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Review Article

Host ADP-ribosylation and the SARS-CoV-2 macrodomain

Nicolas C. Hoch

Department of Biochemistry, University of São Paulo, São Paulo, Brazil

Correspondence: Nicolas C. Hoch (nicolas@iq.usp.br)

The COVID-19 pandemic has prompted intense research efforts into elucidating mechanisms of coronavirus pathogenesis and to propose antiviral interventions. The interferon (IFN) response is the main antiviral component of human innate immunity and is actively suppressed by several non-structural SARS-CoV-2 proteins, allowing viral replication within human cells. Differences in IFN signalling efficiency and timing have emerged as central determinants of the variability of COVID-19 disease severity between patients, highlighting the need for an improved understanding of host–pathogen interactions that affect the IFN response. ADP-ribosylation is an underexplored post-translational modification catalyzed by ADP-ribosyl transferases collectively termed poly(ADP-ribose) polymerases (PARPs). Several human PARPs are induced by the IFN response and participate in antiviral defences by regulating IFN signalling itself, modulating host processes such as translation and protein trafficking, as well as directly modifying and inhibiting viral target proteins. SARS-CoV-2 and other viruses encode a macrodomain that hydrolyzes ADP-ribose modifications, thus counteracting antiviral PARP activity. This mini-review provides a brief overview of the known targets of IFN-induced ADP-ribosylation and the functions of viral macrodomains, highlighting several open questions in the field.

Introduction

Viruses must infect a host cell in order to replicate, co-opting the cellular machinery to aid in the replication of its genetic material and for translation of viral proteins. Once infected, host cells activate innate immune responses, centred around a class of cytokines termed interferons (IFNs), that induce a cellular antiviral state that can inhibit several crucial steps of the viral replication cycle, essentially precluding the establishment of a viral infection [1]. Therefore, viruses have had to evolve intricate mechanisms to evade or suppress host IFN responses, leading to an evolutionary 'arms race' that shapes both host antiviral responses and viral evasion mechanisms [2]. Among the multitude of molecular mechanisms involved in host–pathogen conflicts [3], one recently emerging 'battleground' is the post-translational modification of proteins with ADP-ribose units, termed ADP-ribosylation, and its reversal by a virally encoded enzyme present in SARS-CoV-2 and other viruses [4,5]. This mini-review will describe how ADP-ribosylation, and its hydrolysis by viral macrodomains, can shape antiviral responses and discuss the rationale for the development of SARS-CoV-2 Nsp3 macrodomain inhibitors as novel antiviral therapeutics.

The type I IFN response is activated by a variety of sensor proteins, collectively termed pattern recognition receptors (PRRs), that each recognize the presence of different types of viral nucleic acids or other pathogen-associated molecular patterns (PAMPs) in different compartments within virus-infected cells [6,7]. Each sensor activates different signalling cascades that converge on the activation of the canonical IKK complex, comprised of IKKα, IKKβ and NEMO, and of the IKK-like kinases TBK1 and IKKε [1,8]. These kinases then phosphorylate the IRF3 and IRF7 transcription factors, as well as inducing NF-κB-dependent transcription, resulting in the expression of a range of

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pro-inflammatory cytokines, including interferons α (IFNα) and β (IFNβ), which collectively activate and regulate both innate and adaptive immune responses [9]. Secreted IFNs then bind to transmembrane IFN receptors both on the virus-infected cell itself and on neighbouring cells. This activates a secondary signalling cascade, initiated by IFN receptor-bound JAK kinases, which phosphorylate transcription factors of the STAT family, such as STAT1 and STAT2, leading to their translocation to the nucleus and subsequent induction of hundreds, if not thousands, of genes collectively termed interferon-stimulated genes (ISGs) [10]. Many ISGs participate in feedback mechanisms that regulate the magnitude of both primary and secondary IFN signalling cascades, while others have effector functions that either target viral factors directly, or modify host cell processes to inhibit viral replication or maturation [11].

