Mutations differentially affecting the coronavirus Mac1 ADP-ribose binding and hydrolysis activities indicate that it promotes multiple stages of the viral replication cycle

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ABSTRACT

All coronaviruses (CoVs) encode a conserved macrodomain, termed Mac1, in non-structural

protein 3 (nsp3) that binds and hydrolyzes ADP-ribose covalently attached to proteins. Mac1 is a

key virulence factor that counters antiviral ADP-ribosyltransferase (PARP) activity. Previously, we

found that MHV strain JHM (JHMV) with a mutation in the adenine binding site, JHMV-D1329A,

was extremely attenuated in all tested cell types as opposed to JHMV-N1347A, which only has a

replication defect in bone-marrow derived macrophages (BMDMs). Interestingly, an

N1347A/D1329A double mutant was unrecoverable, indicating an essential role for Mac1 in

JHMV infection. We hypothesized that these mutations may impact different stages of the MHV

lifecycle. First, to clarify how these mutations affected the biochemical activities of Mac1, we

generated Mac1 proteins encoding the same mutations. As expected, the D-A mutation was

extremely defective in ADP-ribose binding but maintained enzyme activity. In contrast, we

previously found that the N-A mutation had WT levels of ADP-ribose binding but low enzyme

activity, confirming that these mutations differentially effect the biochemical functions of Mac1.

Following infection, D1329A displayed a large defect in the accumulation of viral RNA compared

to WT or N1347A in all cells tested. Alternatively, N1347A infection produced normal levels of

viral RNA but produced reduced levels of viral protein in interferon competent bone-marrow

derived macrophages (BMDMs). These results suggest that Mac1 ADP-ribose binding and

enzymatic activities promote different stages of the viral lifecycle, demonstrating the critical

importance of Mac1 for JHMV replication.

IMPORTANCE

Over the last three decades, coronaviruses have repeatedly demonstrated their potential to become significant veterinary and public health threats. Zoonotic transmission of the myriad known coronavirus strains will remain a concern, regardless of the advances in vaccines and treatment. One difficulty in anticipating the next coronavirus outbreak is their diverse lineage and high propensity for mutation and recombination. The coronavirus macrodomain, Mac1, is conserved amongst all known coronaviruses and is also conserved in the *Togaviridae* and *Hepeviridae* families. Mac1 is a key factor in viral replication and pathogenesis, but its role in the replication cycle remains unclear. A deeper investigation of Mac1 function will identify conserved antiviral mechanisms and aid in the development of Mac1 inhibitors that represent a novel strategy for antiviral therapeutics.

INTRODUCTION

Coronaviruses (CoVs) are large, positive-sense ssRNA viruses with unusually large genomes ranging from 28-33 kb. Subdivided into four genera (alpha, beta, gamma, and delta), coronaviruses cause disease of clinical and veterinary significance in mammals and birds. Deadly infections in piglets with porcine endemic diarrhea virus illustrate the significant economic burden coronaviruses impose on the livestock industry (1); however, recent history demonstrates zoonotic transmission of CoVs to be an even greater economic and public health threat. Between 10-30% of circulating common cold viruses in humans are caused by CoVs like hCoV-229E and hCoV-OC43, which have a likely origin in zoonotic transmission from livestock(2, 3). The emergence of three highly pathogenic human CoVs within as many decades has placed the threat of novel zoonotic CoVs near the forefront of pandemic concern (4, 5). Efforts to determine the functions of highly conserved CoV proteins will help aid in developing novel therapies to help mitigate future outbreaks of divergent CoVs.

The CoV genome is organized into two major segments encoding the non-structural and the structural proteins. Structural proteins such as the spike and nucleocapsid protein are encoded in transcriptionally "nested" ORFs at the 3' end of the genome. The non-structural proteins (nsps) are encoded at the 5' end in two large co-terminal ORFs that comprise about two-thirds of the genome. Nsps are translated as large polyproteins from either ORF1a or from ORF1ab which are then proteolytically processed into each of the functional nsps. Translational regulation between these two ORFs is achieved through ribosomal frameshifting bypassing a conserved stop codon. It is thought this strategy is meant to maintain optimal stoichiometric ratios between proteins important for blocking immunity or establishing viral infection and the proteins that constitute the RNA replication machinery (6).

Nsp3 is the largest of the coronavirus nonstructural proteins with a mass of approximately 220

kDa. Nsp3 contains numerous domains with important characterized functions. These include

polyprotein cleavage and deubiquitinase (DUB) activity of PL^{pro}, ssRNA and nucleocapsid binding

of Ubl1, ADP-ribosylhydrolase activity of the conserved macrodomain, and formation of

replication organelles (ROs) (7–10). Most notably, nsp3-4 dimers form large hexamers that, on

one side of the ER double membrane, complex with the replicase holoenzyme and, on the other

side, comprise a pore through which newly synthesized viral RNA is shuttled into the cytoplasm(9,

11–13). These findings establish nsp3 as a platform for multiple key steps of the replication cycle,

including the establishment of the RO, initial RNA replication, viral protein processing, and

packaging. Interestingly, in SARS-CoV-2 infection, nsp3 has also been shown to be distributed at

low levels throughout the cytoplasm, suggesting it may play additional roles in infection (14).

Within nsp3 reside up to three macrodomains termed Mac1, Mac2, and Mac3. Macrodomains are

a class of conserved protein domains present in all forms of life. Their structure, characterized by

a mixed α-β-α sandwich fold, is highly conserved, however their sequences – and therefore their

functions – are diverse (15). Mac2 and Mac3, originally identified in SARS-CoV as part of the

SARS-CoV Unique Domain (SUD), are present only in a subset of CoVs, most notably the

Sarbecoviruses, and were demonstrated to bind nucleic acid (16). Mac1, on the other hand, is

highly conserved across coronaviruses and has been shown to be a key virulence factor. Belonging

to the MacroD-type class of macrodomains, the CoV Mac1 targets and hydrolyses ADP-ribose

moieties, reversing a post-translational modification known as ADP-ribosylation (17–19).

ADP-ribosylation, the process by which ADP-ribose is covalently attached to target proteins, is

facilitated by enzymes known as ADP-ribosyltransferases (ARTs), commonly referred to as

PARPs. With 17 members in mammals, the PARP family regulates a myriad of cellular processes

such as stress granule formation, cellular trafficking, DNA damage repair, gene expression, and

cell cycle progression (20). Additionally, the importance of PARPs in the innate antiviral response

is increasingly clear. Many of the mono-ADP-ribosylating (MARylating) PARPs are IFN-

stimulated genes (ISGs) that modulate the inflammatory response and restrict viral replication (21–

23). Importantly, ADP-ribose can be added to several different types of residues, including basic

and acidic residues, as well as serine and cysteine (24, 25). Importantly, most macrodomains can

only remove ADP-ribose from acidic residues but still bind to ADP-ribose attached at other

residues. Thus, both ADP-ribose binding and hydrolysis can separately impact biological

functions. In CoVs, Mac1 activity is required for IFN repression and efficient viral replication,

though the exact targets remain unclear (26–31). Mac1 catalytic activity relies on a highly

conserved asparagine (residue N1347 in mouse hepatitis virus (MHV) strain JHM (JHMV)) which

coordinates critical hydrogen bonds with the distal ribose (32, 33). Previous characterizations of

an alanine mutation at this site abolished the catalytic activity of Mac1 and sensitized the virus to

type-I IFN (IFN-I) signaling but did not impact its ability to bind ADP-ribose (29, 34). In JHMV,

a N1347A mutant virus replicated normally in IFN-deficient cells such as 17Cl-1 but was

attenuated in IFN-competent bone-marrow derived macrophages (BMDMs). Importantly the

replication defect was lost in IFNAR knockout cells, demonstrating that Mac1 catalytic activity

counters IFN-mediated antiviral responses (34). Soon after, we also generated an alanine mutation

at D1329 in JHMV, a residue predicted to coordinate stabilizing interactions with the adenine

critical for substrate binding. This mutant demonstrated a large replication defect in both IFN-

competent and IFN-deficient cells, suggesting differential roles for the binding and hydrolysis

activity of Mac1 (29). Mutation in both residues resulted in a non-replicating virus that was unable

to be launched *de novo.* Unsurprisingly, a full in-frame deletion of Mac1 from either JHMV or

MERS-CoV resulted in unrecoverable viruses. Interestingly, however, SARS-CoV-2 tolerated the deletion, but was sensitive to IFN-γ pre-treatment, similar to the SARS-CoV-2 N1062A mutant virus (31). This result indicates that JHMV and MERS-CoV have a strict requirement for Mac1 ADP-ribose binding while SARS-CoV-2 may have evolved to encode for a protein with redundant function. We recently found that mutation of a conserved isoleucine in MERS-CoV and SARS-CoV-2 conferred enhanced Mac1 binding affinity to ADP-ribose with no impact on catalytic activity but resulted in an overall reduction in viral replication (35). This data together indicates that Mac1 is a critical factor in CoV replication and fitness during infection but relies on finely tuned biochemical properties to promote virus replication and pathogenesis.

