyr185	Western blot	1:1000		Cell Signaling Technology	9251		Primary
SAPK/JNK	Western blot	1:1000		Cell Signaling Technology	9252		Primary
phospho-p44/42 MAPK (Erk1/2), Thr202/Tyr204	Western blot	1:1000		Cell Signaling Technology	9101		Primary
p44/42 MAPK (Erk1/2)	Western blot	1:1000		Cell Signaling Technology	4695	137F5	Primary
p-MK2 (Thr334)	Western blot	1:1000		Cell Signaling Technology	3007	27B7	Primary
MK2	Western blot	1:1000		Cell Signaling Technology	3042		Primary
p-p53, Ser15	Western blot	1:1000		Cell Signaling Technology	9284		Primary
p-p53	Western blot	1:1000		Cell Signaling Technology	2524	1C12	Primary
ZFP36L1/2	Western blot	1:1000		Proteintech	12306–1-AP	12306–1-AP	Primary
Ubiquitin	Western blot	1:1000		Santa Cruz Biotechnology	sc-8017	P4D1	Primary
HRP- β -actin	Western blot	1:20000	HRP	Abcam	ab49900	AC-15	Primary
HRP anti-rabbit IgG	Western blot	1:3500	HRP	Cell Signaling Technology	7074		Secondary
HRP anti-mouse IgG	Western blot	1:3500	HRP	Cell Signaling Technology	7076		Secondary
TTP	FACS	1:100		Cell Signaling Technology	71632	D113T	Primary
HECTH9	FACS	1:100		Cell Signaling Technology	5695	AX8D1	Primary
TNF alpha	FACS	1:100	APC	eBioscience	17-7321-82	MP6-XT22	Primary
Rat IgG1 kappa Isotype Control	FACS	1:500	APC	eBioscience	17-4301-82	eBRG1	Primary
APC anti-CD90.1/Thy1.1	FACS	1:500	APC	BioLegend	202526		Secondary
Alexa Fluor Plus 594 anti-Mouse IgG	FACS	1:500	Alexa Fluor 594	Thermo Fisher Scientific	A-21201		Secondary

Table 5 continued on next page

Table 5 continued

Target	Application	Dilution	Conjugate	Manufacturer	Catalogue number	Name	Type
Alexa Fluor Plus 680 anti-Rabbit IgG	FACS	1:500	Alexa Fluor 680	Thermo Fisher Scientific	A-21076		Secondary
APC anti-F4/80	FACS	1:100	APC	Thermo Fisher Scientific	17-4801-82	BM8	Secondary
TruStain FcX mouse Fc Receptor CD16/32	FACS	1:100		BioLegend	101319		
IgG Isotype Control	IP	1:300		Cell Signaling Technology	2729		Primary
TTP	IP	1:100		Cell Signaling Technology	71632	D1I3T	Primary
HA tag	IP	1:100		Cell Signaling Technology	3724	C29F4	Primary

300 mM NaCl, and proteins were eluted in 2 X disruption buffer (2.1 M Urea, 667 mM β-mercaptoethanol and 1.4% SDS).

Protein half-life determination

To estimate HA-TTP protein half-life, RAW264.7 Dox-Cas9 cells expressing sgHuwe1 or sgROSA were treated with Dox for 5 days before translational elongation was inhibited using 40 μg/ml of cycloheximide (CHX, Sigma-Aldrich, C1988). At indicated time points, whole cell lysates were prepared, analysed by western blot, quantified, and normalized to ACTIN levels and to time point 0 as indicated. Single exponential decay curves were determined using GraphPad Prism (v9), from which protein half-lives were calculated.

RNA isolation, cDNA synthesis, and qPCR

Total RNA was extracted from mouse bone-marrow-derived macrophages and RAW264.7 Dox-Cas9 cells harboring non-targeting ROSA or Huwe1-targeting sgRNAs. 0.5×10⁶ cells were lysed using Trizol reagent (Thermo-Fisher Scientific, 5596–018) and total RNA was isolated as recommended, and treated with 0.2 U/μl Turbo DNase (Thermo Fisher Scientific, AM2238). cDNA was prepared using Oligo (dT18) Primer (Thermo Fisher Scientific, S0132) or random hexamer primers (Thermo Fisher Scientific, S0142) and RevertAid Reverse Transcriptase (Thermo-Fisher Scientific, EP0441). Real-time PCR experiments were run on a Mastercycler (Biorad), using SYBR Green (Thermo-Fisher Scientific, S7567). Primers for qPCR are listed in Table 6.

Table 6. qPCR primers.		
Target	Primer	Sequence (5' to 3')
Il6	FWD	CCAGAAACCGCTATGAAGTTCC
	REV	TTGTCACCAGCATCAGTCCC
Zfp36	FWD	CTCTGCCATCTACGAGAGCC
	REV	GATGGAGTCCGAGTTTATGTTCC
Tnf	FWD	GATCGGTCCCCAAAGGGATG
	REV	CACTTGGTGGTTTGCTACGAC
pre-Tnf	FWD	GGCAAAGAGGAAGTGTAAAG
	REV	CCATAGAAGTGTATGAGAGG
Gapdh	FWD	ATGGTGAAGGTCGGTGTGA
	REV	TGAAGGGGTCGTTGATGG

Lentivirus production and transduction

Semiconfluent Lenti-X cells were transfected with mixes containing lentiviral transfer plasmids of interest, pCRV1-Gag-Pol (*Hatzioannou et al., 2004*) and pHCMV-VSV-G (*Yee et al., 1994*) using polyethylenimine (PEI, Polysciences, 23966) in a ratio of 1:3 ($\mu\text{g DNA}/\mu\text{g PEI}$) in DMEM without any supplements. Virus containing supernatant was clarified of cellular debris by filtration through a 0.45 μm filter. Virus-like particles were directly used after harvesting or kept at 4 °C for short-term storage. Target cells were infected in the presence of 6 $\mu\text{g}/\text{ml}$ of polybrene (Sigma-Aldrich, TR1003G).

Intracellular staining for flow cytometry

For staining of intracellular proteins, cells were collected and washed twice with PBS and subsequently fixed with 2% PFA for 15 min. at room temperature (RT). After PBS washes, cells were resuspended in ice-cold MeOH for permeabilization. At this point, fixed cells were stored in MeOH at -20 °C for a maximum of 2 days. On the day of the intracellular staining, cells were washed with PBS, and incubated for 10 min. at RT in TruStain FcX mouse Fc Receptor CD16/32 block to inhibit non-specific antibody binding. Cells were then incubated with the primary antibody or left unstained for 1 hr at RT. Following three PBS washes, cells were incubated with the secondary antibody for 15 min at 4 °C. Cells were washed two times and resuspended in FACS buffer for flow cytometric analysis on an LSRFortessa (BD Biosciences) operated by BD FACSDiva software (v8.0). FACS data were analysed in FlowJo (v10.8). Median fluorescence intensities were normalized to the control.

FACS-based CRISPR–Cas9 screens

The genome-wide Vienna sgRNA library was lentivirally packaged in semiconfluent Lenti-X cells (Takara) via PEI transfection. Following double harvest, the collected supernatant was cleared of cellular debris by filtration through a 0.45 μm PES filter and stored at $+4$ °C. The obtained virus was used to transduce RAW264.7 Dox-Cas9 cells at a multiplicity of infection (MOI) of less than 0.2 TU/cell, and 600–1000-fold library representation. The percentage of library-positive cells was determined after 4 days of transduction by immunostaining of the Thy1.1 surface marker, and subsequent flow cytometric analysis. Library-positive cells were selected with G418 (1 mg/ml, Sigma-Aldrich, A1720) and expanded. Genome editing was induced with Dox (500 ng/ml, Sigma-Aldrich, D9891) and Cas9-GFP expression was monitored by FACS. Prior to Cas9 induction with Dox (Day 0), as well as before each FACS sort, an unsorted reference sample was collected. For this, a number of cells corresponding to at least 1000-fold library representation was collected and stored at -80 °C until further processing. After 3 days and 6 days of Cas9 induction, cells were sorted at FACS. Cells were harvested, washed with PBS and stained with Fixable Viability Dye eFluor (1:1,000, eBioscience, 65-0865-14) for 30 min. Subsequently, cells were washed three times with PBS, strained through a 35 μm nylon mesh and sorted in DMEM using the FACS Aria II or FACS Aria III cell sorters operated by BD FACSDiva software (v8.0). For the sort the following gating strategy was used: debris, doublets, dead (Viability Dye positive), Cas9-negative (GFP), mCherry- and BFP-negative cells were excluded. 5% of cells with the lowest and 1% of cells with the highest mCherry-TTP signal were sorted into PBS; same for the BFP internal control. At least 3×10^6 (mCherry^{low} and BFP^{low}) and 5×10^5 (mCherry^{high} and BFP^{high}) cells were collected for each time point. Sorted samples were re-analysed for purity, pelleted and stored at -80 °C until further processing. The gating strategy for flow cytometric cell sorting is shown in **Figure 2—source data 5**.

Generation of next-generation sequencing libraries

Next-generation sequencing (NGS) libraries of sorted and unsorted control samples were processed as previously described (*de Almeida et al., 2021*). Isolated genomic DNA was subjected to two-step PCR. The first PCR allowed the amplification of the integrated sgRNA cassette, the second PCR introduced the Illumina adapters. Purified PCR products size distribution and concentration was measured using a fragment analyzer (Advanced Analytical Technologies). Equimolar ratios of the obtained libraries were pooled and sequenced on a HiSeq 2500 platform (Illumina). Primers used for library amplification are listed in **Table 7**. In primer sequences, NNNNNN denotes random nucleotides, XXXX denotes sample-specific barcodes.

Analysis of pooled CRISPR screens

The analysis of the CRISPR–Cas9 screen was carried out as previously described (*de Almeida et al., 2021*). sgRNAs enriched in day 3 and day 6 sorted samples were calculated against the unsorted

Table 7. NGS library primers.