Some ISGs are members of the poly(ADP-ribose) polymerase (PARP) family, which are a class of enzymes that utilize NAD^+ as a substrate to modify target proteins with ADP-ribose units, either as mono(ADP-ribose) or as poly(ADP-ribose) chains [12]. Although historically associated with DNA damage signalling and repair, ADP-ribosylation is emerging in recent years as an important regulator of cellular antiviral responses [4]. This is underscored by the fact that some of these PARP genes are under strong evolutionary pressure in the primate lineage, as often observed with genes engaged in host–pathogen conflicts [13,14]. The human genome encodes 17 members of the PARP family, characterized by the presence of a conserved PARP catalytic domain that is generally located at the C-terminus [12]. Analysis of publicly available data on gene expression changes induced by the IFN response suggests that of the human PARPs, five are strongly and reproducibly induced by IFN signalling (PARP9, PARP10, PARP12, PARP13 and PARP14) and at least another three (PARP7, PARP8 and PARP11) are often detected as ISGs in these large datasets (Figure 1) [15]. While most of these are bona fide ADP-ribosyl transferases that directly catalyze mono-ADP-ribosylation of target proteins, PARP13 (ZAP/ZC3HAV1) is catalytically inactive [16,17] and PARP9, which is also catalytically inactive in isolation [16], forms a heterodimer with the E3 ubiquitin ligase DTX3L that has mono-ADP-ribosyl transferase activity [18,19]. The next two sections describe the roles of these IFN-responsive PARPs, first in the context of feedback mechanisms that modulate IFN signalling itself, followed by their functions as antiviral effectors.

IFN-responsive PARPs and feedback regulation of IFN signalling

Given the central role of the IFN response as an antiviral signalling cascade, its activity is regulated and fine-tuned at every level by a variety of positive and negative feedback loops, many of which involve PARPs (Figure 2).

One such positive feedback is mediated by the shortest isoform of PARP13, known as ZAPS, which is IFN-responsive and favours IFN signalling by enhancing the activity of the pattern recognition receptor RIG-I, a critical sensor of viral RNA molecules [20]. Mechanistically, ZAPS depletion severely blunted activation of NF-κB and IRF3 in response to the RIG-I ligand 3pRNA, possibly due to reduced RIG-I oligomerization [20].

Similarly, depletion of the IFN-induced PARP14 also severely impairs IFN production in response to PRR agonists [21,22], although the nuclear accumulation of the IRF3 transcription factor was unaffected by PARP14 loss. Since the recruitment of RNA polymerase II to the IFNβ gene was impaired in PARP14 depleted cells [21], PARP14 is likely to promote IRF3-dependent transcriptional activation of its target genes, including the IFNβ gene, although the target protein(s) that it modifies in this context remain(s) unknown.

Downstream of IFN receptor binding, PARP9 and its heterodimeric partner, the E3 ubiquitin ligase DTX3L [23], are thought to promote STAT1-dependent induction of ISGs, potentially through modification of histones, but again the precise mechanism of action is currently unclear [24,25]. Interestingly, PARP9 and PARP14 were shown to have antagonistic roles in IFNγ-induced macrophage activation, with PARP9 promoting IFNγ-induced STAT1 phosphorylation, while PARP14 was shown to mono-ADP-ribosylate STAT1, reducing the transcriptional activation of ISGs [26]. Recently, we have shown that STAT1 phosphorylation and ISG induction are unaffected by PARP9 or DTX3L deletion, suggesting that this feedback mechanism may be context-dependent [27].

Another potential positive feedback mechanism is mediated by PARP12, whose expression is also IFN responsive [28]. PARP12 overexpression was shown to promote NF-κB-dependent transcriptional activation [28], although a more detailed mechanistic understanding of this effect is currently lacking.