In this study, we sought to define the stages of the viral replication cycle reliant on both known biochemical functions of Mac1, binding and catalysis. As predicted, we found that purified SARS-CoV-2 and MERS-CoV D-A mutant proteins demonstrated a stark decrease in substrate affinity which contrasted with the previously published N-A mutant protein. However, it retained substantial enzymatic activity as opposed to the N-A mutant. Thus, the D-A and N-A Mac1 mutants provide suitable models to better understand the role of ADP-ribose bindings versus hydrolysis in the viral lifecycle. In cell culture, the JHMV D1329A mutant virus showed a marked defect in RNA production, while the N1347A mutant virus resembled WT virus in RNA replication kinetics but was significantly reduced for protein accumulation in IFN-competent cells. These results provide a more refined understanding of the multiple mechanisms that Mac1 utilizes to promote CoV replication.

RESULTS

Mutation of conserved aspartic acid and asparagine residue to alanine dramatically reduces ADP-ribose binding but maintains ADP-ribosylhydrolase activity. Two highly conserved residues in JHMV Mac1, N1347 and D1329 **(Fig. 1A)** were previously demonstrated to impact viral fitness and virulence, though to different degrees and in a cell-type specific manner (29). To compare the biochemical properties of the D-A mutation with the previously characterized N-A mutation (29, 35), we performed several previously established ADP-ribose binding and hydrolysis assays using purified SARS-CoV-2 and MERS-CoV Mac1 WT and D-A proteins. We and others have been unable to produce MHV Mac1 protein, so SARS-CoV-2 and MERS-CoV proteins were used instead. ADP-ribose binding assays include a differential scanning fluorimetry (DSF) assay that measures thermal stability, a proximity-based AlphaScreen assay, and isothermal calorimetry (ITC) which measures the thermodynamic properties of binding. Thermal stability of the WT-Mac1 protein was demonstrated to be approximately 6°C higher than that of the D-A protein at the endpoint concentration of 1M ADP-ribose **(Fig. 1B)**. In the AlphaScreen assay, the D-A protein had a multi-log reduction in alpha counts compared to the WT protein **(Fig. 1C)**. Finally, isothermal titration calorimetry (ITC) data showed that the K_D of Mac1 D-A to ADP-ribose was ~16-fold higher than the WT-ADP-ribose interaction **(Fig. 1D)**. We also saw similar results for each of these assays using MERS-CoV Mac1 WT and D-A proteins **(Fig. S1)**. These data together demonstrate a marked defect in the Mac1 D-A protein's ability to bind to ADP-ribose and distinguishes the D-A mutant from the N-A mutant, which was previously shown to bind ADP-ribose at near WT levels (35). In addition, we performed an *in vitro* ADP-ribosylhydrolase assay by co-incubating purified SARS-CoV-2 Mac1-WT and D-A proteins with mono-ADP-ribosylated (MARylated) PARP10 and measured the loss of MAR signal from PARP10 by Western blotting,

as previously described (27). We found that Mac1-D-A retained substantial enzyme activity which

contrasts with Mac1-N-A which we previously found to be largely devoid of catalytic activity **(Fig.**

1E) (35) These results are consistent with results from alphavirus macrodomain studies. These

biochemical studies provide a stark delineation between the D-A and N-A mutant proteins as being

deficient in either binding or hydrolysis, respectively.

MHV D1329A/D1330A virus is attenuated in cell culture and *in vivo*. Previous work with the

JHMV single-nucleotide D1329A mutant virus revealed a high propensity for reversion *in vivo*,

indicating a high fitness cost compared to other viable Mac1 mutants (29). To prevent this

reversion, the JHMV-D1329A mutant virus was regenerated to contain a two-nucleotide

substitution using a bacterial artificial chromosome (BAC) based reverse genetic system (36). We

produced this virus in cell culture and confirmed that it had no reverting mutations. Furthermore,

to confirm that the defect is not due to second-site mutations produced during cloning, we

reintroduced the WT Mac1 sequence into the JHMV-D1329A BAC which was termed the

"repaired" D1329A (*rep*D1329). In addition, we fully sequenced the MHV genome from terminal

passages of D1329A and found no additional mutations in the viral genome. At low (0.1) and high

(1.0) MOI, this new JHMV-D1329A mutant virus demonstrated the same multi-log replication

defect in DBTs when compared to *rep*D1329 and N1347A viruses as previously described **(Fig.**

2A-B) (29). Importantly, intranasal infection of C57BL/6 with JHMV-D1329A resulted in no

lethality or weight loss as compared to infection with *rep*D1329 which resulted in ~25% mouse

survival and an average of more than 10% weight loss. These results indicate that this two-base

pair mutant D1329A virus, unlike the single-base pair mutant, was unable to revert to WT *in vivo*

(Fig. 2C-D). We also engineered the orthologous mutation into MHV-A59, D1330A. The A59-

D1330A virus had a mild replication defect in cell culture **(Fig. 2E-G)** but replicated poorly *in*

vivo compared to WT virus **(Fig. 2H)**, indicating that Mac1 ADP-ribose binding is critical across

multiple strains of MHV.

JHMV-D1329A produces small plaques and demonstrates reduced infectivity in mouse cells

compared to *repD1329* and N1347A viruses. The first notable hallmark of the JHMV-D1329A

virus infection was a substantial reduction in overall plaque size in both DBT and L929 cells **(Fig.**

3A-B). We quantitated the size of D1329A plaques as compared to *rep*D1329 and N1347A viruses

and found that the D1329A plaques were on average about half the size of the plaques produced

by *rep*D1329 and N1347A on both DBT and L929 cells **(Fig. 3C-D)**. JHMV plaques are formed

by cell-cell syncytia mediated by the S protein (37). Thus, this phenotype is likely due to a severe

reduction in the production of S protein or its ability to traffic to the cell surface. A severe lack of

S protein production could be due to the inability to initiate infection in some cells. To determine

if JHMV-D1329A has a defect in initiating a productive infection we performed a plaquing

efficiency assay using DBT, L929, and HeLa cells expressing the MHV receptor (Hela-MVR).

Equal plaque forming units (PFU) of *rep*D1329, D1329A, and N1347A were plated on each cell

type and the number of plaques were determined. D1329A produced ~5-10-fold fewer plaques on

both DBT and L929 cells as compared to *rep*D1329 and N1347A, indicating that D1329A has a

substantial defect in the ability to initiate a productive infection. Interestingly, this defect may be

species specific, as no defect was observed in Hela-MVR cells which are of human origin but

express the MHV receptor **(Fig. 3E)**.

JHMV-D1329A has no defect in genomic RNA delivery into cells. To determine if the inability

of D1329A virus to initiate infection stems from a reduction in the entry of viral RNA into the

cytoplasm of infected cells, we measured intracellular genomic RNA levels at 2 hpi via RT-qPCR.

Each of the three indicated cell lines were infected with JHMV-*rep*D1329 and D1329A for two

hours; after which, the cells were trypsinized and washed three times with PBS to remove non-

infective virions then resuspended in TRIzol™ Reagent (Invitrogen). RT-qPCR revealed no

significant reduction in D1329A gRNA levels when compared with *rep*D1329, suggesting that the

plaquing defect is not due to a defect in viral entry **(Fig. 4)**.

JHMV-D1329A virus is defective in genomic and sub-genomic RNA accumulation compared

to *rep*D1329 and N1347A viruses. Next, we assayed the accumulation of viral genomic and sub-

genomic RNAs in both high and low MOI infections of DBT and L929 cells. Using RT-qPCR,

viral genomic- RNA (gRNA) and sub-genomic RNA6 (sgRNA6) levels were quantified over a 20-

hour period **(Fig. 5A-B)**. We used sgRNA6 as a marker for sub-genomic RNAs as the primers for

sgRNA6 gave the most robust results in our qPCR assay. At high MOI, the N1347A virus closely

resembled the *rep*D1329 virus, as viral sgRNA6 and gRNA levels increased substantially between

4 and 12 hpi in DBTs, while D1329A infected cells failed to produce any new viral transcripts

before 12hpi. This difference was most notable in sgRNA6, as *rep*D1329 and N1347A transcripts

were increased by nearly 3-logs over the D1329A virus. The D1329A infected cells did show

increasing RNA production after 12hpi, indicating a delay in transcription initiation, though the

levels never reached that of *rep*D1329 or N1347A infected cells **(Fig. 5A)**. Similarly, infection of

L929s showed approximately 10 to 100-fold increases in gRNA and sgRNA6 levels in the

*rep*D1329 and N1347A infected cells compared to D1329A throughout the course of infection

(Fig. 5B). Similar results were seen at low MOI **(Fig. S2)**. To determine whether the defect in

D1329A RNA accumulation was due to targeted degradation of viral transcripts, infected L929

cells were treated with the Remdesivir metabolite GS-441524 at 10 hpi to arrest RdRp-mediated

transcription (38), after which viral transcripts were quantified at four-hour intervals until 22hpi

(Fig. 5C). GS-441524 was not added until 10 hpi as earlier addition may have limited the amount

of RNA produced during D1329A infection making it unfeasible to detect RNA degradation. Viral

RNA synthesis increased in GS-441524 treated cells for a few hours, after which they stabilized,

as compared to RNA levels in untreated cells, which continued to increase. In both the *rep*D1329

and D1329A infections treated with GS-441524, there was no evidence of RNA degradation as

RNA levels did not significantly decrease through the course of the infection, indicating that there

is no increased degradation of the D1329A transcripts compared to *rep*D1329.