PCR 1			
Primer_name	Direction	Sequence	Comments
sgDeepSeq_rev_XXXX	Rv	CTCTTTCCCTACACGACGCTCTTCCGATCT NNNNNNCTCATTCCAGCATAGCTCTTAAAC	Library preparation 1st PCR
sgDeepSeq_rev_XXXX	Rv	CTCTTTCCCTACACGACGCTCTTCCGATCT NNNNNNTCGATTCCAGCATAGCTCTTAAAC	Library preparation 1st PCR
sgDeepSeq_rev_XXXX	Rv	CTCTTTCCCTACACGACGCTCTTCCGATCT NNNNNNCCTATTCCAGCATAGCTCTTAAAC	Library preparation 1st PCR
sgDeepSeq_rev_XXXX	Rv	CTCTTTCCCTACACGACGCTCTTCCGATCT NNNNNNNGAACTTCCAGCATAGCTCTTAAAC	Library preparation 1st PCR
sgDeepSeq_rev_XXXX	Rv	CTCTTTCCCTACACGACGCTCTTCCGATCT NNNNNNNATCCTTCCAGCATAGCTCTTAAAC	Library preparation 1st PCR
sgDeepSeq_rev_XXXX	Rv	CTCTTTCCCTACACGACGCTCTTCCGATCT NNNNNNNACTCTTCCAGCATAGCTCTTAAAC	Library preparation 1st PCR
sgDeepSeq_rev_XXXX	Rv	CTCTTTCCCTACACGACGCTCTTCCGATCT NNNNNNCTTCTTCCAGCATAGCTCTTAAAC	Library preparation 1st PCR
sgDeepSeq_rev_XXXX	Rv	CTCTTTCCCTACACGACGCTCTTCCGATCT NNNNNNCAAGTTCCAGCATAGCTCTTAAAC	Library preparation 1st PCR
sgDeepSeq_rev_XXXX	Rv	CTCTTTCCCTACACGACGCTCTTCCGATCT NNNNNNTGAGTTCCAGCATAGCTCTTAAAC	Library preparation 1st PCR
sgDeepSeq_rev_XXXX	Rv	CTCTTTCCCTACACGACGCTCTTCCGATCT NNNNNNTTCTGTTCCAGCATAGCTCTTAAAC	Library preparation 1st PCR
sgDeepSeq_rev_XXXX	Rv	CTCTTTCCCTACACGACGCTCTTCCGATCT NNNNNNTAGGTTCCAGCATAGCTCTTAAAC	Library preparation 1st PCR
sgDeepSeq_rev_XXXX	Rv	CTCTTTCCCTACACGACGCTCTTCCGATCT NNNNNNTCTGTTCCAGCATAGCTCTTAAAC	Library preparation 1st PCR
Fwd1_hybrid_P7_Nras	Fwd	GCATACGAGATAGCTAGCCACC	Library preparation 1st PCR
PCR 2			
Primer_name	Direction	Sequence	Comments
Rev2_p5_sgDeepSeq	Rv	AATGATACGGCGACCACCGAGATCTACACT CTTCCCTACACGACGCT	Library preparation 2nd PCR
Fwd2_p7_sgDeepSeq	Fwd	CAAGCAGAAGACGGCATACGAGATAGCTAGCCACC	Library preparation 2nd PCR

population control harvested on the respective day of sorting. In addition, the dropout over time of sgRNAs was calculated by comparing the unsorted populations to the initial day 0 population.

Quantitative proteomics

To systematically assess protein changes after *Huwe1* or *Psemb7* knockout, in RAW264.7-Dox-Cas9 cells *ROSA*-, *Huwe1*- and *Psemb7*-KO was induced for 2 days. Cells were incubated with LPS for the indicated times, after which 5×10⁵ cells were washed with PBS, pelleted and snap-frozen and stored at −80 °C. Sample protein concentration was measured using standard Pierce Protein Assay Kit (Thermo Fisher Scientific, 23225), after which 40 µg of protein were processed using the iST PreOmics Sample Preparation kit 8 x (P.O. 00001) according to the manufacturer’s instructions.

Peptides were separated on an Ultimate 3000 RSLC nano-flow chromatography system (Thermo Fisher Scientific), using a pre-column for sample loading (Acclaim PepMap C18, 2 cm ×0.1 mm, 5 µm, Thermo Fisher Scientific), and a C18 analytical column (Acclaim PepMap C18, 50 cm ×0.75 mm, 2 µm, Thermo Fisher Scientific), applying a segmented linear gradient from 2% to 35% and finally 80% solvent B (80% acetonitrile, 0.1% formic acid; solvent A 0.1% formic acid) at a flow rate of 230 nL/min over 120 min. Eluting peptides were analyzed on an Exploris 480 Orbitrap mass spectrometer (Thermo

Fisher Scientific), which was coupled to the column with a FAIMS pro ion-source (Thermo Fisher Scientific) using coated emitter tips (PepSep, MSWil). The mass spectrometer was operated in DIA mode with the FAIMS CV set to -45 , the survey scans were obtained in a mass range of 400 – 900 m/z , at a resolution of 120 k at 200 m/z and a normalized AGC target at 300% . 31 MS/MS spectra with variable isolation width between 13 and 24 m/z covering 399.5 – 899.5 m/z range including 1 m/z windows overlap, were acquired in the HCD cell at 30% collision energy at a normalized AGC target of 1000% and a resolution of 30 k. The max. injection time was set to auto.

Raw data were processed using Spectronaut software (version 15.4.210913.50606, <https://biognosys.com/software/spectronaut/>) with the DirectDIA workflow. The Uniprot mouse reference proteome (version 2021.03, <https://www.uniprot.org/>), as well as a database of most common contaminants were used. The searches were performed with full trypsin specificity and a maximum of 2 missed cleavages at a protein and peptide spectrum match false discovery rate of 1% . Carbamidomethylation of cysteine residues were set as fixed, oxidation of methionine and N-terminal acetylation as variable modifications. The global normalization and imputation were done in Spectronaut - all other parameters were left at default. Spectronaut output tables were further processed using Cassiopeia_LFQ in R (https://github.com/moritzmadern/Cassiopeia_LFQ; Madern, 2021). Contaminant proteins, protein groups identified only by one peptide and protein groups with less than two quantitative values in one experimental group, were removed for further analysis. Differences between groups were statistically evaluated using the LIMMA package (Ritchie et al., 2015) in Cassiopeia_LFQ at 5% FDR (Benjamini-Hochberg).

To generate A375 AAVS1- and HUWE1-KO cells, inducible Cas9 clones of both cell lines were transduced with an sgRNA construct targeting the respective gene by lentiviral delivery. Cells were antibiotic selected for genomic integration and expanded. A375 samples were further FACS sorted for sgRNA + cells to obtain purity of $>99\%$. Cells were harvested 72 hr after Cas9 induction with Dox at final concentration of 0.2 $\mu\text{g}/\mu\text{l}$. A total number of 3×10^6 SW620 or 2.5×10^6 A375 cells were harvested, washed with PBS, pelleted, snap-frozen, and stored at -70°C . Protein concentrations were measured using standard BCA assay and normalized to 50 micrograms. The protein samples were prepared with iST preOmics Sample Preparation kit 96 x (P.O. 00027) according to the manufacturer's protocol.

The nano HPLC system (UltiMate 3000 RSLC nano system, Thermo Fisher Scientific) was coupled to an Orbitrap Eclipse Tribrid mass spectrometer equipped with a FAIMS pro interfaces and a Nanospray Flex ion source (all parts Thermo Fisher Scientific). Peptides were loaded onto a trap column (PepMap Acclaim C18, 5 mm $\times$ 300 μm ID, 5 μm particles, 100 Å pore size, Thermo Fisher Scientific) at a flow rate of 25 $\mu\text{l}/\text{min}$. using 0.1% TFA as mobile phase. After 10 minutes, the trap column was switched in line with the analytical column (PepMap Acclaim C18, 500 mm $\times$ 75 μm ID, 2 μm , 100 Å, Thermo Fisher Scientific) operated at 30°C . Peptides were eluted using a flow rate of 230 nL/min , starting with the mobile phases 98% A (0.1% formic acid in water) and 2% B (80% acetonitrile, 0.1% formic acid) and linearly increasing to 35% B over the next 180 minutes. The Eclipse was operated in data-dependent mode, performing a full scan (m/z range 350 – 1500 , resolution $120,000$, target value $1\text{E}6$) at 4 different compensation voltages (CV- 45 , -55 , -65 , -75), followed each by MS/MS scans of the most abundant ions for a cycle time of 0.75 sec per CV. MS/MS spectra were acquired using an isolation width of 1.2 m/z , target value of $3\text{E}4$ and intensity threshold of $5\text{E}4$, maximum injection time 20ms , HCD with a collision energy of 30 , using the Iontrap for detection in the rapid scan mode. Precursor ions selected for fragmentation (include charge state 2 – 6) were excluded for 60 s. The monoisotopic precursor selection filter and exclude isotopes feature were enabled.