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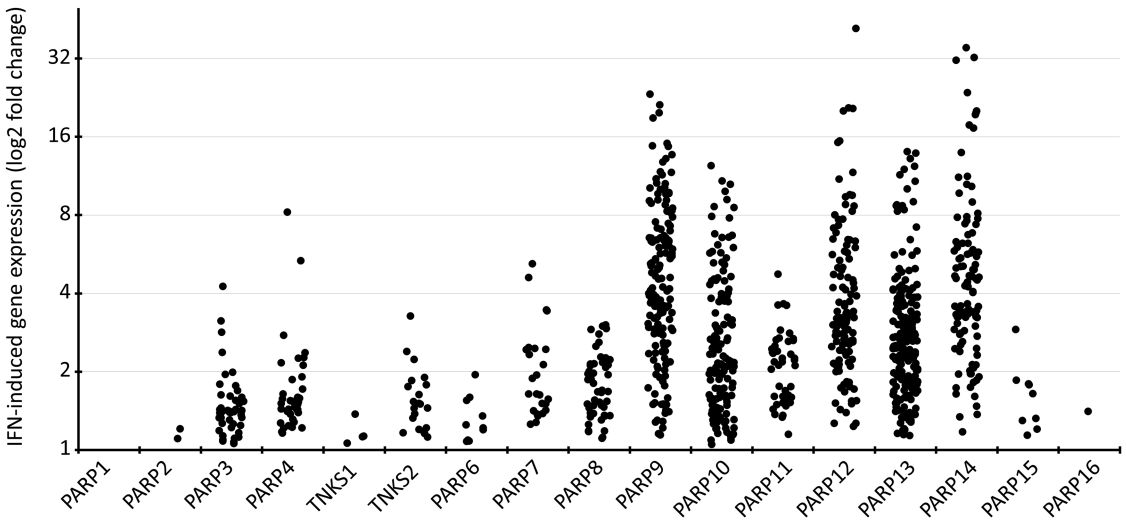

Figure 1. IFN-induced gene expression changes of PARP family members.

Data for each of the 17 human PARP genes was downloaded from the Interferome 2.0 database [13], which contains manually curated microarray datasets of type I, II or III IFN-induced genes. Each dot represents an entry in which a given PARP gene was found to be significantly up-regulated ($P < 0.05$) within 24 h of IFN treatment in human cells, irrespective of the treatment time, cell type or type of IFN used. Datapoints in which PARP gene expression was not significantly changed or down-regulated relative to baseline are omitted for clarity. Multiple data points from the same dataset are often present.