As a separate method to analyze viral RNA production, we performed confocal immuno-

fluorescence microscopy with probes for dsRNA, the hallmark RNA replication intermediate **(Fig.**

6A). DBT cells were infected with *rep*D1329, D1329A, and N1347A at an MOI of 0.5 and cells

were fixed and stained for nsp3 and dsRNA at 8 hpi. While mock infected cells were devoid dsRNA

signal, *rep*D1329 and N1347A infected cells demonstrated robust dsRNA staining that co-localized

with nsp3, indicating the dsRNA represented viral RNAs. In contrast, D1329A infected cells had

minimal dsRNA despite having significant nsp3 staining. Quantitation of these images confirmed

that the D1329A virus produced significantly reduced levels of dsRNA but only mildly reduced

levels of nsp3 **(Fig. 6B)**. In addition, we measured the proportion of dsRNA that overlapped with

nsp3 (labeld as M1) and conversely the proportion of nsp3 that overlapped with dsRNA (labeled

as M2) in each image using a Mander's coefficient. We found that wherever dsRNA staining was

present, it almost always overlapped with nsp3 staining from each virus **(Fig. 6C, M1-green)**.

However, there were a couple of cases in D1329A infected cells where nsp3 staining did not

contain any overlapping dsRNA staining, unlike *rep*D1329 or N1347A **(Fig. 6C, M2-red)**. This

result indicates that D1329A infected cells produced nsp3 but were unable to efficiently produce

RNA. N1347A, conversely, appeared to produce similar levels of nsp3 and dsRNA staining

compared to *rep*D1329. To further determine if D1329A can efficiently translate non-structural

proteins, we stained for nsp3 at 6 hpi, the earliest time point that we could detect it. Imaging

demonstrated that D1329A produced nsp3 at these early time points at levels that were reduced

but not significantly different from *rep*D1329 and N1347A **(Fig. S3A-B)**. The reduction in nsp3 is

likely due to already reduced RNA production in these cells, as more genomic RNA would lead to

increased production of nsps for the *rep*D1329 or N1347A viruses. In combination, these results

suggest that Mac1 ADP-ribose binding is required for efficient viral transcription initiation in

murine cells, whereas hydrolysis activity is likely dispensable in the earliest stages of replication.

N1347A infected primary bone-marrow derived macrophages (BMDMs) produce WT levels

of viral RNA but have reduced N protein accumulation compared to *rep*D1329 virus. We

previously demonstrated that both N1347A and D1329A mutants have similar replication defects

in primary macrophages when infected at low MOI, which could be partially rescued by treatment

with PARP inhibitors (29). However, N1347A replication could also be rescued in IFNAR and

PARP12 knockout cells, while D1329A replication was not, indicating these viruses may impact

different parts of the viral lifecycle in these cells (31, 34). First, we tested whether these viruses

had replication defects at high MOI so we could evaluate the viral lifecycle within a single cycle

of replication. As expected, both viruses had replication defects in BMDMs when infected at an

MOI of 1.5 **(Fig.7A)**. Specifically, D1329A virus titers were reduced almost 14-fold from

*rep*D1329, while N1347A titers were reduced 5-fold. Next, we evaluated the accumulation of viral

gRNA and sgRNA6. D1329A infection resulted in a significant reduction in both gRNA and

sgRNA6 levels at 9 hpi, while gRNA and sgRNA6 levels following N1374A infection were similar

to those of *rep*D1329, as seen in both DBT and L929 cells **(Fig. 7B)**. Specifically, sgRNA and

gRNA levels were mildly reduced at 9 hpi but increased at 12 hpi following N1347A infection,

though these differences were not significant. However, when we evaluated the production of viral

structural proteins, N1347A infected cells produced reduced levels of nucleocapsid (N) and spike

(S) proteins at 9 and 12 hpi **(Fig. 7C)**. When quantitating viral protein levels from multiple

experiments, the amount of N protein was significantly reduced almost 4-fold at 9 hpi compared

to *rep*D1329 infection, while at 12 hpi N protein was reduced 1.5-fold **(Fig. 7D)**.

Additionally, we evaluated nsp3 and N protein abundance by confocal

immunofluorescence in BMDMs. BMDMs were infected at an MOI of 0.5 before fixation and

staining for nsp3 and N protein at 9 hpi **(Fig. 8A)**. Nsp3 puncta were again observed across all

three viruses. Quantification showed the D1329A infected cells had 7.5-fold less nsp3 staining

compared to *rep*D1329, while nsp3 levels were only reduced 1.6-fold in N1347A infected cells,

neither of which were significant **(Fig. 8B, middle)**. In fact, in several cells infected with D1329A,

the level of nsp3 was at or just below the mean of the *rep*D1329 infected cells, again demonstrating

that D1329A can produce sufficient levels of nsp3 early in infection. This is in contrast to N protein

staining, which was reduced nearly 100-fold in D1329A infected cells and almost 10-fold in

N1347A infected cells **(Fig. 8B, right)**. However, the level of N protein staining in N1347A

infected cells was highly variable within individual cells, with some cells producing normal levels

of N protein, while others were dramatically reduced. As N1347A is restricted by IFN-induced

PARPs (34), these results suggest that the levels of PARP induction between individual cells may

fluctuate, resulting in inconsistent reductions in N protein production compared to *rep*D1329. But

in general, N1347A infected cells produced less N protein, but not nsp3, than the *rep*D1329

infected cells. These observations suggest that initial nsp3 synthesis is not substantially impacted

by Mac1, but that subsequent viral RNA or late protein production relies on the binding and

hydrolysis activities, respectively, of Mac1 to proceed efficiently.

DISCUSSION

The current understanding of the conserved CoV macrodomain relies largely on its characterization as an ADP-ribosylhydrolase. Previous work has highlighted the importance of PARP activity and Mac1's de-ADP-ribosylating activity on viral fitness and pathogenesis in both coronaviruses and alphaviruses (39). We have previously generated alanine mutations in several conserved residues of the Mac1 binding pocket. We found that mutation in a conserved asparagine residue important for hydrolysis activity, N1347A in JHMV, attenuated the virus in primary macrophages and sensitized it to PARP activity. We also found that mutating another conserved residue predicted to coordinate binding affinity, D1329, conferred a greater replication defect for this virus. Finally, we have been unable to recover JHMV that incorporates both mutations or a full Mac1 deletion. These findings led us to hypothesize that Mac1 is critical for JHMV infection and serves multiple roles in the viral replication cycle.

In this study, we sought to determine the stages of the viral life cycle most reliant on the binding and hydrolysis activity of Mac1 in an effort to better understand the roles Mac1 plays in infection. To characterize the biochemical impacts of the conserved aspartic acid and asparagine residues in the ADP-ribose binding pocket, we utilized purified SARS-CoV-2 and MERS-CoV Mac1 proteins with each mutated to alanine and then performed *in vitro* ADP-ribose binding and hydrolysis assays. These data demonstrated a stark delineation between these mutant Mac1 proteins and their ADP-ribose binding and hydrolysis activities. Previous work from our group established the N-A mutant Mac1 suffered little to no loss in substrate binding affinity but had greatly reduced hydrolysis (35) while here we found that the D-A mutant Mac1 had a multi-log reduction in binding affinity, but only a slight reduction in hydrolysis. Importantly, similar results were demonstrated by other groups with SARS-CoV-2 mutant proteins and with recombinant

alphavirus macrodomain proteins as well (33, 40). Next, we sought to compare the impact of both

mutations on viral replication in the early viral life cycle. To achieve this, we utilized the previously

generated JHMV-N1347A virus and re-generated a new clone of the previously studied D1329A

virus. This new clone was generated via a two base-pair substitution in the D1329 codon rather

than one to reduce the likelihood of reversion. As expected, this virus demonstrated severe

attenuation in cell culture and *in vivo*. Through the detection of dsRNA by confocal microscopy

and specific determination of viral genomic and sub-genomic RNA levels by qPCR, we found that

the D1329A virus does not efficiently initiate viral RNA production following infection of any

murine cell line that is susceptible to JHMV **(Figs. 5-6)**. Furthermore, the D1329A virus produced

similar amounts of nsp3 in BMDMs as *rep*D1329 virus, and puncta of nsp3 staining in D1329A

infected cells often lacked any notable dsRNA staining. In contrast, the N1347A virus replicated

viral RNA at levels equal to that of WT virus in all cell lines tested but had a notable defect in viral

protein production in primary BMDMs **(Fig. 7)**. These data indicate that the binding activity of

Mac1 is key for early RNA production, while the hydrolysis activity is important for accumulation

of viral proteins later on in the replication cycle. However, we cannot rule out the possibility that

other defects, such as in polyprotein processing or assembly, may also be present. In fact,

considering the level of N protein in N1347A infected BMDMs was almost back to WT levels at

12 hpi, it seems likely that there may be further defects in the assembly process for this virus.

Below we present several hypotheses for how each activity may promote replication.

ADP-ribosylhydrolase activity. We have demonstrated that PARP12 and PARP14 are both required

to restrict the replication of CoVs with catalytically inactive Mac1 (21, 31, 41). Others have found

that PARP14 is required for the production of ADP-ribose puncta following IFNγ or poly(I:C)

treatment (34). Within these puncta, both PARP14 and DTX3L, an E3 ubiquitin ligase, are likely

ADP-ribosylated, with DTX3L/PARP9 regulating the activity of PARP14 (42, 43). As an E3

ubiquitin ligase, it's possible that DTX3L ADP-ribosylation enhances its ability to target viral

proteins for degradation. However, there are many other potential targets of PARP14, such as p62,

an autophagy regulator, and RACK1, a ribosome binding protein, that could impact viral protein

production through autophagic degradation or repression of translation, respectively (44, 45).