For peptide identification, the RAW-files were loaded into Proteome Discoverer (version 2.5.0.400, Thermo Fisher Scientific). All MS/MS spectra were searched using MS Amanda v2.0.0.16129 (Dorfer et al., 2014). The peptide mass tolerance was set to ± 10 ppm, the fragment mass tolerance to ± 400 mmu, the maximal number of missed cleavages was set to 2 , using tryptic enzymatic specificity without proline restriction. Peptide and protein identification were performed in two steps. For an initial search, the RAW-files were searched against the database human_uniprot_reference_2021-06-30.fasta ($20,531$ sequences; $11,395,157$ residues), supplemented with common contaminants, using the following search parameters: alkylation of cysteine by $\text{C}_6\text{H}_{11}\text{NO}$ was set as a fixed modification, oxidation of methionine as variable modification. The result was filtered to 1% FDR on protein level using the Percolator algorithm (Käll et al., 2007) integrated in Proteome Discoverer. A

sub-database of proteins identified in this search was generated for further processing. For the second search, the RAW-files were searched against the created sub-database using the same settings as above plus considering additional variable modifications: phosphorylation on serine, threonine and tyrosine, deamidation on asparagine and glutamine, and glutamine to pyro-glutamate conversion at peptide N-terminal glutamine, acetylation on protein N-terminus were set as variable modifications. The localization of the post-translational modification sites within the peptides was performed with the tool ptmRS, based on the tool phosphoRS (Taus et al., 2011). Identifications were filtered again to 1% FDR on protein and PSM level, additionally an Amanda score cut-off of at least 70 was applied. Peptides were subjected to label-free quantification using IMP-apQuant (Doblmann et al., 2019). Proteins were quantified by summing unique and razor peptides and applying intensity-based absolute quantification (iBAQ; Schwanhäusser et al., 2011) with subsequent normalisation based on the MaxLFQ algorithm (Cox et al., 2014). Proteins were filtered to be identified by a minimum of 3 quantified in at least 1 sample. Protein-abundance-normalization was done using sum normalization. Statistical significance of differentially expressed proteins was determined using limma (Smyth, 2004).

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Author contributions

Sara Scinicariello, Conceptualization, Formal analysis, Validation, Investigation, Visualization, Methodology, Writing – original draft, Project administration, Writing – review and editing; Adrian Soderholm, Formal analysis, Investigation, Methodology, Writing – review and editing; Markus Schäfer, Conceptualization, Methodology, Writing – review and editing; Alexandra Shulkina, Formal analysis, Investigation, Methodology; Irene Schwartz, Robert Kalis, Investigation, Methodology, Writing – review and editing; Kathrin Hacker, Valentina Budroni, Investigation, Methodology; Rebeca Gogova, Investigation, Writing – review and editing; Kimon Froussios, Formal analysis; Annika Bestehorn, Tim Clausen, Pavel Kovarik, Johannes Zuber, Conceptualization, Writing – review and editing; Gijs A Versteeg, Conceptualization, Supervision, Funding acquisition, Writing – original draft, Writing – review and editing

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Ethics

All animals were maintained in the pathogen-free animal facility of the Research Institute of Molecular Pathology, and all procedures were carried out according to an ethical animal license that is approved and regularly controlled by the Austrian Veterinary Authorities (License Number: GZ: 516079/2017/14).

Decision letter and Author response

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Additional files

Supplementary files

- MDAR checklist

Data availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifiers PXD036714, PXD036715, and PXD038892. All other data generated or analyzed during this study are included in the manuscript and supporting files.

The following datasets were generated:

Author(s)	Year	Dataset title	Dataset URL	Database and Identifier
Versteeg GA	2023	HUWE1 controls tristetraprolin proteasomal degradation by regulating its phosphorylation	https://www.ebi.ac.uk/pride/archive/projects/PXD036714	PRIDE, PXD036714
Versteeg GA	2023	HUWE1 controls tristetraprolin proteasomal degradation by regulating its phosphorylation	https://www.ebi.ac.uk/pride/archive/projects/PXD036715	PRIDE, PXD036715
Versteeg GA	2023	HUWE1 controls tristetraprolin proteasomal degradation by regulating its phosphorylation	https://www.ebi.ac.uk/pride/archive/projects/PXD038892	PRIDE, PXD038892

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6. Discussion

6.1.1 Uncovering novel regulatory factors of innate immunity by CRISPR/Cas9 screening

The innate immune system is essential for human health and must adapt dynamically to contexts ranging from homeostasis and immunosurveillance to active inflammation and resolution. This adaptability relies on multiple layers of regulation, spanning transcription, co/post-transcriptional RNA processing, and post-translational control of protein abundance. The critical importance of understanding these regulatory mechanisms is underscored by severe consequences of dysregulated innate immune activity, including autoimmune diseases and cancer.

A central aim of this thesis was to uncover novel regulators of innate immune signalling, with a focus on RNA processing factors. To this end, a tailored genome-wide CRISPR/Cas9-based genetic screen served as the foundation for all three studies, each optimised for a distinct readout:

1. Endogenous protein levels – IFN γ signalling
2. Exogenously expressed, fluorescently tagged protein – IRF1 regulation
3. Exogenously expressed, fluorescently tagged protein – TTP regulation

The versatility of the CRISPR/Cas9 system was critical for all projects. By introducing flexibly designed sgRNAs into an inducible Cas9 cell line, we could systematically disrupt nearly any gene. Using recently developed design algorithms³³⁶ and six independent sgRNAs per target gene increased hit confidence, while inducible Cas9 expression allowed investigation of essential genes by limiting the time window between knockout and phenotypic measurement^{337,338}. Each screen was further adapted to its specific goal: intracellular staining of endogenous protein for IFN γ signalling to uncover RNA regulatory factors, or measuring exogenously expressed proteins with fluorescent tags for IRF1 and TTP to isolate post-translational regulation from transcriptional and other RNA processing effects.

While powerful, CRISPR/Cas9 screening has limitations. Variability in sgRNA efficiency, off-target effects, limited Cas9 access to certain genomic regions^{634–637}, activation of compensatory pathways or redundancy among factors^{638,639}, and secondary effects can produce false negatives or false positives. We addressed these

issues in part by using multiple sgRNAs per gene, validating hits in follow-up experiments, and performing parallel screens with complementary readouts. Nonetheless, potential biases remain, and future studies could integrate orthogonal approaches, such as siRNA-based screens, to strengthen confidence in accurate hit identification.

Overall, this strategy provided a systematic and adaptable platform to identify novel regulators of IFN γ signalling and innate immune factors herein. The three studies in this thesis exemplify how CRISPR/Cas9 screening approaches can be tuned to address diverse biological questions, from co/post-transcriptional regulation of *JAK2* by ERH to post-translational control of IRF1 and TTP by SPOP and HUWE1, respectively.

6.2 Regulation of IFN γ signalling by ERH and other co/post-transcriptional RNA processing factors

The primary focus of this thesis was to identify and characterise novel RNA regulators of IFN γ signalling. Using an inducible CRISPR/Cas9 screen that measured upregulated IRF1 protein abundance as a proxy for pathway activity, we complemented a previous gene-trap screen⁷¹ and identified regulatory factors independent of their role in cellular viability. In this way, we could for the first time identify ERH, the splicing/export factor SRSF1, the EJC, and to a lesser extent the export adaptor ALYREF, as important factors for IFN γ signalling. Loss of any of these factors impaired *JAK2* expression, a core pathway component, by disrupting co- and post-transcriptional *JAK2* mRNA processing.

My research focused primarily on ERH, which we identified to play a critical role in preventing AU-rich *JAK2* IR. I also aimed to define ERH's broader cellular and molecular functions. Since the screen identified ERH alongside several of its known protein cofactors, including its direct interactor ALYREF, and as we had the necessary tools to study these essential proteins, we saw a valuable opportunity to uncover novel regulatory mechanisms of IFN γ signalling and to shed light on the enigmatic ERH protein. In the following section, I examine the evolutionary history of ERH and its protein cofactors to provide context for its specialised role in metazoan RNA processing.

6.2.1 Evolution of ERH and its protein cofactors supports a metazoan-specific role in IR distinct from its function in yeast

ERH is highly evolutionary conserved in eukaryotes and was discovered over thirty years ago, yet its molecular function has remained largely unknown. The gene is practically identical in vertebrates³⁷⁸ and its high level of identity across species is similar to that of ultra-conserved DNA replication factors, for example the scaffolding factor proliferating cell nuclear antigen (PCNA)⁶⁴⁰. Indeed, despite about one billion years of independent evolution⁶⁴¹, the size of ERH has remained comparable from fission yeast to human, ranging from 100 to 110 amino acids (104 in humans), with no domain removed or acquired³⁷⁸. This structural conservation is further reflected by its conserved 3D architecture: ERH forms a homodimer structure with four β -sheets towards the interaction surface and three α -helices pointing outwards^{642–648}. Its amino acid sequence is also particularly unique, lacking any identifiable domains shared with other proteins³⁷⁸, making it difficult to infer ERH function or localisation from its sequence alone. As a result, functional studies of ERH have relied primarily on phenotypic analyses and interaction assays.

Despite this deep structural conservation, its functional activity can diverge. It has been proposed that ERH's 3D structure is nearly "bulletproof"³⁷⁸ since tagging it^{499,649,650}, for example with GFP, or mutating many of its residues (mainly to alanine)⁶⁵¹ did not disrupt its general nuclear localisation in various studies. However, localisation alone may not reflect function. Indeed, some amino acid substitutions altered ERH recruitment to sub-nuclear structures such as nuclear speckles or replication foci in a manner dependent on ERH-binding partners⁶⁵¹. This suggests that while the overall structure is maintained, specific interactions – and thus functions – can be disrupted by sequence changes.

Notably, ERH's amino acid sequence and functional role differs between species. While conserved, human *ERH* only shares 28% identity to its yeast homolog (*erh1*) in *S. pombe*³⁷⁸. This difference in amino acids likely contributes to species-specific interaction profiles and functional roles. Indeed, whilst human ERH is essential for cellular viability, *erh1* deletion in *S. pombe* is not lethal^{648,652}. These observations suggest that ERH function may have diversified through co-evolution with distinct binding partners.

This divergence is evident from the differing role ERH plays in regulating IR in yeast and in humans. Yeast *erh1* has been shown to promote the nuclear decay of transcripts with IR^{583,648,652}. In contrast, our data show that human ERH prevents IR in AU-rich regions, such as those in the *JAK2* mRNA. Importantly, the yeast Erh1 partner Mmi1, which mediates its role in RNA decay, is not conserved in humans³⁷⁸. Moreover, *ERH* ablation in human cells decreased cytoplasmic levels of affected IR transcripts, suggesting impaired splicing or export rather than nuclear stabilisation. These IR transcripts did not accumulate in the nucleus either, indicating that they are detected and degraded by the nuclear quality control machinery, such as the RNA exosome⁴⁸⁰. This points toward a mechanism by which ERH loss causes the generation of aberrant IR transcripts (for example through altered splicing) rather than failed decay of existing ones. Thus, while ERH is involved in regulating IR in both species, its function appears mechanistically distinct due to divergence in species-specific binding partners.