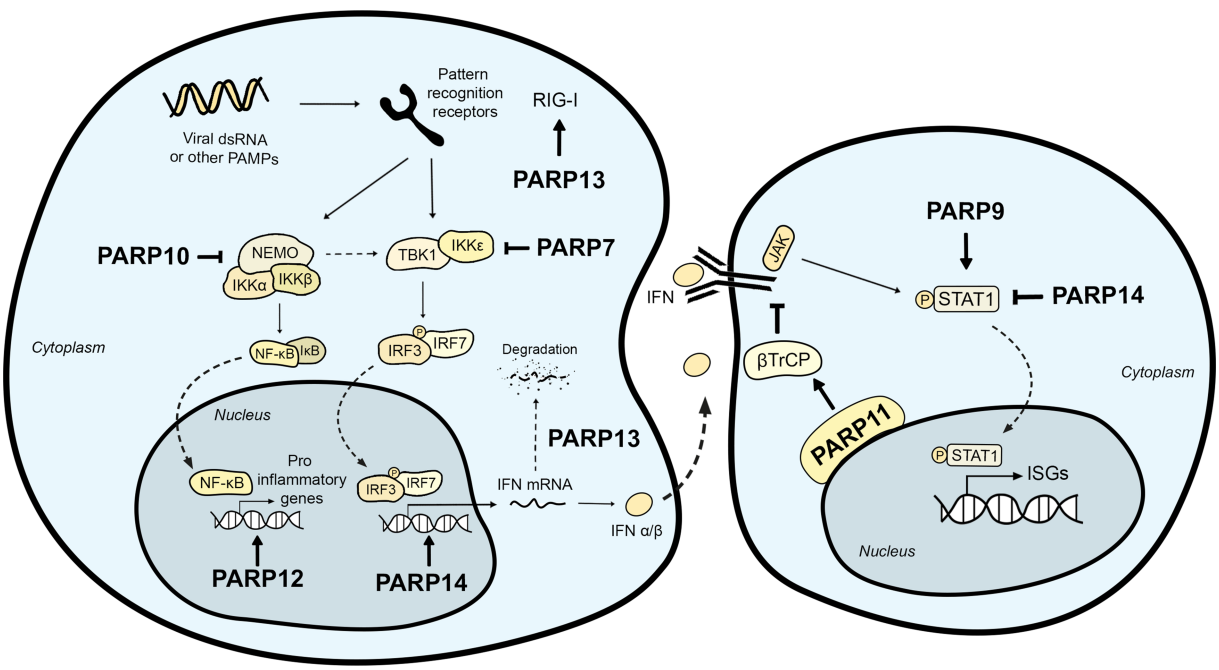

Figure 2. Schematic representation of the primary and secondary IFN signalling cascades, highlighting steps that are regulated by PARPs.

In the primary signalling cascade, PARP13 promotes RIG-I activation, PARP7 inhibits TBK1 and PARP10 inhibits NEMO-dependent signalling, whereas PARP12 facilitates NF-κB-dependent transcription, PARP14 promotes IRF3/7-dependent production of interferons and PARP13 restricts IFN mRNA levels. In the secondary cascade, PARP11 reduces IFN receptor levels, while PARP9 and PARP14 have antagonistic roles in STAT1-mediated induction of ISGs. Refer to the main text for further clarification.

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Conversely, PARP7 is one of a few PARPs shown to negatively regulate IFN signalling. PARP7 expression is strongly induced by activation of the aryl hydrocarbon receptor (AhR), but is recurrently observed as an IFN-responsive gene as well [22,29] and can be induced in virus-infected cells even in the presence of AhR inhibitors [30], suggesting that its expression is regulated by multiple pathways. Importantly, PARP7 depletion was shown to enhance IFN signalling, increasing type I IFN production and thus reducing the replication of several viruses [31]. Mechanistically, PARP7 seems to mono-ADP-ribosylate and inhibit the TBK1 kinase, thus reducing IRF3 phosphorylation and subsequent IFN production [31].

Likewise, PARP10, which is also an IFN-responsive PARP [32], negatively regulates signalling events in the innate immune response, although its effects on the primary IFN response have not been directly demonstrated yet. PARP10 is known to mono-ADP-ribosylate and inhibit NEMO, which is a central component of the IKK kinase complex required for the activation of NF-κB signalling [33]. Since NEMO can also promote TBK1-dependent phosphorylation of IRF3 in certain contexts [34], PARP10 may suppress IRF3-dependent IFN production in a similar fashion to PARP7.

Interestingly, the short isoform of PARP13, ZAPS, already mentioned above as a positive regulator, has also been implicated in a negative feedback affecting IFN signalling. ZAPS was found to bind to the mRNA of several of the IFN genes, promoting their degradation, such that ZAPS-depleted cells displayed a more prolonged IFN response [35].

Another negative feedback mechanism is mediated by PARP11, which was recently implicated in the regulation of type I IFN receptor levels. PARP11 suppresses the secondary IFN signalling cascade in response to type I IFN by mono-ADP-ribosylation and stabilization of β-TrCP, which is a ubiquitin ligase that targets the type I IFN receptor subunit IFNAR1 for proteasomal degradation [36]. Thus, PARP11 promoted the degradation of IFNAR1, reducing the responsiveness of cells to IFNα [36].

ADP-ribosylation and IFN-induced effector functions

While the above examples illustrate the diverse roles of ADP-ribosylation in modulating IFN signalling cascades (Figure 2), several PARPs can also have direct antiviral functions, modifying both host or viral proteins to supress viral replication (Figure 3).