Furthermore, PARP12 is localized to the Golgi and cell stress can trigger PARP12 movement from

the Golgi and a block in anterograde-membrane trafficking, which could impact viral assembly

(46). Further work is required to decipher which PARP12/14 target(s) restrict viral protein

production or assembly following infection with Mac1 catalytic-mutant CoVs.

ADP-ribose binding activity. We envision two separate but not mutually exclusive hypotheses for

how this Mac1 function promotes viral RNA production. Recent work has demonstrated that nsp3

plays a major role in forming a hexameric complex that constitutes a pore in the replication

organelle, through which viral RNA is thought to pass (9, 11). As part of the larger pore structure,

the N-terminal domains of nsp3 extend into the cytoplasm, where one or more subunits may

interact with a number of host and viral factors. Previous work has established the association of

nsp3 with the nucleocapsid protein as a critical factor in establishing CoV infection (8, 47, 48).

Current models postulate that, since CoV gRNA serves as both the initial mRNA for nsp translation

and the template for RNA replication, N protein may shuttle gRNA into the replication organelle

via the nsp3 pore (8). Mutational analysis from several groups have demonstrated this relies largely

on the interaction of the N-terminal Ubl1 of nsp3 with multiple phosphorylated residues in the

serine and arginine-rich regions N1b and N2a of N protein (47–49). Other characterizations of the

CoV nucleocapsid have revealed that it is also ADP-ribosylated (50). From the current data, it

appears that the N protein is ADP-ribosylated only during infection, and the modification is

retained in the virion. Moreover, the modification was shown to be unaffected by catalytically

active Mac1. Thus, in our first hypothetical model, Mac1 would interact with an ADP-ribosylated

N protein, to promote the translocation of either the viral genome or N protein through the pore

(Fig. 9A). Since N protein phosphorylation is reliant on host kinases (51) and ADP-ribosylation

may compete with phosphorylation on some residues, perhaps Mac1 binding to ADP-ribosylated

N evolved as an alternative measure to maintain the interaction between N protein and nsp3.

Identification of the sites of N protein ADP-ribosylation and mutation analysis would help clarify

how ADP-ribosylation affects N protein's ability to interact with nsp3.

However, the hypothesis best supported by the current data is that the ADP-ribose binding

affinity of Mac1 serves to prevent a key antiviral host factor from disrupting the initiation of viral

transcription. Plaquing efficiency assays from this study showed that the D1329A mutant virus

more readily establishes productive infections in HeLa-MVR cells than in murine DBT or L929

cells, suggesting a host-dependent anti-viral response. Furthermore, treatment with PARP

inhibitors partially rescues D1329A virus replication, and treatment with the PARP substrate NAD$^+$

further reduces the replication of the D1329A virus (29). These results in combination suggests

there is a murine-specific host factor that restricts virus replication in an ADP-ribosylation

dependent manner. Binding of Mac1 to an unknown ADP-ribosylated host factor or factors may

prevent it from traversing the nsp3 pore to access the replication transcription complex (RTC) and

repress viral RNA production **(Fig. 9B)**. Structural analysis of the pore complex predicts a central

channel of around 17 Å (52) that may be large enough for small proteins to access the RTC.

Previous studies of related alphavirus macrodomain mutant viruses found that macrodomain/ADP-

ribose binding is also required for initiating efficient viral RNA synthesis, indicating that there

may be a conserved host mechanism that represses the initial transcription of multiple viruses (40,

53). Thus, future research into these mechanisms could identify novel methods for developing anti-

viral therapeutics targeting multiple classes of RNA viruses.

METHODS

Cell Culture. Delayed Brain Tumor (DBT), L929, 17CL-1, and HeLa cells expressing the MHV receptor CEACAM1 (HeLa-MVR) were cultured in Dulbecco's modified Eagle medium (Corning) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin, 10mM HEPES, 1mM sodium pyruvate, 0.1mM nonessential amino acids, and 2mM L-glutamine. Bone marrow-derived macrophages (BMDMs) were sourced from WT C57BL/6 mice and differentiated to M0 via culturing for 7 days in Roswell Park Memorial Institute medium (RPMI) supplemented with 10µg/ml Macrophage Colony Stimulating Factor (MCSF) (Genscript) in addition to 10% fetal bovine serum 100 U/ml penicillin, 100 mg/ml streptomycin, 10mM HEPES, 1mM sodium pyruvate, 0.1mM nonessential amino acids, and 2mM L-glutamine. Differentiation into M2 macrophages was conducted was achieved by the further addition of 10µg/ml IL-4 (Peprotech Inc.) preceding infection by 24 hours.

Mice. Pathogen-free C57BL/6 mice originally sourced from Jackson Laboratories were bred and maintained in the animal care facility at the University of Kansas as approved by the University of Kansas Institutional Animal Care and Use Committee (IACUC) following guidelines set forth in the *Guidelines for the Care and Use of Laboratory Animals.*

Generation of Recombinant JHMV and A59 Constructs. Recombinant pBAC-JHMV D1329A constructs were generated using Red recombination. Double-base pair mutations in the nsp3 Mac1 were engineered using the Kan^r-I-SceI marker cassette as previously described, using primers listed in **Table 1**. Purified BAC DNA from Cml^r Kan^s colonies were analyzed by restriction enzyme digest, PCR and full plasmid sequencing for validation of correct clones. Primers used for validation of correct clones are also listed in **Table 1**. The recombinant MHV-A59 D1330A mutant was generated as described previously (54, 55). The D1330A mutation was introduced using site-

direct mutagenesis (Q5 Site-Directed Mutagenesis Kit, E0554S, NEB England Biolabs) and

sequencing-validated. The full-length viral cDNA was assembled using T4 DNA ligase (M0202L,

NEB England Biolabs). The full-length viral RNA was synthesized using an in vitro transcription

kit (mMESSAGE mMACHINE T7 Transcription Kit, AM1344, Thermo Fisher Scientific)

Reconstitution of recombinant pBAC-JHMV-derived virus. Approximately 1×10^6 BHK-MVR

cells were transfected with approximately 0.5-1 µg of pBAC-JHMV DNA and 1 µg of pcDNA-

MHV-N plasmid or 1 µg of full-length A549 RNA using Polyjet (SignaGen) as a transfection

reagent. Virus stocks were created by infecting $\sim1.5\times10^7$ 17Cl-1 cells at an MOI of 0.1 plaque-

forming units (PFU)/cell and collecting both the cells and supernatant at 16-20 hpi. The cells were

freeze-thawed, and debris was removed prior to collecting virus stocks. Virus stocks were

quantified by plaque assay on Hela-MVR cells and sequenced by collecting infected 17Cl-1 or

L929 cells using TRIzol™ Reagent (Invitrogen). The RNA from JHMV virions was likewise

isolated from infected cells and submitted for full genome sequencing (SeqCenter).

Virus Replication Assay. Cells were infected at the specified multiplicities of infection (MOIs)

via a 1-hour adsorption phase with a minimum working volume of DMEM containing virus

particles incubated at 37°C and periodically rocked to ensure complete coverage. Infected cells

and supernatants were collected by freeze-thaw at -80°C. Viral titers were determined via plaque

assay using HeLa-MVR cells.

Plaque Size Measurements. JHMV was serially diluted and plated onto monolayers of indicated

cell types in 24-well tissue culture plates. At 18hpi (DBT) or 20hpi (L929), cells were imaged

using an Accu-Scope® EXI-310 with an attached Excelis™ HDS Digital Imaging System. Plaque

diameters were measured using CaptaVision software calibrated with a stage micrometer.

Plaque Reduction Assay. Stocks of JHMV *rep*D1329, D1329A, and N1347A were serially diluted and plated in triplicate on cell monolayers of HeLa, DBT, or L929 cells in 24-well tissue culture plates. Plaque formation was assayed at 20hpi.

Viral Entry Assay. In a 24-well plate, DBT, L929, and HeLa cells were seeded at 2.25e5 cells/well and infected with a 100μl volume of MHV-JHM at an MOI of 1 using a 1-hour adsorption phase with regular shaking at 4°C. After the adsorption phase, cells were rinsed twice with PBS before fresh media was added to each well and plates were subsequently returned to the incubator at 37°C. At 2hpi, cells were removed from the incubator, rinsed with PBS, trypsinized, and the resulting cell suspension was transferred to 1.5ml microcentrifuge tubes. Tubes were centrifuged at 7,500 rpm for 5 minutes and the resulting cell pellets were resuspended in fresh PBS. Cells were rinsed in this fashion three times before a final resuspension in TRIzol™ Reagent (Invitrogen) frozen at -80°C. RNA extraction was carried out following the manufacturer's recommended protocol.

RT-qPCR Analysis. Cell monolayers were treated with ice cold TRIzol™ Reagent (Invitrogen) and RNA was extracted via the manufacturer's recommended protocol. Synthesis of cDNA was conducted using random hexamers (Roche) with M-MuLV reverse transcriptase (NEB) via the manufacturer's recommended instructions. Quantitation of cDNA transcripts was conducted using the Quantstudio 3 Real-Time PCR system (ThermoFisher) using the SYBR™ Green Universal Master Mix (ThermoFisher) with primers listed in **Table 2**.