Supporting this view, the other IFN γ -regulatory factors we identified that could either directly or indirectly cooperate with ERH for *JAK2* mRNA processing are also highly conserved in metazoans. SRSF1, for instance, is part of the SR protein family, which is generally conserved in eukaryotes, but it lacks a direct orthologue in yeast^{653–655}. Furthermore, SR-like proteins like Npl3 have limited functions such as regulating conventional splicing⁶⁵⁶, as yeast lack alternative splicing mechanisms⁶⁵³. In contrast, SRSF1 is highly conserved among metazoans⁶⁵⁷, and it together with other SR proteins likely diversified in higher eukaryotes to accommodate more complex alternative splicing, such as regulating IR⁵⁰⁷.

Similarly, the complete EJC with its closely interacting factors seems to be a metazoan innovation that is tied to complex co- and post-transcriptional processing like alternative splicing and NMD⁵¹⁹. ALYREF, another key ERH-interacting factor, has a functional homolog in yeast (*Yra1*)⁶⁵⁸, but in metazoans, its recruitment and function are linked to the abundant presence of EJCs in metazoan transcripts^{544,659}, reflecting the divergence in splicing complexity. These distinctions underscore that while some components are ancestrally conserved, their molecular roles have diversified in line with increased transcriptomic complexity in multicellular organisms, driven largely by splicing processes.

To summarise, the evolution of ERH and its associated factors regulating *JAK2* processing supports a model in which ERH regulates a metazoan-specific function: alternative splicing through IR. The distinct mechanism of ERH-regulated IR in human

cells, compared to yeast, underscores the importance of species-specific interactomes in shaping RNA processing functions over evolutionary time.

6.2.2 ERH-regulated intron retention is distinct from detained introns and may involve dynamic regulation

Given the high conservation of ERH and its associated factors, their particular involvement in regulating a vertebrate-specific process like IFN γ signalling⁶⁶⁰ is intriguing. We found that many ERH-regulated IR events occur in mRNAs encoding essential and conserved cellular functions, including DNA replication and DNA damage response genes such as *ATR*. In contrast, *JAK2* and other JAK/STAT pathway components are restricted to vertebrates⁶⁶⁰ and are not essential for cellular viability. These findings suggest that rather than ERH evolving to regulate immunity, more recent transcripts, such as *JAK2*, may have evolved to depend on pre-existing ERH-mediated RNA processing mechanisms.

IR has previously been linked to dynamic gene expression regulation, particularly in response to stress and immune stimuli. A subset of these events involves so-called “detained introns”, which are stably retained in the nucleus and form transcript reservoirs that can be rapidly spliced upon environmental cues such as cellular stress, DNA damage, and inflammatory stimuli (e.g. viral infections)^{475,481,482}. This raises the possibility that ERH may play a dynamic regulatory role in stimulus-responsive IR. Indeed, ERH has previously been implicated in the splicing of transcripts involved in the DNA damage response and cell cycle regulation, supporting the idea that it may contribute to regulated intron removal under specific stress conditions³⁷⁸.

However, several observations suggest that ERH-regulated IR is mechanistically distinct from detained intron regulation. First, ERH is ubiquitously and constitutively expressed at high levels across all human tissues^{499,661}, and we found no change in its abundance in response to IFN γ stimulation. Its RNA levels also appear stable throughout the cell cycle stages⁶⁶¹, suggesting that ERH regulation is unlikely to occur via transcriptional or translational changes. This argues against a model in which ERH activity is dynamically modulated through expression control.

Still, dynamic regulation of ERH activity may occur at the post-translational level, but little is known in this regard. In vitro, the *Drosophila* ERH has been shown to

be phosphorylated by the highly conserved casein kinase 2 (CK2) at two residues that are also conserved in human ERH⁶⁶². Further functional experiments in *Drosophila* using phosphomimetic and non-phosphorylatable mutants suggest that single phosphorylation activates ERH, whereas dual phosphorylation or absence of phosphorylation inactivates it⁶⁶³. While structural modelling based on ERH crystal structures suggests that this phosphorylation could alter ERH's dimerisation or interaction with binding partners^{642,645,646}, these hypotheses remain unconfirmed in mammalian systems. Thus, although intriguing, the possibility that ERH activity is dynamically modulated via phosphorylation or other post-translational mechanisms remains speculative.

Functionally, ERH-regulated IR transcripts behave differently from detained intron-containing mRNAs. Following ERH depletion, these transcripts did not accumulate in the nucleus as would be expected for detained introns which localise to nuclear speckles and become resistant to nuclear decay^{476,482}. Nor did they significantly stabilise in the cytoplasm following NMD inhibition with cycloheximide (CHX), indicating that they were not exported and degraded via NMD. Instead, these transcripts appear to be rapidly targeted for nuclear exosomal degradation, consistent with prior observations of inefficiently spliced mRNAs^{480,664}. Moreover, most ERH-regulated introns showed low retention in WT cells (Results 1, Fig. 5G), in contrast to detained introns which are constitutively retained^{476,482}. Taken together, these results suggest that ERH plays a constant, rather than inducible, role in the efficient removal of these introns.

In summary, while ERH-regulated IR shares surface similarities with detained intron mechanisms, several features such as low constitutive expression and susceptibility to RNA decay, distinguish it mechanistically. Nevertheless, it remains possible that ERH's activity could be altered in response to stimuli that regulate IR in stress or immune contexts, subsequently affecting expression of DNA damage response factors (e.g. ATR) or key innate immune factors (e.g. JAK2). Future studies examining the subnuclear localisation of ERH and its transcript targets under different stimuli may shed light on whether ERH contributes to a regulated splicing switch or functions exclusively as a constitutive RNA processing factor.

6.2.3 ERH interactions with POLDIP3 and ALYREF: implications for localisation, RNA export, and functional specificity

ERH's small, highly conserved structure may allow it to fit adaptably into diverse protein complexes, enabling roles in multiple RNA processing pathways despite lacking enzymatic or RNA-binding domains³⁷⁸. Understanding its protein–protein interactions is therefore key to define its molecular function.

ERH is mainly nuclear, yet has no nuclear localisation signal³⁷⁸. While its small size (~12 kDa) would allow nuclear entry via passive diffusion⁶⁶⁵, its localisation is likely also controlled by interactions to partners that are actively imported to the nucleus⁶⁶⁶. For example, ERH localisation to nuclear speckles can be induced by co-overexpression with POLDIP3⁶⁵¹, one of ERH's first identified direct interaction partners in human cells⁴⁹⁹. In our co-IP MS/MS, POLDIP3 emerged as a top-scoring ERH-interactor; however, loss of POLDIP3 did not phenocopy ERH depletion in reducing JAK2 levels or altering global mRNA expression. Although we used multiple sgRNAs with consistent effects on *JAK2* expression, we did not confirm *POLDIP3* KO by western blotting, and thus cannot exclude incomplete depletion. Assuming similar KO efficiency to other factors, our data suggest that POLDIP3 is not a major functional partner, or that its function is redundant.

Still, the strong ERH-POLDIP3 affinity raises the possibility of indirect regulation: POLDIP3 might modulate ERH's activity by sequestering it away from other partners, or by retaining it in nuclear speckles near specific splicing and export partners. Conversely, ERH might influence POLDIP3 complexes by competing with its protein cofactors, as reported for ERH with SAFB and the SRPK1 kinase³⁸⁸.

In contrast, ALYREF, a well-characterised RNA export adaptor^{544,658}, showed stronger functional overlap with ERH in our RNA sequencing analysis. While ALYREF loss did not induce IR like *ERH* ablation, both KOs phenocopied each other most closely relative to the other KOs for transcripts with reduced cytoplasmic abundance. This indicates that they likely do not function together in splicing, but rather cooperate in RNA export for a subset of transcripts. One model is that ERH contributes to mRNP packaging and compaction during export. Crosslinking MS data of TREX–mRNP complexes indicate that ERH binds ALYREF and CHTOP⁵⁴⁴, and could thus potentially stabilise ALYREF/CHTOP dimers. Given ALYREF's role in facilitating multimerisation of EJC and recruiting TREX complexes^{498,544,561}, ERH may also help stabilise these

higher-order structures, thereby promoting mRNP compaction and efficient RNA export.

Future studies could employ complementary methods to dissect ERH's role in RNA export and compaction. Examining non-spliced histone mRNAs⁶⁶⁷ could help separate its export function from effects on splicing. Since ALYREF supports export of such transcripts via interactions with the 5' CBC⁵⁴¹ and other export-adaptors⁶⁶⁸, ERH might similarly contribute in this context. However, because non-spliced transcripts lack EJC's deposited at exon-exon junctions^{529,530}, they may not fully capture export mechanisms used for spliced mRNAs.

Assessing ERH's role in mRNP compaction is more technically challenging to do in cells. One available method is single molecule fluorescence in situ hybridisation (smFISH) coupled to super resolution microscopy, which measures distances between differently labelled regions of the same mRNA in 3D space. This approach has illuminated compaction changes during mRNP transit to the nuclear envelope^{559,562,669} and the involvement of specific factors such as SR proteins⁵⁶². However, technical constraints, including the need for very long and abundant transcripts, have limited application to only a few endogenous or artificial transcripts. This method may currently not be representative for most transcripts and applying it for ERH-regulated mRNAs would be challenging.

Overall, ERH's interactions with POLDIP3 and ALYREF highlight how it may be embedded in multiple RNA processing machineries, which influence both its localisation and function. Its possible involvement in RNA packaging and export highlights the need for improved methods to visualise and quantify these processes in living cells.