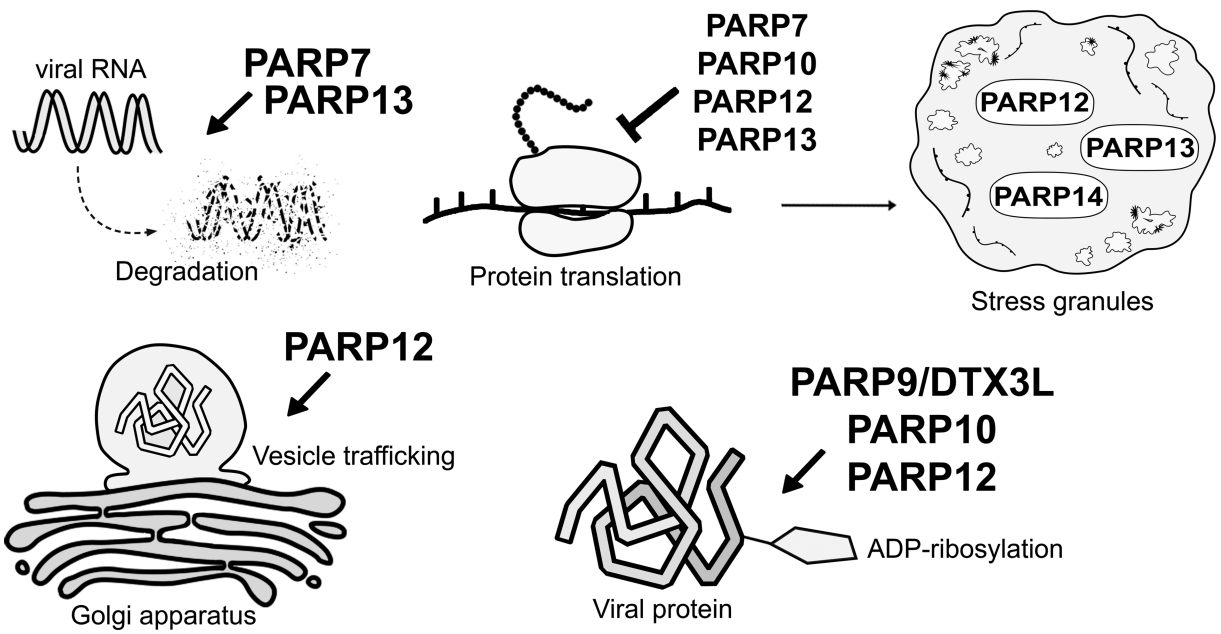

Figure 3. Schematic representation of the antiviral effector functions of PARPs.

PARP7 and PARP13 promote the degradation of viral RNA, and PARPs 7, 10, 12 and 13 restrict protein translation. PARPs 12, 13 and 14 localize to stress granules containing stalled translation complexes, and PARP12 promotes vesicle trafficking. PARP9/DTX3L, PARP10 and PARP12 can directly ADP-ribosylate and inhibit viral proteins.

One important strategy to restrict viral replication is the recognition and targeted degradation of viral RNAs. The best-studied example of this is PARP13, which can specifically bind viral RNAs, often based on their higher CpG content relative to host-derived RNA [37], targeting them for degradation by several mechanisms [38,39]. Interestingly, the long isoform of PARP13, ZAPL, seems to have a more prominent antiviral role than the shorter ZAPS isoform [35,40], potentially due to a different subcellular localization of the long isoform caused by a prenylation site [41]. Although these roles of PARP13 do not involve ADP-ribosyl transferase activity [42], recent efforts to identify PARP14 ADP-ribosylation targets identified several modification sites on PARP13, suggesting that PARP14 may regulate PARP13-dependent viral RNA decay [43]. Similar to PARP13, the N-terminal zinc fingers of PARP7 have also been shown to bind viral RNA for exosome-mediated degradation [44] without the requirement for PARP7 catalytic activity.