Mouse Infections. For MHV mouse infections, 5-8 week-old male and female mice were anesthetized with isoflurane and inoculated intranasally with 1×10^4 PFU recombinant JHMV or intraperitoneally with 500 PFU of A59. To obtain tissue for A59 virus titers mice were euthanized on different days post challenge, livers were removed and homogenized in phosphate buffered saline (PBS), and titers were determined by plaque assay on either Hela-MVR (MHV) cells.

Immunoblotting. Total cell extracts were lysed in SDS-based lysis buffer containing Pierce™ Protease Inhibitor Cocktail (Thermo Scientific), PhosStop phosphatase inhibitor (Roche), 2% β-mercaptoethanol, and 1 mM PMSF. Proteins were resolved on a 10% SDS-PAGE gel, transferred to a polyvinylidene difluoride (PVDF) membrane, hybridized with a primary antibody, reacted with an infrared (IR) dye-conjugated secondary anti- body, visualized using a Li-COR Odyssey Imager (Li-COR), and analyzed using Image Studio software. Primary antibodies used for immunoblotting included anti-MHV N monoclonal antibody (1:10,000), anti-MHV S monoclonal antibody (1:5,000) (29), and anti-actin monoclonal antibody (1:10,000) (clone AC15; Abcam, Inc.). Secondary IR antibodies were purchased from Li-COR.

Immunofluorescence Staining. Cells were seeded at approximately 1×10^5 cells/ well in 8 Well Chamber, removable slides (ibidi) that were pre-coated with 50μg/ml collagen for 30 minutes. Cells were infected at an MOI of 0.5. At each time point, cells were rinsed with Hank's Balanced Salt Solution with 0.01% Sucrose (HBSS/Su) and fixed with the addition of 2% paraformaldehyde in HBSS/Su. Permeabilization was conducted by rinsing 3x with HBSS/Su containing 0.1% Saponin (HBSS/Su/Sap) before blocking with 3% normal goat serum in HBSS/Su/Sap. Primary antibodies (mouse α-dsRNA clone rJ2, Millipore) (rabbit α-nsp3, in-house), each diluted 1:1,000 in HBSS/Su/Sap, were added at room temperature for 3 hours followed by rinsing 3× with HBSS/Su/Sap. Goat-derived secondary antibodies (AlexaFluor 488/555) diluted 1:200 in HBSS/Su/Sap were incubated 1 hour at room temperature protected from light followed by rinsing twice with HBSS/Su/Sap, once with HBSS/Su, and once with HBSS. Nuclear staining was achieved via the addition of 300nM DAPI in HBSS for 30 minutes followed by rinsing twice with HBSS. Slides were finalized with the addition of Vectashield® hardening Mounting Media (VectorLabs).

Confocal Microscopy. Images were collected with a Leica DM6-SPE upright laser-scanning confocal microscope using a 63x oil-immersion objective lens with Type F oil. DAPI fluorescence was achieved with a 405 excitiation and 430/80 emission. Goat anti-mouse ALEXA 488 was imaged with 488 excitation 500/40 emission. Goat anti-rabbit ALEXA 555 was imaged with 561 excitation and 570/90 emission. Images were acquired using Leica LasX software.

Quantification of Confocal Immunofluorescence. Quantification of protein staining was automated using a custom macro written in FIJI (ImageJ v.1.54f) (56). For each image, a median 3D filter was applied to multi-channel Z-stacks (x=1, y=1, z=1 for DBT; x=2, y=2, z=2 for BMDM) after which maximum intensity Z-projections were generated, and the channels were split. Regions of Interest (ROIs) were generated from these single-channel duplicates by automated thresholding via the Moments, Triangle, and Renyientropy methods for the DAPI, dsRNA/N protein, and nsp3 channels, respectively. Resulting binary masks were applied to the original Z-projections and fluorescence intensity of staining was measured within regions of interest (ROIs) on the relevant channel. The total area of each ROI was also recorded, and the Integrated Density (IntDen) displayed is a product of the mean fluorescence intensity and the ROI area.

Colocalization Analysis

Manders' coefficients were obtained using the BIOP JACoP plugin for FIJI (ImageJ v.1.54f) (56, 57). Median filters (x=1, y=1) were applied to maximum projection z-stacks, after which Triangle or RenyiEntropy auto thresholds were applied to the dsRNA and nsp3 channels, respectively. Resulting ROIs were cropped to produce proportions of total dsRNA signal overlapped (M1) and total nsp3 signal overlapped (M2).

Protein Expression and Purification. Recombinant proteins were produced as previously described (35).

***In vitro* de-MARylation Assay.** MARylated PARP10 was generated via the incubation of purified GST-PARP10 with β-Nicotinamide Adenine Dinucleotide (β NAD$^+$) (Millipore-Sigma) in a reaction buffer (50 mM HEPES, 150 mM NaCl, 0.2 mM DTT, and 0.02% NP-40). MARylated PARP10 was aliquoted and stored at -80°C. Purified SARS-CoV-2 Mac1 WT and D-A were co-incubated with MARylated PARP10 for indicated times at 37°C with a ratio of 1 μM Mac1 to 5 μM PARP10 in reaction buffer (50 mM HEPES, 150 mM NaCl, 0.2 mM DTT, and 0.02% NP-40). The reaction was halted with the addition of 2X Laemmli sample buffer containing 10% β-mercaptoethanol. Samples were then heated for five minutes at 95°C before loading into a Bolt™ 4%-12% Bis-Tris Plus Gel in MES running buffer (ThermoFisher). Direct protein detection was conducted via staining on a separate, identical gel using ReadyBlue® Protein gel stain (Sigma). Transfer to polyvinylidene difluoride (PVDF) membrane was achieved via the iBlot™ 2 Dry Blotting System (ThermoFisher). Immunoblotting of the MAR and GST signal was conducted with anti-mono ADP-ribose binding reagent MABE1076 (Sigma) and an anti-GST monoclonal antibody MA4-004 (ThermoFisher). Detection of primary antibodies was conducted using secondary infrared anti-rabbit and anti-mouse antibodies (LI-COR Biosciences). Samples were visualized using the Odyssey M® Imaging System (LI-COR Biosciences).

Isothermal Titration Calorimetry. All ITC titrations were performed on a MicroCal PEAQ-ITC instrument (Malvern Pananalytical Inc., MA). All reactions were performed in 20 mM Tris pH 7.5, 150 mM NaCl using 100 μM of all macrodomain proteins at 25°C. Titration of 2 mM ADP-ribose or ATP (MilliporeSigma) contained in the stirring syringe included a single 0.4 μL injection, followed by 18 consecutive injections of 2 μL. Data analysis of thermograms was analyzed using one set of binding sites model of the MicroCal ITC software to obtain all fitting model parameters for the experiments. MERS-CoV and SARS-CoV-2 WT protein ITC data were previously

published (35). These experiments were performed alongside the mutant proteins, thus serving as

appropriate controls.

Differential Scanning Fluorimetry. Thermal shift assay with DSF involved use of LightCycler®

480 Instrument (Roche Diagnostics). In total, a 15 µL mixture containing 8X SYPRO Orange

(Invitrogen), and 10 µM macrodomain protein in buffer containing 20 mM Hepes, NaOH, pH 7.5

and various concentrations of ADP-ribose were mixed on ice in 384-well PCR plate (Roche).

Fluorescent signals were measured from 25 to 95 °C in 0.2 °C/30-s steps (excitation, 470-505 nm;

detection, 540-700 nm). Data evaluation and Tm determination involved use of the Roche

LightCycler® 480 Protein Melting Analysis software, and data fitting calculations involved the

use of single site binding curve analysis on GraphPad Prism.

AlphaScreen Assay. The AlphaScreen reactions were carried out in 384-well plates (Alphaplate,

PerkinElmer, Waltham, MA) in a total volume of 40 µL in buffer containing 25 mM HEPES (pH

7.4), 100 mM NaCl, 0.5 mM TCEP, 0.1% BSA, and 0.05% CHAPS. All reagents were prepared

as 4X stocks and 10 µL volume of each reagent was added to a final volume of 40 µL. After 1h

incubation at RT, streptavidin-coated donor beads (7.5 µg/mL) and nickel chelate acceptor beads

(7.5 µg/mL); (PerkinElmer AlphaScreen Histidine Detection Kit) were added under low light

conditions, and plates were shaken at 400 rpm for 60 min at RT protected from light. Plates were

kept covered and protected from light at all steps and read on BioTek plate reader using an

AlphaScreen 680 excitation/570 emission filter set. For data analysis, the percent inhibition was

normalized to positive (DMSO + labeled peptide) and negative (DMSO + macrodomain + peptide,

no ADPr) controls.

Statistical Analysis. A Student's *t* test was used to analyze differences in mean values between 2

groups, for multiple group comparisons, an ordinary one-way ANOVA was used. All results are

expressed as means ± standard errors of the means (SEM) unless stated as standard differentiation

(SD) or as geometric means ± 95% confidence internal. P values of ≤0.05 were considered

statistically significant (*, P≤0.05; **, P≤0.01; ***, P≤0.001; ****, P ≤0.0001; ns, not significant).

Data and Materials Availability. All data associated with this manuscript will be available

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593

594 A.R.F. was named as an inventor on a patent filed by the University of Kansas for a live-attenuated
595 SARS-CoV-2 vaccine

REFERENCES

1. Lin C-M, Saif LJ, Marthaler D, Wang Q. 2016. Evolution, antigenicity and pathogenicity of global porcine epidemic diarrhea virus strains. Virus Res 226:20–39.