6.2.4 ERH-regulated AU-rich intron retention is linked to splicing factors and processing in the nuclear periphery

Our data indicate that ERH is critical for proper RNA splicing, with its depletion causing IR in AU-rich regions, like those within the *JAK2* mRNA. SRSF1 depletion produced similar IR in a couple of transcripts, although notably not in *JAK2*, suggesting that SRSF1 may cooperate with ERH to prevent IR in certain contexts. These introns tended to have weaker 3'ss, rendering them more reliant on RBPs like SR proteins that aid splice site recognition^{476,482,507}. ERH also interacts with other splicing/export

factors, such as THRAP3 and BCLAF1^{496,497}, which were not essential for JAK2 processing but may cooperate with ERH in regulating splicing of other transcripts. Collectively, these observations support a model in which ERH partners with multiple splicing and export factors to regulate distinct mRNA subsets.

Unlike the purine (A/G) rich SR-binding motif enrichment seen in SRSF1-regulated IR⁵⁰⁷, ERH-regulated IR is predominantly AU-rich, with no detectable enrichment for SRSF1 motifs. This suggests that SRSF1's effects on these transcripts may be indirect, for example, via ERH's cooperation with SAFB to regulate SRPK1 and thereby control SR protein phosphorylation and activity³⁸⁸. The AU-rich bias also points toward a different regulatory network involving AU-RBPs.

Consistent with this, we detected local enrichment of motifs bound by AU-RBPs such as hnRNPC, hnRNPL, KHDRBS1/2/3, and TIA1 in ERH-regulated splicing regions. These proteins were absent from our ERH co-IP-MS/MS, suggesting that they may interact only transiently during splicing, or that ERH does not directly bind them. Many of these AU-RBPs did not score in our IFN γ signalling screen, likely reflecting redundancy due to multiple paralogs⁶⁷⁰.

Recent work has linked AU/GC content to distinct splicing mechanisms in specific subnuclear regions⁶⁷⁰. Using Hi-C sequencing, ChIP sequencing, RNA FISH, and available enhanced crosslinking and immunoprecipitation (eCLIP) sequencing data, investigators found that transcripts processed in the nuclear periphery had high AU content – particularly in long introns – and were associated with splicing factors such as KHDRBS1 and hnRNPC⁶⁷⁰. Notably, SAFB and SAFB2, both ERH interactors³⁸⁸, were enriched in this peripheral splicing network⁶⁷⁰ and could connect ERH to AU-RBP-mediated splicing at the periphery (Fig. 4).

Interestingly, by using genome integrated minigene reporters and RNA sequencing in cells with chromatin rearrangements, this study also showed that perturbed splicing in the periphery was mostly associated with exon skipping, whereas IR was more prevalent in the centre and near nuclear speckles⁶⁷⁰. This highlights that ERH-regulated AU-rich IR may follow a distinct mechanistic path compared to canonical GC-rich IR⁵⁰⁷. Confirming the subnuclear localisation of ERH-regulated splicing, using approaches like smFISH, will be an important next step toward defining its mechanistic basis.

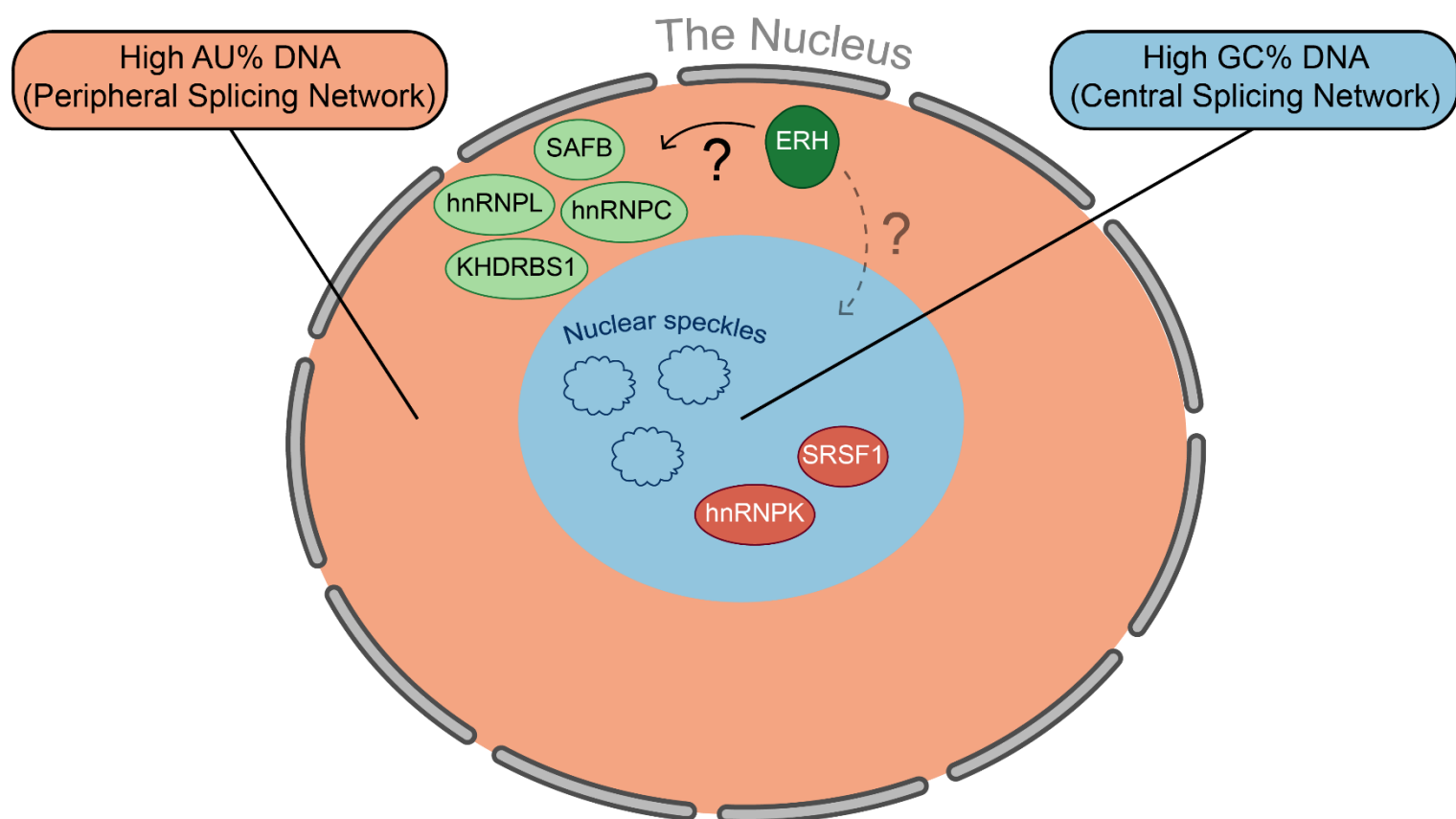


Figure 4 ERH as part of a peripheral splicing network. Distinct RNA processing networks localise to different subnuclear regions, with AU-rich transcripts preferentially processed at the nuclear periphery, whereas GC-rich transcripts are more commonly processed in the centre and at nuclear speckles, involving factors such as SRSF1⁶⁷⁰. Our findings support a model in which ERH regulates splicing of AU-rich transcripts, potentially by cooperating with the peripheral splicing machinery components such as SAFB.

Overall, our results support a model in which ERH predominantly regulates splicing of AU-rich transcripts, likely through cooperation with splicing machinery positioned in the nuclear periphery.

6.2.5 General RNA processing machinery can be rate-limiting for specific mRNAs

While ERH-regulated transcripts were generally AU-rich, not all AU-rich mRNAs were sensitive to ERH loss. This indicates that susceptibility depends not only on sequence features, for example motif abundance, but also on broader aspects of RNA metabolism. A similar pattern is seen for broadly essential processing factors such as ALYREF and the EJC, the latter deposited at virtually all exon–exon junctions^{529,530},

with many transcripts appearing relatively unaffected by their loss. Such observations suggest that additional determinants modulate processing dependence.

Key aspects may include transcript turnover rates, what RNA processing step is rate-limiting, and the degree of redundant processing. For example, short-lived mRNAs may be more sensitive to even minor delays in nuclear processing, with effects accumulating more readily than in long-lived transcripts. As such, highly stable and abundant mRNAs may buffer against transient processing defects, making changes harder to detect in the time window of our Cas9-induced KOs. Future studies using approaches such as SLAM sequencing^{671,672} could more precisely track how transcript half-life interacts with processing factor depletion.

The nature of the rate-limiting step in RNA maturation may also shape susceptibility: transcripts with rapid transcription but complex splicing may be highly sensitive to splicing defects, whereas intronless transcripts may be more dependent on export adaptors such as ALYREF. In the case of *JAK2*, the loss of multiple individual factors severely impaired processing and expression, suggesting both an absence of redundant mechanisms and a strong reliance on specific co/post-transcriptional regulators.

Overall, these observations highlight that even general RNA processing machinery can be rate-limiting for select transcripts, thereby exerting pathway-specific effects. Understanding why certain mRNAs are disproportionately sensitive to specific RNA regulators remains an important question for future research.

6.2.6 Outlook of underpinning the molecular function of ERH

Pinpointing the precise molecular function of ERH in regulating AU-rich IR will require further work. In our study, we utilised multiple approaches to exclude alternative explanations. For example, short timepoint 4-thiouridine (4sU) labelling allowed us to isolate nascent transcripts and demonstrate that transcription of *JAK2* was unaffected by ERH depletion. Techniques based on metabolic labelling, such as 4sU, can also be used to assess additional RNA kinetics, including splicing rates^{673,674}. Recent studies have combined these methods with subcellular fractionation to also examine RNA export and decay^{675,676}. Although technically challenging, applying such approaches to ERH-regulated IR events could help resolve their splicing dynamics and subcellular

distribution, though it would still not reveal whether ERH acts directly in the RNA maturation process.