Another important antiviral mechanism is the inhibition of protein translation from viral mRNAs. The binding of PARP13 to viral RNAs can reduce their association with ribosomes, reducing their translation [45,46], and recent evidence suggests that specific ribosome frameshifting events, such as the ORF1a/b slippage event in SARS-CoV-2 can be regulated by PARP13 binding as well [47]. Strong inhibition of protein synthesis was also observed in cells overexpressing PARP7, PARP10 or PARP12L (the long isoform of PARP12) from virally encoded constructs, although a clear mechanistic understanding of this effect is currently lacking [28,29,48]. Interestingly, the IFN-responsive PARPs 12, 13 and 14 have been shown to localize to stress granules [28,49,50], which are thought to be cytoplasmic aggregates of stalled translation complexes induced by several forms of cellular stress, including viral infections [51]. While the function of stress granules, and the ADP-ribosylation of its components, are poorly understood, there is growing evidence for a central role of this structure in host antiviral mechanisms [52].

During their intracellular replication cycle, many viruses hijack cellular membrane trafficking mechanisms, such as the endocytic and the secretory pathways, for the transport of viral genomes or proteins throughout the cell [53]. Recently, PARP12 was shown to affect the intracellular trafficking of viral proteins, by promoting transport from the Golgi to the plasma membrane via ADP-ribosylation of Golgin-97 [49,54,55].

Direct modification of viral proteins with ADP-ribose has also been observed in several instances. PARP12 was shown to ADP-ribosylate Zika virus proteins NS1 and NS3, promoting their proteasomal degradation [56]. Similarly, the PARP9/DTX3L complex can modify the encephalomyocarditis virus 3C protease, leading to its degradation by the proteasome [24]. While ADP-ribosylation of the nucleocapsid protein was detected in several coronaviruses (CoVs), both the function of this modification and the PARP responsible for catalyzing it remain elusive [57]. Recently, PARP10 was shown to ADP-ribosylate the chikungunya virus Nsp2 protein, inhibiting its protease activity [58].

The viral macrodomain

Given the breadth of ADP-ribosylation targets that regulate cellular antiviral mechanisms (Figures 2 and 3), and the likely multitude of targets yet to be discovered, it is perhaps unsurprising that viruses have evolved mechanisms to counteract the activity of host PARPs. The catalytically inactive PARP13 is targeted for degradation or otherwise inhibited by a variety of viral mechanisms [4], while some viral families, such as CoVs and members of the alphavirus-like superfamiliy, encode a macrodomain that can bind and hydrolyze the ADP-ribose modifications catalyzed by host PARPs [5,59,60].

One open question is why particular families of viruses evolved a macrodomain to suppress host ADP-ribosylation, while others did not. A clear separation can be observed, even between closely related viruses, based on the presence of PARP enzymes that participate in innate immune signalling in the host species [61], but the question remains as to why other single-sense positive-strand RNA viruses that infect mammalian species have not evolved macrodomains, such as flaviviruses and picornaviruses, or the coronavirus-related arteriviruses. The presence of a macrodomain does not correlate with the type of vesicle these (+)ssRNA viruses utilize to protect replication intermediates from detection by host PRRs [62], but one possibility is that differences in viral mRNA capping mechanisms could account for this distinction. Alphavirus-like viruses (macro+) have a non-canonical mechanism of 5′RNA capping that differs both from the canonical pathway utilized by flaviviruses (macro−) and from the protein-capped structure employed by picornaviruses (macro−) [63]. For nidoviruses, including arteriviruses (macro−) and coronaviruses (macro+), the capping mechanism is incompletely understood, mainly due to the lack of an identifiable guanylyltransferase activity, although a recent study suggests that the RNA polymerase itself could catalyze this step in SARS-CoV-2 [64]. Interestingly, arteriviruses (macro−) and coronaviruses (macro+) likely have different cap

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methylation mechanisms [65], but a more precise definition of these differences in capping, and their effects on defining the PRRs involved in sensing nucleic acids produced during the lifecycle of these viral families will be required to clarify this issue.