2. Vijgen L, Keyaerts E, Lemey P, Maes P, Van Reeth K, Nauwynck H, Pensaert M, Van Ranst M. 2006. Evolutionary History of the Closely Related Group 2 Coronaviruses: Porcine Hemagglutinating Encephalomyelitis Virus, Bovine Coronavirus, and Human Coronavirus OC43. J Virol 80:7270–7274.

3. Corman VM, Eckerle I, Memish ZA, Liljander AM, Dijkman R, Jonsdottir H, Juma Ngeiywa KJZ, Kamau E, Younan M, Al Masri M, Assiri A, Gluecks I, Musa BE, Meyer B, Müller MA, Hilali M, Bornstein S, Wernery U, Thiel V, Jores J, Drexler JF, Drosten C. 2016. Link of a ubiquitous human coronavirus to dromedary camels. Proc Natl Acad Sci U S A 113:9864–9.

4. Paules CI, Marston HD, Fauci AS. 2020. Coronavirus Infections—More Than Just the Common Cold. JAMA 323:707.

5. Menachery VD, Yount BL, Debbink K, Agnihothram S, Gralinski LE, Plante JA, Graham RL, Scobey T, Ge X-Y, Donaldson EF, Randell SH, Lanzavecchia A, Marasco WA, Shi Z-L, Baric RS. 2015. A SARS-like cluster of circulating bat coronaviruses shows potential for human emergence. Nat Med 21:1508–13.

6. Hartenian E, Nandakumar D, Lari A, Ly M, Tucker JM, Glaunsinger BA. 2020. The molecular virology of coronaviruses. Journal of Biological Chemistry 295:12910–12934.

7. Lei J, Kusov Y, Hilgenfeld R. 2018. Nsp3 of coronaviruses: Structures and functions of a large multi-domain protein. Antiviral Res. Elsevier B.V. https://doi.org/10.1016/j.antiviral.2017.11.001.

8. Koetzner CA, Hurst-Hess KR, Kuo L, Masters PS. 2022. Analysis of a crucial interaction between the coronavirus nucleocapsid protein and the major membrane-bound subunit of the viral replicase-transcriptase complex. Virology 567:1–14.

9. Zimmermann L, Zhao X, Makroczyova J, Wachsmuth-Melm M, Prasad V, Hensel Z, Bartenschlager R, Chlanda P. SARS-CoV-2 nsp3 and nsp4 are minimal constituents of a pore spanning replication organelle https://doi.org/10.1038/s41467-023-43666-5.

10. Hagemeijer MC, Monastyrska I, Griffith J, van der Sluijs P, Voortman J, van Bergen en Henegouwen PM, Vonk AM, Rottier PJM, Reggiori F, De Haan CAM. 2014. Membrane rearrangements mediated by coronavirus nonstructural proteins 3 and 4. Virology 458–459:125–135.

11. Wolff G, Limpens RWAL, Zevenhoven-Dobbe JC, Laugks U, Zheng S, de Jong AWM, Koning RI, Agard DA, Grünewald K, Koster AJ, Snijder EJ, Bárcena M. 2020. A molecular pore spans the double membrane of the coronavirus replication organelle. Science (1979) 369:1395–1398.

12. Snijder EJ, Limpens RWAL, de Wilde AH, de Jong AWM, Zevenhoven-Dobbe JC, Maier HJ, Faas FFGA, Koster AJ, Bárcena M. 2020. A unifying structural and functional model of the coronavirus replication organelle: Tracking down RNA synthesis. PLoS Biol 18.

13. Chen A, Lupan AM, Quek RT, Stanciu SG, Asaftei M, Stanciu GA, Hardy KS, de Almeida Magalhães T, Silver PA, Mitchison TJ, Salic A. 2024. A coronaviral pore-replicase complex links RNA synthesis and export from double-membrane vesicles. Sci Adv 10:eadq9580.

14. Andronov L, Han M, Zhu Y, Balaji A, Roy AR, Barentine AES, Patel P, Garhyan J, Qi LS, Moerner WE. 2024. Nanoscale cellular organization of viral RNA and proteins in SARS-CoV-2 replication organelles. Nat Commun 15:1–17.

15. Rack JGM, Perina D, Ahel I. 2016. Macrodomains: Structure, Function, Evolution, and Catalytic Activities. Annu Rev Biochem 85:431–454.

16. Johnson MA, Chatterjee A, Neuman BW, Wüthrich K. 2010. SARS Coronavirus Unique Domain: Three-Domain Molecular Architecture in Solution and RNA Binding. J Mol Biol 400:724–742.

17. Fehr AR, Jankevicius G, Ahel I, Perlman S. 2018. Viral Macrodomains: Unique Mediators of Viral
Replication and Pathogenesis. Trends Microbiol. Elsevier Ltd
https://doi.org/10.1016/j.tim.2017.11.011.
18. Jankevicius G, Hassler M, Golia B, Rybin V, Zacharias M, Timinszky G, Ladurner AG. 2013. A family
of macrodomain proteins reverses cellular mono-ADP-ribosylation. Nat Struct Mol Biol 20:508–14.
19. Egloff M-P, Malet H, Putics Á, Heinonen M, Dutartre H, Frangeul A, Gruez A, Campanacci V,
Cambillau C, Ziebuhr J, Ahola T, Canard B. 2006. Structural and Functional Basis for ADP-Ribose and
Poly(ADP-Ribose) Binding by Viral Macro Domains. J Virol 80:8493–8502.
20. Lüscher B, Ahel I, Altmeyer M, Ashworth A, Bai P, Chang P, Cohen M, Corda D, Dantzer F, Daugherty
MD, Dawson TM, Dawson VL, Deindl S, Fehr AR, Feijs KLH, Filippov D V., Gagné JP, Grimaldi G,
Guettler S, Hoch NC, Hottiger MO, Korn P, Kraus WL, Ladurner A, Lehtiö L, Leung AKL, Lord CJ,
Mangerich A, Matic I, Matthews J, Moldovan GL, Moss J, Natoli G, Nielsen ML, Niepel M, Nolte F,
Pascal J, Paschal BM, Pawłowski K, Poirier GG, Smith S, Timinszky G, Wang ZQ, Yélamos J, Yu X,
Zaja R, Ziegler M. 2021. ADP-ribosyltransferases, an update on function and nomenclature. FEBS
Journal https://doi.org/10.1111/febs.16142.
21. Fehr AR, Singh SA, Kerr CM, Mukai S, Higashi H, Aikawa M. 2020. The impact of PARPs and ADP-
ribosylation on inflammation and host-pathogen interactions. Genes Dev 34:341–359.
22. Atasheva S, Frolova EI, Frolov I. 2014. Interferon-Stimulated Poly(ADP-Ribose) Polymerases Are
Potent Inhibitors of Cellular Translation and Virus Replication. J Virol 88:2116–2130.
23. Li L, Zhao H, Liu P, Li C, Quanquin N, Ji X, Sun N, Du P, Qin CF, Lu N, Cheng G. 2018. PARP12
suppresses Zika virus infection through PARP-dependent degradation of NS1 and NS3 viral proteins.
Sci Signal 11:535.
24. Hendriks IA, Larsen SC, Nielsen ML. 2019. An Advanced Strategy for Comprehensive Profiling of
ADP-ribosylation Sites Using Mass Spectrometry-based Proteomics. Mol Cell Proteomics 18:1010–
1026.
25. Buch-Larsen SC, Hendriks IA, Lodge JM, Rykær M, Furtwängler B, Shishkova E, Westphall MS, Coon
JJ, Nielsen ML. 2020. Mapping Physiological ADP-Ribosylation Using Activated Ion Electron Transfer
Dissociation. Cell Rep 32:108176.
26. Leung AKL, Griffin DE, Bosch J, Fehr AR. 2022. The Conserved Macrodomain Is a Potential
Therapeutic Target for Coronaviruses and Alphaviruses. Pathogens 11(1):94.
27. Alhammad YMO, Kashipathy MM, Roy A, Gagné J-P, McDonald P, Gao P, Nonfoux L, Battaile KP,
Johnson DK, Holmstrom ED, Poirier GG, Lovell S, Fehr AR. 2021. The SARS-CoV-2 Conserved
Macrodomain Is a Mono-ADP-Ribosylhydrolase. J Virol 95(3):e01969-20.
28. Alhammad YMO, Fehr AR. 2020. The viral macrodomain counters host antiviral ADP-ribosylation.
Viruses 12:1–12.
29. Voth LS, O'Connor JJ, Kerr CM, Doerger E, Schwarting N, Sperstad P, Johnson DK, Fehr AR. 2021.
Unique Mutations in the Murine Hepatitis Virus Macrodomain Differentially Attenuate Virus
Replication, Indicating Multiple Roles for the Macrodomain in Coronavirus Replication. J Virol
95(15):e0076621.
30. Heer CD, Sanderson DJ, Voth LS, Alhammad YMO, Schmidt MS, Trammell SAJ, Perlman S, Cohen
MS, Fehr AR, Brenner C. 2020. Coronavirus infection and PARP expression dysregulate the NAD
metabolome: An actionable component of innate immunity. Journal of Biological Chemistry
295:17986–17996.
31. Alhammad YM, Parthasarathy S, Ghimire R, Kerr CM, O'Connor JJ, Pfannenstiel JJ, Chanda D, Miller
CA, Baumlin N, Salathe M, Unckless RL, Zuñiga S, Enjuanes L, More S, Channappanavar R, Fehr AR.
2023. SARS-CoV-2 Mac1 is required for IFN antagonism and efficient virus replication in cell culture
and in mice. Proceedings of the National Academy of Sciences 120(35):e2302083120.