A major limitation of our work is that the effects caused by the induced KOs could be either direct or indirect. Nuclear RNA processing steps are highly interconnected; disruption of one process can affect others and cause indirect effects. In addition, because the factors we studied influence the expression of many protein-coding mRNAs, their loss may indirectly alter processing through perturbed protein activity. Our inducible Cas9 system helps limit such secondary effects, but as KO establishment still requires several days, indirect contributions cannot be ruled out.

To overcome this, degron-based systems offer the ability to deplete proteins within minutes, enabling the observation of immediate direct effects. A notable example is the acute degradation of MYC, which revealed that despite its widespread DNA binding, only a small subset of genes are direct transcriptional targets⁶⁷¹, resolving years of uncertainty from slower knockdown/knockout approaches. Inspired by this, I attempted to implement the improved auxin-inducible degron (AID) system⁶⁷⁷ by expressing sgRNA-resistant ERH tagged with the mAID degron at either terminus (data not shown). However, unlike a previous study in which HA-FLAG-tagged ERH rescued the lethality of siRNA knockdown³⁸¹, my mAID-tagged ERH failed to rescue the *ERH* KO despite similar expression to endogenous levels. We thus concluded that our protein-tag significantly impaired the function of ERH, potentially due to its bulkier size in comparison to the small HA-FLAG tag. Using longer linkers than the three amino acids I used might allow a degron tag to escape the crowded ERH complex environment. If optimised, such a system could allow rapid ERH depletion and assessment of its direct involvement in splicing and RNA export.

We also explored dissecting ERH function by disrupting its binding to specific protein partners via point mutations (data not shown). However, these mutants accumulated at much lower protein levels than WT ERH, suggesting that loss of binding may destabilise ERH and trigger its degradation. As a result, we could not cleanly isolate and discern their rescue on splicing of AU-rich transcript regions. This observation raises the intriguing possibility that ERH homeostasis is partly regulated post-translationally through a stoichiometric quality-control mechanism that eliminates excess, non-complexed ERH. Such regulation could allow cells to fine-tune ERH availability in response to changes in the abundance of its binding partners, dynamically modulating splicing and RNA processing outputs. Future work dissecting

ERH turnover pathways could clarify whether this instability represents a dedicated regulatory mechanism or simply a by-product of impaired protein folding.

Given that ERH engages multiple partners through each of its binding pockets³⁷⁸, mutating ERH itself may have inadvertently disrupted several interactions at once. To cleanly dissect ERH function, an alternative approach would be to mutate the ERH-interacting partners rather than ERH, ideally in a way that preserves their native function while preventing ERH binding. Such a strategy would enable selective disruption of individual ERH-partner interactions without globally destabilising ERH, thereby clarifying ERH's pathway-specific roles in preventing AU-rich intron retention.

6.2.7 Targeting ERH in the context of IFN γ -related diseases

Given ERH's role in mRNA processing of transcripts encoding factors involved in DNA replication and the DNA damage response, its reported involvement in multiple cancer-related pathways is unsurprising^{378,678,679}. Several studies have reported elevated ERH expression in diverse cancers^{680–682}, and its silencing has been associated with reduced cell proliferation, migration, epithelial–mesenchymal transition (EMT), impaired recovery from replication stress and DNA damage, and increased apoptosis^{382,383,650,680,681,683,684}. ERH's functional importance appears heightened in cancers harbouring mutations in the *KRAS* oncogene^{381,685}, where elevated ERH expression correlates with shorter patient survival³⁸¹. These observations suggest that ERH inhibition could be therapeutically beneficial, particularly in *KRAS*-driven tumours.

Our findings that ERH is essential for IFN γ signalling through *JAK2* mRNA processing raise important considerations for its therapeutic targeting. While IFN γ signalling is often tumour-suppressive, chronic activation can promote tumourigenesis via inhibitory feedback mechanisms^{272–279}. This is exemplified by constitutive activation of the JAK/STAT pathway^{287–289}, such as in the chronically active *JAK2* mutant V617F that drives myeloproliferative neoplasms^{287–289}. In such contexts, ERH inhibition could offer a dual therapeutic advantage: reducing tumour cell proliferation while also dampening pro-tumourigenic IFN γ signalling.

However, the complexity of JAK/STAT signalling in autoimmunity and cancer cautions against broad ERH inhibition. JAK/STAT dysregulation contributes to many autoimmune diseases, yet IFN γ -targeting therapies have had limited clinical

success^{228,230–232}. Moreover, as ERH also regulates essential DNA replication factors alongside JAK2, total inhibition could cause substantial toxicity and side effects.

Rather than fully blocking it, a more refined approach may be to modulate ERH activity and selectively impairing specific ERH functions while preserving others. Currently, CK2-mediated phosphorylation is the only reported post-translational modification linked to ERH activity^{662,663}. Since CK2 itself is a promising anticancer target, with selective inhibitors such as Silmitasertib (CX-4945) currently undergoing clinical trials for cancers^{686,687}, targeting CK2 could indirectly modulate ERH. Alternatively, ERH's unique sequence and particular structure makes it amenable to the development of protein-protein interaction inhibitors to block binding with specific protein partners, thus tailoring its activity to inhibit specific ERH functions. Alternatively, small molecules that destabilise the ERH homodimer could be used to target all ERH activity.

Overall, ERH's multifaceted roles create both opportunities and challenges for therapeutic targeting. Context-specific inhibition, guided by a detailed understanding of ERH's pathway-specific functions, will likely offer the greatest potential for clinical benefit.

6.3 Regulation of IRF1 protein degradation by SPOP

Transcriptional regulation governed by IRF1 is critical for innate immune responses that protect against invading pathogens. Its activity appears to be primarily regulated through protein degradation, with properly balanced IRF1 baseline levels being key to prevent auto-inflammation^{392,393} while maintaining effective tumour surveillance^{207,391}. We aimed to discover novel IRF1 protein regulators and identified the E3 ligase SPOP as a direct mediator of IRF1 ubiquitination and its subsequent proteasomal degradation.

Interestingly, IRF1 degradation has been reported to be regulated by multiple E3 ligases, including FBXW7³⁹⁶, MDM2³⁹⁷, CHIP³⁹⁷, and SPOP, which was simultaneously also identified by other researchers⁶⁸⁸. Their importance for IRF1 regulation seems to vary in a cell-context dependent manner similar to that described for other transcription factors, e.g. MYC⁶⁸⁹. This was evident in our study, as the E3 ligase FBXW7 did not contribute to IRF1 degradation in RKO cells, whereas it has a prominent role in COS7 and MRC5 cells³⁹⁶.

Mechanistically, we found both IRF1 and SPOP to be nuclear, with IRF1 mainly in the nucleoplasm and SPOP enriched in nuclear speckles. While we could not directly determine whether IRF1 was targeted by SPOP in the nucleoplasm or nuclear speckles, prior work showed that SPOP can recruit IRF1 to nuclear speckles⁶⁸⁸. Since these clusters are hubs for RNA processing factors^{514,690,691}, SPOP-mediated IRF1 degradation may be coupled to IRF1's transcriptional activity, as reported for another IRF1 turnover pathway³⁹⁶. However, most of the SPOP-targeted IRF1 degrons are in an IRF1 domain that is key for its heterodimerisation with other transcriptional regulators⁶⁹². As such, functionally active IRF1 might instead be partly shielded, rather than accessible, from SPOP-mediated degradation. Testing this with DNA-binding-deficient IRF1 mutants could clarify the relationship between SPOP activity and IRF1's functional state.

Post-translational modifications, particularly phosphorylation, may be major determinants of which E3 ligases target IRF1 in different immune contexts. In this regard, SPOP may exert opposing functions in resting, virally infected, and bacterially activated immune cells. In myeloid cells, SPOP can stabilise IRF1 by inhibiting its phosphorylation by the kinase LYN, which otherwise promotes its ubiquitination by an unidentified E3 ligase and its subsequent proteasomal degradation in the nucleus⁶⁹³. This protective effect, mediated by recruiting and activating the CSK kinase within the MyD88 complex, requires active TLR signalling from inflammatory bacterial stimuli (LPS or CpG-DNA)⁶⁹³. These observations suggest that SPOP promotes IRF1 activity at the onset of bacterial infections by inhibiting LYN, but later during resolution or in the absence of inflammation suppresses IRF1 by targeting it for degradation.

In contrast, during viral infection, phosphorylation of IRF1 by IKK ϵ may instead promote SPOP-mediated degradation. SPOP's substrate recognition can be phosphorylation-sensitive, preferring non-phosphorylated substrates in some cases and phosphorylated degrons in others^{694–696}. In breast cancer cells, two residues within a known SPOP-targeted degron of IRF1 were shown to be phosphorylated by IKK ϵ , marking IRF1 for proteasomal degradation⁶⁹⁷, potentially via SPOP. Since IKK ϵ is primarily induced by viral triggers, such as TLR3 activation, and plays a key role in the type I interferon response^{698,699}, this mechanism could explain why SPOP appears to inhibit some types of TLR signalling^{700,701}. In this way, SPOP may promote IRF1 stability in bacterial infections by blocking LYN-mediated phosphorylation but facilitate its turnover in viral infections by recognising IKK ϵ -phosphorylated degrons.