In alphaviruses, the macrodomain is present at the N-terminus of non-structural protein 3 (Nsp3), which also contains a central zinc-binding domain and a C-terminal hypervariable domain (HVD) involved in protein–protein interactions [66]. Given that several PARPs localize to stress granules (above), and that the core stress granule component G3BP1 is ADP-ribosylated [50], it is particularly interesting that the alphavirus Nsp3 HVD binds to stress granule components [67]. Recently, the catalytic activity of the Nsp3 macrodomain was shown to regulate the composition of these stress granules [68], presumably to promote the release of host translation factors required for viral protein production. Alphaviruses harbouring macrodomain mutations that impair ADP-ribose binding or hydrolysis are severely attenuated due to reduced viral replication [69–71], although the mechanisms by which the macrodomain aids viral RNA replication are currently unclear. Interestingly, the Hepatitis E virus macrodomain is also required for viral RNA replication [72] and is located on ORF1, in close proximity to the helicase and RNA polymerase domains, suggesting a more direct role in the replication process itself.

In coronaviruses (CoVs), the macrodomain is contained in Nsp3, which is a large multidomain protein with slightly different domain architectures in different CoVs. Eight domains are strictly conserved: two ubiquitin-like domains (Ubl1 and Ubl2), a hypervariable region, a macrodomain (Mac1), a papain-like protease ($PL2^{Pro}$), the Nsp3 ectodomain, as well as the Y1 and CoV-Y domains of unknown function [73]. Nsp3 also contains two transmembrane regions that anchor the protein to the double-membrane vesicle (DMVs) that envelops the viral replication–transcription complex and segregates viral RNA replication from the host cell cytosol [74]. Nsp3 is essential for the formation of DMVs [75] and thus for viral replication, but only the Nsp3 ectodomain is positioned towards the interior of these vesicles, while all other domains — including the macrodomain — are thought to be cytosolic [73]. Consistent with that, Nsp3 macrodomain mutations that abrogate ADP-ribosyl hydrolase activity do not appreciably impair viral RNA replication itself [76,77]. Nonetheless, lack of macrodomain activity still severely attenuates mouse hepatitis virus (MHV) due to increased IFN signalling in host cells infected with the mutant virus compared with WT [22,76,78], suggesting a critical role for this domain in suppressing host IFN signalling. PARP12 and PARP14 were shown to be required for this increased IFN signalling in cells infected with macrodomain-mutated MHV, suggesting that the ADP-ribose modifications they catalyze may be the principal targets of this CoV macrodomain [22]. Interestingly, a recent study challenged this view via the identification of MHV macrodomain mutations that severely impaired viral replication, while only modestly affecting IFN suppression [79], likely due to differences between the effect of these mutations on ADP-ribose binding compared with ADP-ribosyl hydrolase activity [69–71,79].

In this context, it is important to note that both mono- or poly-ADP-ribose can be attached to different aminoacid residues, including glutamates, aspartates, lysines, arginines, serines or cysteines [80] and that the MacroD-type viral macrodomains discussed here are thought to selectively hydrolyze mono-ADP-ribose-modified glutamates and aspartates [70,81,82]. While the aminoacid preference of many PARPs is insufficiently defined, particularly for the less-well studied PARPs discussed here, there is evidence for the SARS-CoV-2 macrodomain acting on PARP10, PARP12 and PARP14-catalyzed modifications *in vitro* [58,61,83].

Recently, we have shown that ectopic expression of the SARS-CoV-2 macrodomain in isolation is sufficient to hydrolyze ADP-ribose modifications induced by IFN signalling in human cells [27]. Interestingly, this IFN-induced and macrodomain-sensitive ADP-ribosylation signal was completely dependent on the PARP9/DTX3L heterodimer and had no effect on either the primary or the secondary IFN signalling cascades, suggesting that this modification was an effector of IFN signalling. Since the PARP9/DTX3L heterodimer is known to ADP-ribosylate the C-terminus of ubiquitin [18] and Nsp3 contains ubiquitin-like domains and a deubiquitinating $PL2^{Pro}$ domain [84,85] in addition to the macrodomain, coronavirus Nsp3 may modulate host ADP-ribosylation and ubiquitination in a concerted manner.