32. Correy GJ, Kneller DW, Phillips G, Pant S, Russi S, Cohen AE, Meigs G, Holton JM, Gahbauer S, Thompson MC, Ashworth A, Coates L, Kovalevsky A, Meilleur F, Fraser JS. 2022. The mechanisms of catalysis and ligand binding for the SARS-CoV-2 NSP3 macrodomain from neutron and x-ray diffraction at room temperature Sci. Adv 8(21):eabo5083.

33. Rack JGM, Zorzini V, Zhu Z, Schuller M, Ahel D, Ahel I. 2020. Viral macrodomains: a structural and evolutionary assessment of the pharmacological potential. Open Biol 10(11):200237.

34. Grunewald ME, Chen Y, Kuny C, Maejima T, Lease R, Ferraris D, Aikawa M, Sullivan CS, Perlman S, Fehr AR. 2019. The coronavirus macrodomain is required to prevent PARP-mediated inhibition of virus replication and enhancement of IFN expression. PLoS Pathog 15(5):e1007756.

35. Kerr CM, Pfannenstiel JJ, Alhammad YM, O'Connor JJ, Ghimire R, Shrestha R, Khattabi R, Saenjamsai P, Parthasarathy S, McDonald PR, Gao P, Johnson DK, More S, Roy A, Channappanavar R, Fehr AR. 2024. Mutation of a highly conserved isoleucine residue in loop 2 of several β-coronavirus macrodomains indicates that enhanced ADP-ribose binding is detrimental for replication. J Virol 98(11):e0131324.

36. Fehr AR. 2020. Bacterial artificial chromosome-based lambda red recombination with the I-Scei homing endonuclease for genetic alteration of MERS-CoV. Methods in Molecular Biology 2099:53–68.

37. Yamada Y, Liu XB, Fang SG, Tay FPL, Liu DX. 2009. Acquisition of cell-cell fusion activity by amino acid substitutions in spike protein determines the infectivity of a coronavirus in cultured cells. PLoS One 4(7):e6130.

38. Yin W, Mao C, Luan X, Shen D-D, Shen Q, Su H, Wang X, Zhou F, Zhao W, Gao M, Chang S, Xie Y-C, Tian G, Jiang H-W, Tao S-C, Shen J, Jiang Y, Jiang H, Xu Y, Zhang S, Zhang Y, Xu HE. Structural basis for inhibition of the RNA-dependent RNA polymerase from SARS-CoV-2 by remdesivir Science 368(6498):1499-1504.

39. Ryan AP, Delgado-Rodriguez SE, Daugherty MD. 2025. Zinc-finger PARP proteins ADP-ribosylate alphaviral proteins and are required for interferon-γ–mediated antiviral immunity. Sci Adv 11(5):eadm6812.

40. Abraham R, Hauer D, McPherson RL, Utt A, Kirby IT, Cohen MS, Merits A, Leung AKL, Griffin DE. 2018. ADP-ribosyl-binding and hydrolase activities of the alphavirus nsP3 macrodomain are critical for initiation of virus replication. Proc Natl Acad Sci U S A 115:E10457–E10466.

41. Kerr CM, Parthasarathy S, Schwarting N, O'Connor JJ, Pfannenstiel JJ, Giri E, More S, Orozco RC, Fehr AR. 2023. PARP12 is required to repress the replication of a Mac1 mutant coronavirus in a cell- and tissue-specific manner. J Virol 97(9):e0088523.

42. Saleh H, Liloglou T, Rigden DJ, Parsons JL, Grundy GJ. 2024. KH-like Domains in PARP9/DTX3L and PARP14 Coordinate Protein–Protein Interactions to Promote Cancer Cell Survival. J Mol Biol 436:168434.

43. Zhang Y, Mao D, Roswit WT, Jin X, Patel AC, Patel DA, Agapov E, Wang Z, Tidwell RM, Atkinson JJ, Huang G, McCarthy R, Yu J, Yun NE, Paessler S, Lawson TG, Omattage NS, Brett TJ, Holtzman MJ. 2015. PARP9-DTX3L ubiquitin ligase targets host histone H2BJ and viral 3C protease to enhance interferon signaling and control viral infection. Nat Immunol 16:1215–1227.

44. Challa S, Nandu T, Kim HB, Gong X, Renshaw CW, Li W-C, Tan X, Aljardali MW, Camacho C V., Chen J, Kraus WL. 2025. RACK1 MARylation regulates translation and stress granules in ovarian cancer cells. Journal of Cell Biology 224(2):e202401101.

45. Kubon D, Leslie Pedrioli DM, Hottiger MO. 2024. PARP14 mediated SQSTM1/p62 cysteine ADP-ribosylation is counteracted by the SARS-CoV-2 macrodomain BioRxiv https://doi.org/10.1101/2024.06.29.601315.

46. Catara G, Grimaldi G, Schembri L, Spano D, Turacchio G, Lo Monte M, Beccari AR, Valente C, Corda
D. 2017. PARP1-produced poly-ADP-ribose causes the PARP12 translocation to stress granules and
impairment of Golgi complex functions. Sci Rep 7(1):14035.
47. Hurst KR, Ye R, Goebel SJ, Jayaraman P, Masters PS. 2010. An Interaction between the Nucleocapsid
Protein and a Component of the Replicase-Transcriptase Complex Is Crucial for the Infectivity of
Coronavirus Genomic RNA. J Virol 84:10276–10288.
48. Cong Y, Ulasli M, Schepers H, Mauthe M, V'kovski P, Kriegenburg F, Thiel V, de Haan CAM, Reggiori
F. 2020. Nucleocapsid Protein Recruitment to Replication-Transcription Complexes Plays a Crucial
Role in Coronaviral Life Cycle. J Virol 94(4):e01925-19.
49. Keane SC, Giedroc DP. 2013. Solution Structure of Mouse Hepatitis Virus (MHV) nsp3a and
Determinants of the Interaction with MHV Nucleocapsid (N) Protein. J Virol 87:3502–3515.
50. Grunewald ME, Fehr AR, Athmer J, Perlman S. 2018. The coronavirus nucleocapsid protein is ADP-
ribosylated. Virology 517:62–68.
51. Wu C-H, Yeh S-H, Tsay Y-G, Shieh Y-H, Kao C-L, Chen Y-S, Wang S-H, Kuo T-J, Chen D-S, Chen P-
J. 2009. Glycogen Synthase Kinase-3 Regulates the Phosphorylation of Severe Acute Respiratory
Syndrome Coronavirus Nucleocapsid Protein and Viral Replication. Journal of Biological Chemistry
284:5229–5239.
52. Huang Y, Wang T, Zhong L, Zhang W, Zhang Y, Yu X, Yuan S, Ni T. 2024. Molecular architecture of
coronavirus double-membrane vesicle pore complex. Nature 633:224–231.
53. Park E, Griffin DE. 2009. The nsP3 macro domain is important for Sindbis virus replication in neurons
and neurovirulence in mice. Virology 388:305–314.
54. Deng X, StJohn SE, Osswald HL, O'Brien A, Banach BS, Sleeman K, Ghosh AK, Mesecar AD, Baker
SC. 2014. Coronaviruses resistant to a 3C-like protease inhibitor are attenuated for replication and
pathogenesis, revealing a low genetic barrier but high fitness cost of resistance. J Virol 88:11886–98.
55. Yount B, Denison MR, Weiss SR, Baric RS. 2002. Systematic assembly of a full-length infectious
cDNA of mouse hepatitis virus strain A59. J Virol 76:11065–78.
56. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C,
Saalfeld S, Schmid B, Tinevez J-Y, White DJ, Hartenstein V, Eliceiri K, Tomancak P, Cardona A. 2012.
Fiji: an open-source platform for biological-image analysis. Nat Methods 9:676–682.
57. BOLTE S, CORDELIÈRES FP. 2006. A guided tour into subcellular colocalization analysis in light
microscopy. J Microsc 224:213–232.
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 FIGURE LEGENDS

Fig. 1. Mutation of a highly conserved aspartic acid in the ADP-ribose binding pocket of

Mac1 dramatically reduces ADP-ribose binding but largely maintains its enzyme activity.

(A) Highly conserved aspartic acid (red) and asparagine (green) residues highlighted in alignments

of coronavirus Mac1 and other related macrodomain proteins. **(B)** SARS-CoV-2 Mac1-WT and

D-A proteins were incubated with increasing quantities of ADP-ribose and then assayed for

thermal stability by differential scanning fluorimetry (DSF). **(C)** SARS-CoV-2 Mac1 WT and D-

A proteins were tested for their ability to interact with an ADP-ribosylated peptide using an

AlphaScreen assay. The results in B-C are representative of 2 independent experiments. **(D)**

Isothermal titration calorimetry (ITC) of purified SARS-CoV-2 WT and D-A proteins in the

presence of increasing concentrations of ADP-ribose. *SARS-CoV-2 WT protein was previously

published (27). The results are the combined averages of 4 independent experiments. **(E)** WT and

D-A proteins were incubated with MARylated GST-PARP10 CD for the indicated time and

MARylation levels were measured by Western blotting for mono-ADP-ribose. GST antibody was

used as a loading control. Results are from one experiment representative of 2 independent

experiments.