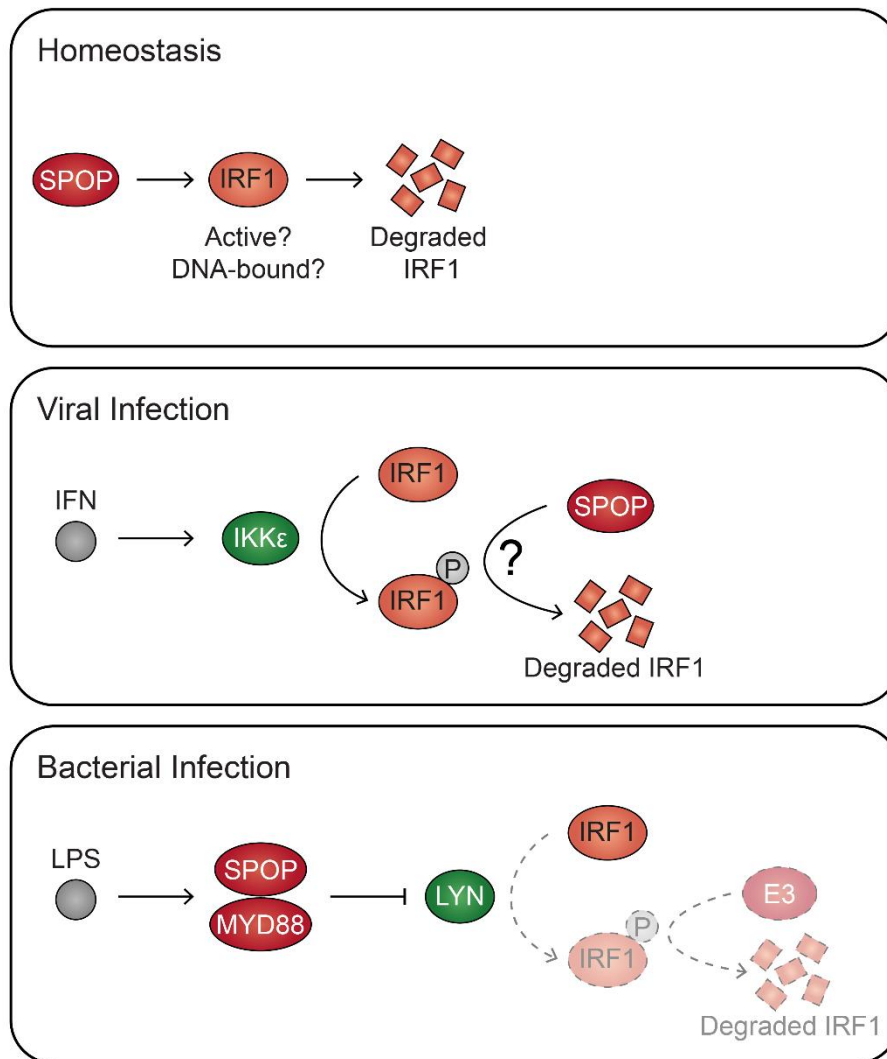


Figure 5 Context-dependent regulation of IRF1 by SPOP. SPOP likely exerts distinct effects on IRF1 depending on cellular context. At homeostasis, our findings support a direct role for SPOP in promoting IRF1 degradation, although whether inactive or DNA-bound active IRF1 is targeted remains unresolved. During viral infection, SPOP may similarly contribute to the degradation of IKKε-phosphorylated IRF1, which is unstable⁶⁹⁷. In contrast, during bacterial infection, LPS-induced MYD88 cooperates with SPOP to inhibit LYN, thereby preventing IRF1 phosphorylation and subsequent degradation⁶⁹³.

Taken together, comparing our findings with other studies reveals a model in which SPOP may regulate IRF1 degradation through opposing mechanisms depending on its phosphorylation (Fig. 5). Interestingly, while IRF1 phosphorylation seems to primarily regulate its degradation, and thereby its activity, the function of constitutively expressed IRFs, such as IRF3, is directly controlled by activating phosphorylation rather than proteasomal turnover⁷⁰². Overall, the regulation of IRF1 protein degradation highlights how multiple aspects are involved in determining targeting of innate immune factors by E3 ligases, including cell context, protein

localisation, functional substrate engagement, and the inflammatory state. IRF1 also exemplifies how phosphorylation can indirectly regulate activity by affecting protein stability. This concept seems to also extend to other innate immune RNA regulators such as TTP, which is discussed below.

6.4 Intricate post-translational regulation of TTP by HUWE1

6.4.1 HUWE1 as a master regulator of TTP stability

TTP is a key RBP involved in the resolution of inflammation, mediating the well-timed decay of mRNAs encoding major innate immune factors like TNF. Our genetic screen and follow-up validations revealed that the E3 ligase HUWE1 is a major regulator of TTP protein levels. Surprisingly, HUWE1 does not seem to be a direct mediator of TTP ubiquitination, but is rather regulating TTP phosphorylation, thereby modulating both its stability and functional activity.

A couple other E3 ligases, including VHL and its adaptor TCEB1 (which links VHL to the complete cullin-E3 ubiquitin ligase complex^{703–705}) also influenced TTP levels. As they affected the ratio of non-phosphorylated to phosphorylated TTP, they may preferentially target specific phosphor-forms or indirectly affect stability through phosphorylation. However, their effects were considerably weaker compared to the effects from *HUWE1* ablation, suggesting redundancy among E3 ligases, with HUWE1 acting as a dominant regulator. This redundancy may explain why few additional major E3s emerged from our screen, and further work could explore this by simultaneous knockout of multiple candidate E3s.

Previous reports have suggested that TTP can be degraded through a ubiquitin-independent mechanism via its intrinsically disordered regions (IDRs)^{625,633}, a niche mechanism described for some proteins^{706–708}. However, these conclusions heavily rely on a failure to detect TTP ubiquitination in cells, and that TTP is degraded in vitro by proteasomes, likely reflecting random proteolysis of IDRs rather than a physiologically relevant process in cells. Our data instead show that TTP is heavily ubiquitinated and that its degradation strongly depend on this process, as TAK-234 (ubiquitin-dependent proteasome inhibitor) maximised TTP stabilisation similar to epoxomicin (total proteasome inhibitor). Thus, ubiquitin-dependent degradation is the predominant physiological pathway, and HUWE1's major role likely lies in regulating this process.

Consistent with this, HUWE1 depletion strongly stabilised exogenously expressed mCherry-TTP in LPS-treated cells, but had little effect in untreated cells, suggesting that its action is closely tied to LPS-induced phosphorylation events. Even if HUWE1 directly ubiquitinates TTP, a possibility we could not test *in vitro* due to difficulties purifying full-length TTP, this would likely occur in a phosphorylation-dependent manner. These findings point toward phosphorylation as the key mechanism underlying HUWE1's control of TTP stability, which will be examined in the following section.

6.4.2 HUWE1 regulates TTP stability through a distinct phosphorylation-dependent mechanism

If HUWE1 modulates kinases or phosphatases that control TTP phosphorylation, one would expect it to also influence other highly phosphorylated proteins, given the typically broad substrate specificity of these enzymes. Consistent with this, TTP's closely related family member ZFP36L1 was strongly stabilised in multiple cell lines following HUWE1 depletion, as measured by protein abundance MS, suggesting that its degradation is similarly phosphorylation-dependent. Furthermore, supporting a role for HUWE1 in regulating phosphorylation, HUWE1 loss in LPS-treated RAW 264.7 macrophages led to a substantial reduction (~58% of WT levels) in NOTCH2 abundance, a known LPS-induced⁷⁰⁹ and phosphor-degron-containing protein subject to phosphorylation-dependent degradation⁷¹⁰. NOTCH2 ranked among the top 20 most downregulated proteins in this dataset, and since it is not a TTP target⁷¹¹, this reduction is likely a direct consequence of phosphorylation-induced turnover rather than an indirect effect from LPS-mediated TTP activity.

Confirming HUWE1's impact on global and TTP-specific phosphorylation events will require further phosphoproteomic profiling in *HUWE1* KO cells. We identified the C-terminal region of TTP to be a hotspot for phosphorylation events, but the specific residues critical for HUWE1-dependent regulation remain unknown. TTP is phosphorylated at multiple sites by different kinases, with each site potentially affecting distinct aspects of its function, such as RNA or protein partner binding, subcellular localisation, and protein stability. Focused C-terminal phospho-mutagenesis or even a mutagenic screen could possibly pinpoint the key residues which mediate HUWE1's effects. Given this multi-site regulation, HUWE1 is likely to