SARS-CoV-2, IFN responses and the search for macrodomain inhibitors

In the course of the current COVID-19 pandemic, it has become increasingly evident that the adequate timing and robustness of the host IFN response is an important determinant of clinical outcome. Severe COVID-19 is often associated with defective IFN responses, such as the presence of autoantibodies against type I IFN [86] or

genetic predisposition caused by mutations in components of the IFN signalling cascade [87,88]. Similarly, reduced or delayed timing of IFN production relative to peak viral loads and/or aberrantly sustained IFN signalling in late stage disease are associated with poorer clinical outcomes [89–93]. Taken together with the fact that SARS-CoV-2 employs several strategies for suppression of host IFN signalling [89,94–96] and that early treatment with recombinant IFN can prevent disease in animal models of COVID-19 [97,98], it is clear that the competition between effective induction of IFN responses by host cells and viral mechanisms to counteract IFN signalling has important clinical consequences. Therefore, the search for pharmaceutical interventions that shift this balance via inhibition of viral IFN suppression mechanisms, such as inhibition of the Nsp3 papain-like protease [99], has garnered significant attention.

In this context, several efforts to develop SARS-CoV-2 Nsp3 macrodomain inhibitors are underway, on the assumption that the macrodomain is similarly critical for SARS-CoV-2 pathogenicity as previously described for related CoVs, although direct evidence for this is currently lacking. Our group has screened drugs already approved for human consumption [27], which would allow immediate application, but could not identify repurposed macrodomain inhibitors. Schuller et al. [100] undertook a large screen using both crystallographic and computational methods to identify low molecular mass chemical fragments that bind different portions of the SARS-CoV-2 macrodomain active site, which can subsequently be combined into larger molecules, whereas Brosey et al. [101] have modified inhibitors for the human ADP-ribosyl hydrolase PARG in search of compounds that target the related CoV macrodomain. Ni et al. [102] co-crystalized the macrodomain with a series of nucleotide analogues, identifying a remdesivir metabolite, while Virdi et al. [103] performed a high-throughput screen in libraries of drug-like compounds using differential scanning fluorimetry and Sowa et al. [104] developed a FRET-based assay for high-throughput screening of compound libraries. Despite some encouraging results, none of these efforts identified macrodomain inhibitors with sub-micromolar binding affinities yet.

Critically, to develop compounds that have broad activity against many if not all viral macrodomains, while sparing macrodomains contained in human proteins, it is important to identify structural differences between these macrodomains that can be exploited. Evolutionary divergence in active site residues substantially alters the mode of binding to the adenosine ring and distal ribose moieties, while different macrodomain families seem to employ completely different reaction mechanisms [61], suggesting that this should be achievable.

Conclusion

As highlighted throughout this mini-review, much remains to be learned about the roles of ADP-ribosylation in the regulation of IFN signalling and downstream antiviral mechanisms, as well as the importance of viral macrodomain-dependent reversal of these modifications for viral replication and pathogenesis. A better understanding of these mechanisms and the development of potent and specific compounds will be critical to test the clinical potential of viral macrodomain inhibitors to treat viral diseases and to prepare humankind for the next CoV pandemic that will inevitably strike in the coming years or decades.

Perspective

- ADP-ribosylation is emerging as an important regulator of innate immunity, that is targeted by some viruses to counteract host antiviral mechanisms.
- Much remains to be understood about the molecular targets of IFN-induced host ADP-ribosylation, and how viral macrodomain activity affects viral replication and pathogenesis.
- A better understanding of these processes will be necessary to assist the development of macrodomain inhibitors as a novel class of antivirals.

Competing Interests

The author declares that there are no competing interests associated with this manuscript.

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Abbreviations

CoV, coronavirus; DMVs, double-membrane vesicle; HVD, hypervariable domain; IFN, interferon; ISGs, interferon-stimulated genes; MHV, mouse hepatitis virus; PARPs, poly(ADP-ribose) polymerases; PRRs, pattern recognition receptors.

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