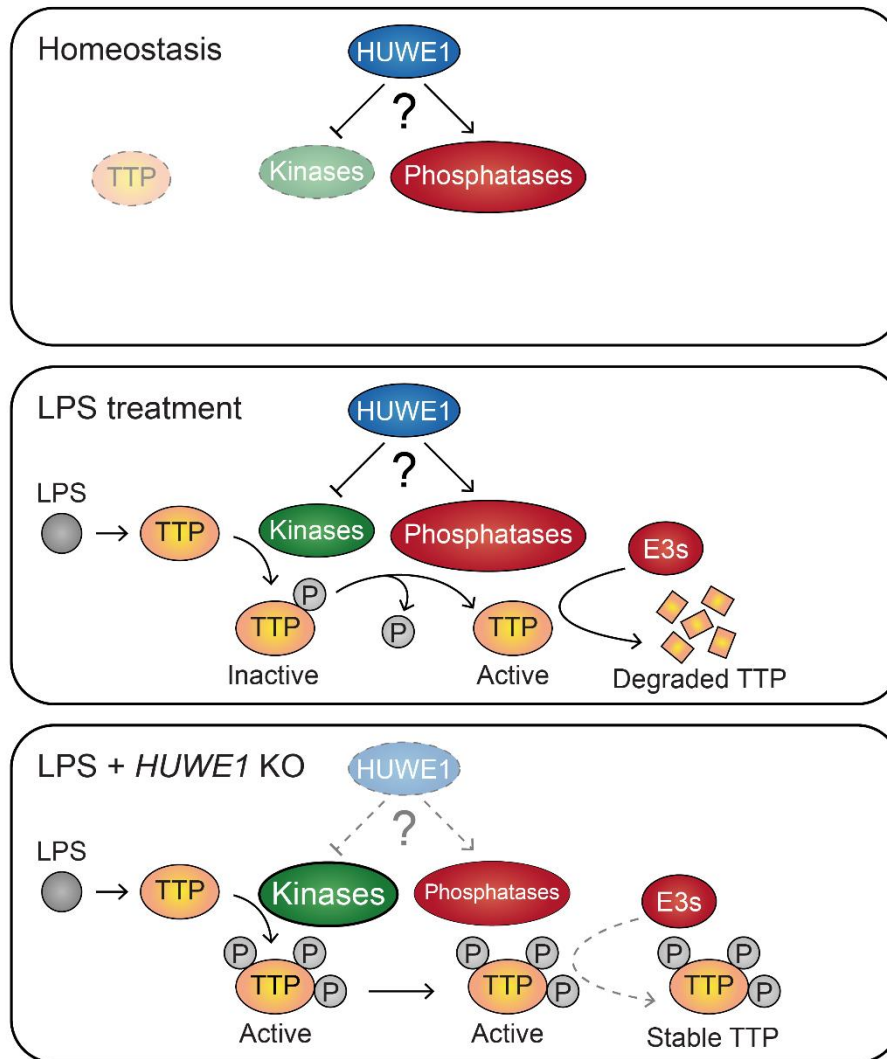


Figure 6 HUWE1 as a master regulator of TTP abundance. HUWE1 indirectly regulates TTP protein abundance via its phosphorylation, likely via the activity of multiple kinases and phosphatases. Under homeostatic conditions, TTP and immune-activated kinases are largely absent. Following LPS stimulation, TTP is phosphorylated by stress-activated kinases, which transiently stabilizes the protein but reduces its mRNA decay activity, before it is subsequently dephosphorylated and degraded. In contrast, in LPS-treated *HUWE1* knockout (KO) cells, TTP becomes extensively phosphorylated yet remains both stable and functionally active, underscoring the importance of previously underappreciated phosphorylation sites in controlling TTP turnover and activity.

control TTP phosphorylation events that is mechanistically distinct from previously described pathways.

Importantly, HUWE1 regulates TTP in a manner distinct from the only functionally well-characterised TTP phosphorylation, mediated by MK2. MK2 phosphorylates two serine residues in TTP, enabling 14-3-3 protein binding that sequesters TTP in the cytoplasm, stabilises it, and reduces its mRNA decay

activity^{628–631,712}. In contrast, our data suggest that the stabilised, phosphorylated TTP generated upon HUWE1 depletion remains functionally active. In LPS-treated cells, *HUWE1* ablation also stabilised a TTP-binding mutant previously shown to relocate from the nucleus to the cytoplasm upon stress-induced p38/MAPK-mediated phosphorylation⁶¹⁵, indicating that LPS-triggered phosphorylation promoted cytoplasmic localisation of this mutant similar to WT TTP. Notably, TTP binding to its mRNA targets was recently demonstrated to occur in the nucleus by analysing TTP mutants, including an NLS mutant restricted to the cytoplasm⁶¹⁵. This raises the possibility that phosphorylated TTP-forms in *HUWE1* KO cells may still access the nucleus to some extent, thereby retaining its activity. Together, our findings support a model in which HUWE1-regulated TTP phosphorylation events differs fundamentally from sole MK2-driven regulation, allowing TTP to remain active either through partial nuclear access or through atypical RNA binding in the cytoplasm (Fig. 6).

Adding to this complexity, LPS stimulation also alters p38/MAPK localisation, with phosphorylation exposing their NLSs that promote their nuclear import^{713–715}. These dynamic changes in kinase localisation could influence which phosphorylation sites on TTP are modified and thus how HUWE1 intersects with this regulation. Since we only measured total TTP levels, it will be important for future studies to separately analyse nuclear and cytoplasmic pools, alongside their phosphorylation states, degradation rates, and functional activities. This will be essential to fully dissect how HUWE1-driven regulation integrates into TTP's multi-site, multi-kinase regulatory network.

6.4.3 Timing, specificity, and remaining questions in TTP regulation

Our results suggest that HUWE1-regulated phosphorylation stabilises TTP without abolishing its activity, raising broader questions about how phosphorylation influences the timing and specificity of TTP-mRNA interactions. Although TTP is well established as a regulator of mRNAs with AU-rich elements in their 3'UTRs, it remains unclear exactly when it binds its targets and how this binding translates into functional outcomes.

One study proposed that TTP preferentially binds pre-mRNA in the nucleus, mainly based on enrichment of intronic reads among TTP-bound RNAs⁶¹⁵. However, TTP has also been found to bind AU-rich elements in introns^{201,610–612}, with the

biological significance for this binding remaining unresolved^{201,594}. While the study also reported examples of mRNAs bound by TTP in their 3'UTR that nonetheless showed intronic enrichment⁶¹⁵, this pattern may largely reflect intron binding rather than pre-mRNA preference. Moreover, as TTP was crosslinked to 4sU-labeled RNAs after a rather short pulse (1 hour), the pulldown data may have been biased towards nascent transcripts. Thus, whether TTP predominantly engages transcripts co-transcriptionally or post-splicing remains uncertain.

Adding to the complexity, many mRNAs bound by TTP in their 3'UTRs appear unaffected in stability and translation, suggesting that binding alone is insufficient to predict regulatory outcomes⁵⁹⁴. TTP's functional activity and binding capacity may instead be shaped by its phosphorylation state, interaction with protein cofactors, or its subcellular localisation, processes that HUWE1-driven regulation likely influence.

Taken together, our findings and previous work highlight TTP as a valuable model for dissecting the regulation and function of RBPs, particularly within innate immunity. TTP exemplifies how post-translational modifications such as phosphorylation can impact nearly every aspect of RBP biology, including RNA binding, subcellular localisation, ubiquitination, proteasomal turnover, and functional activity. By uncovering HUWE1 as a master regulator of TTP stability through phosphorylation-dependent mechanisms, our work expands existing models of TTP regulation beyond the well-described MK2 pathway. Still, key gaps remain in understanding how TTP engages and acts on specific mRNA targets, and how its multiple layers of regulation integrate to shape its activity. Addressing these questions will be critical to fully understand TTP's role as a post-transcriptional checkpoint in innate immune responses.

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8. Abbreviations

3'ss	3' Splice site
3'UTR	3' Untranslated region
4sU	4-Thiouridine
5'ss	5' Splice site
AID	Auxin-inducible degron
APA	Alternative polyadenylation
APC	Antigen presenting cell
AP-MS	Affinity purification mass spectrometry
ARE	AU-rich element
ASAP	ACIN1-RNPS1-SAP18 complex
AU-RBP	AU rich element RNA binding protein
Bp	Base pairs
CBC	Cap-binding complex
CBP	CREB-binding protein
CDK8	Cyclin-dependent kinase 8
CHX	Cycloheximide
ChIP	Chromatin immunoprecipitation
CK2	Casein kinase 2
CTL	Cytotoxic T lymphocyte
DAMP	Damage-associated molecular pattern
Dox	Doxycycline
EAE	Experimental autoimmune encephalomyelitis
eCLIP	Enhanced crosslinking and immunoprecipitation
EIF4A3	Eukaryotic initiation factor 4A-3
EJC	Exon junction complex

EMT	Epithelial–mesenchymal transition
ERH	Enhancer of Rudimentary Homolog
ERV	Endogenous retrovirus
GAS	Gamma IFN activation site
hnRNP	Heterogeneous nuclear RiboNucleoProtein
ICB	Immune checkpoint blockade
IDO	Indoleamine-2,3-dioxygenase
IDR	Intrinsically disordered region
IFN	Interferon
IL	Interleukin
ILC	Innate lymphoid cell
IR	Intron retention
IRF	Interferon regulatory factor
ISG	IFN-stimulated gene
ISRE	Interferon-stimulated response element
JAK	Janus kinase
KO	Knockout
LPS	Lipopolysaccharide
m7G	7-Methylguanosine
MAGOH	Mago-nashi homolog
MAPK	Mitogen-activated protein kinase
mRNA	Messenger RNA
mRNP	Messenger ribonucleoprotein
MS	Multiple sclerosis
NAR	Nucleic Acids Research
NEXT	Nuclear exosome-targeting

NHEJ	Non-homologous end joining
NK cells	Natural killer cells
NMD	Nonsense-mediated decay
NPC	Nuclear pore complex
p21	CDK inhibitor 1
PAMP	Pathogen-associated molecular pattern
PAS	Polyadenylation signal
PAXT	Poly(A) exosome-targeting
P-bodies	Processing bodies
PCNA	Proliferating cell nuclear antigen
PD-L1	Programmed death-ligand 1
PIAS	Protein inhibitor of activated STAT
Pol II	RNA polymerase II
POLDIP3	DNA polymerase delta interacting protein 3
PRR	Pattern recognition receptor
PSAP	PNN-RNPS1-SAP18 complex
PTC	Premature termination codon
PTP	Protein tyrosine phosphatase
RA	Rheumatoid arthritis
RBM8A	RNA binding motif protein 8A
RBP	RNA-binding protein
RNAi	RNA interference
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SAFB	Scaffold attachment factor B
SF1	Splicing factor 1

sgRNA	Single guide RNA
siRNA	Short interfering RNA
SjS	Sjögren's syndrome
SLE	Systemic lupus erythematosus
snRNP	Small nuclear ribonucleoprotein
smFISH	Single molecule fluorescence in situ hybridisation
SOCS	Suppressor of cytokine signalling
SPOP	Speckle type BTB/POZ protein
srHC	Sonication resistant chromatin
SRSF	Serine/arginine-rich splicing factor
S/T	Serine/threonine
STAT	Signal transducer and activator of transcription
TC45	T cell protein tyrosine phosphatase isoform
TCR	T cell receptor
TF	Transcription factor
Th1 cells	T helper 1 cells
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
Tregs	Regulatory T cells
TREX	Transcription-export complex
TSS	Transcription start site
TTP	Tristetraprolin
XL-MS	Cross-linking mass spectrometry