Fig. 2. MHV-D1329A/D1330A virus is attenuated in cell culture and *in vivo*. (A-B) DBT cells

were infected with JHMV-*rep*D1329, N1347A, and D1329A viruses at an MOI of 0.1 (A) and 1.0

(B). Progeny virus was collected at indicated times and was titered by plaque assay. The results

are from a single experiment representative of 2 independent experiments. **(C-D)** C57BL/6 mice

were infected with 10^4 PFU JHMV-*rep*D1329 and D1329A virus and monitored for survival (C)

and weight loss (D) over 12 days. n=9 mice, *rep*D1329; n=10 mice, D1329A. The results are the

combined data from two independent experiments. **(E-G)** DBT (E), L929 (F), and 17Cl-1 (G) cells

were infected with MHV-A59 WT and D1330A viruses and progeny virus was measured at 24 hpi.

The results are from a single experiment representative of 2 independent experiments. **(H)**

C57BL/6 mice were infected with 500 PFU A59-WT and D1330A viruses. Livers were collected

at 3 dpi in PBS and viral loads were measured by plaque assay. Graphs represent the geometric

mean with 95% confidence internal. n=7 mice, WT; n=9 mice, D1330A.

Fig. 3. JHMV-D1329A virus produces small plaques and demonstrates reduced infectivity in

mouse cells compared to *rep*D1329 and N1347A viruses. (A-B) DBT (A) and L929 (B) cells

were infected with JHMV-*rep*D1329 and D1329A at an MOI of 0.1 and were examined at 18

(DBT) or 20 (L929) hpi to determine relative plaque sizes between mutants. **(C)** Plaque sizes were

measured using CaptaVision software calibrated with a stage micrometer n=27, *rep*D1329; n=41,

D1329A; n=45, N1347A **(D)** Plaque sizes were measured using CaptaVision software calibrated

with a stage micrometer n=25, *rep*D1329; n=21, D1329A; n=33, N1347A The results represent

the combined results of 2 independent experiments. **(E)** Hela-MVR, DBT, and L929 cells were

infected with equal PFUs of each virus and the number of plaques were counted 20 hpi. The results

are from a single experiment representative of 3 independent experiments.

Fig. 4. JHMV-D1329A virus has no defect in genomic RNA delivery into cells. Indicated cell

lines were infected with JHMV *rep*D1329 and D1329A viruses for 1 hr, then trypsinized for 5

minutes and rinsed 3 times with PBS. The cells were then collected, RNA was harvested, and

genomic RNA levels were determined by qPCR using the ΔCt method and normalized to HPRT

mRNA. These data are from a single experiment representative of 2 independent experiments.

Fig. 5. JHMV-D1329A virus is defective in the production of viral genomic RNA (gRNA) and

sub-genomic RNA6 (sgRNA6) compared to *rep*D1329 and N1347A viruses. (A-B) DBT (A)

and L929 (B) cells were infected with indicated viruses at an MOI of 3.0. Cells were collected at

indicated times and gRNA (top) and sgRNA6 (bottom) were measured by qPCR using the $\Delta\Delta$CT

method and normalized to HPRT. The data in A-B is from a single experiment representative of 2

independent experiments. **(C)** L929 cells were infected with either *rep*D1329 or D1329A viruses

at an MOI of 0.1. At 10 hpi cells were treated with Remdesivir metabolite GS-441524 or DMSO

control. Cells were collected at indicated time points and viral gRNA (left) or sgRNA6 (right)

levels were determined by qPCR using the $\Delta\Delta$Ct method and normalized to HPRT mRNA. The

results are from a single experiment representative of 2 independent experiments.

Fig. 6. JHMV-D1329A virus has reduced dsRNA levels early in infection. (A) DBTs were

infected with indicated virus at an MOI of 0.5, fixed at 8 hpi, stained for dsRNA and nsp3, and

analyzed by confocal microscopy. **(B)** Quantification of fluorescence signal displayed as a product

of mean pixel intensity and thresholded area (IntDen). Error bars indicate geometric mean with

95% CI, combined data from two separate experiments. **(C)** Mander's coefficients displaying the

proportional overlap between regions where dsRNA overlaps nsp3 staining (M1 – green), and

where nsp3 staining overlaps dsRNA (M2 – red). Error bars indicate mean with 95% CI, combined

data from two separate experiments.

Fig. 7. N1347A infected BMDMs produce WT levels of viral RNA but have reduced N protein

accumulation compared to *rep*D1329 virus. (A) M2 differentiated BMDMs were infected with

indicated viruses at an MOI of 1.5. Cells and supernatants were collected at 18 hpi and progeny

virus was measured by plaque assay. The results are from a single experiment representative of 2

independent experiments. **(B)** BMDMs were infected as in (A) and cells were collected at indicated

times points and gRNA (left) and sgRNA6 (right) were measured by qPCR using the ΔCt method

and normalized to HPRT mRNA. The data in B represent the combined results of 3 (sgRNA6) or

4 (gRNA) independent experiments. **(C)** BMDMs were infected as in (A) and cells were collected

in sample buffer at indicated times points and viral spike (S) and nucleocapsid (N) proteins were

analyzed by Western blotting. Actin was used as a loading control. The results are from a single

experiment representative of 2-4 independent experiments. **(D)** Quantitation of the western blot

signal in C normalized to actin using Image J. The data in (D) represent the combined results from

4 (*rep*D1239 & N1347A) or 3 (D1329A) independent experiments.

Fig. 8. D1329A and N1347A infected BMDMs have near *rep*D1329 levels of nsp3 but have reduced N protein abundance. (A) BMDMs were infected with indicated virus at an MOI of 0.5 and fixed at 9 hpi, stained for nsp3 and N protein, and analyzed by confocal microscopy. **(B)** Quantification of fluorescence signal displayed as a product of mean pixel intensity and thresholded area (IntDen) for all three fluorescent channels. Combined data from two independent experiments presented as geometric mean with 95% confidence intervals.

Fig. 9. Potential roles for Mac1 in regulating early RNA transcription. (A) The Ubl1 domain of nsp3 interacts with phosphorylated residues on the RNA-bound nucleocapsid protein which is a key factor in shuttling viral RNA through the nsp3 pore to the site of transcription. Mac1 binding affinity may enhance this interaction between nsp3 and N protein, without which there is a delay in transcription initiation. **(B)** Mac1 binding affinity may sequester or block ADP-ribosylated host factors that target and inhibit viral transcription early in the replication cycle.

SUPPLEMENTAL FIGURE LEGENDS

Fig S1. MERS-CoV Mac1 D-A protein has poor ADP-ribose binding affinity. (A) Isothermal titration caliorimetry of purified MERS-CoV WT D-A Mac1 protein incubated with ADP-ribose. *MERS-CoV WT protein data was previously published (27). **(B)** MERS-CoV Mac1 WT and DA proteins were tested for their ability to interact with an ADP-ribosylated peptide using an AlphaScreen assay. The results in A-B are from a single experiment representative of at least 2 independent experiments.

Fig S2. JHMV-D1329A virus is defective in the production of viral genomic RNA (gRNA) and sub-genomic RNA6 (sgRNA6) compared to *rep*D1329 and N1347A viruses. (A-B) L929 (A) and DBT (B) cells were infected with indicated viruses at an MOI of 0.1. Cells were collected at indicated times and genomic and RNAs were measured by qPCR using the $\Delta\Delta$CT method normalized to HPRT. Results are from one experiment representative of two independent experiments.

Fig. S3. JHMV-D1329A produces nsp3 in the early stages of infection. (A) DBTs were infected with indicated virus at an MOI of 0.5, fixed at 6 hpi, stained for nsp3, and analyzed by confocal microscopy. **(B)** Quantification of fluorescence signal displayed as a product of mean pixel intensity and total thresholded area (IntDen), representative of two independent experiments.

872 **TABLE 1. Linear recombination primers for engineering JHMV-D1329A**

		5' – 3'
		Mac1 D1329A Mutant Generation
MHV-JHM DA	F	TTCATGTATAACACCAAATGTTTGTTTTGTTAAAGGAGCGGTTATAAAGGTTTTGCGC**AGAGGATGACGACGATAAGTAGGG**
	R	CGATGACTTCAGCACCAACTCTGCGCAAAACCTTTATAACCGCTCCTTTAACAAAACAAA**GCCAGTGTTACAACCAATTAACC**

873 Nucleotides mutated are designated in red

875 **TABLE 2. qPCR and Sequencing primers**

		5' – 3'
		qPCR Primers
M. musculus HPRT	F	GCGTCGTGATTAGCGATGATG
	R	CTCGAGCAAGTCTTTCAGTCC
MHV gRNA	F	AGGGAGTTTGACCTTGTTGC
	R	ATAATGCACCTGTCATCCTCG
MHV sgRNA	F	TATAAGAGTGATTGGCGTCC
MHV sgRNA6	R	GTGGGGCCACATTAACCACAAG
MHV sgRNA4	R	CTAATGCTAGGCGCAGAA
		Sequencing Primers
MHV Mac1	F	GGCTGTTGTGGATGGCAAGCA
	R	GCTTTGGTACCAGCAACGGAG
MERS Mac1	F	CCGTCTGCACCTCAGACTATC
	R	GCAGAGTTGTCTGTGCACAC

Fig. 1. Mutation of a highly conserved aspartic acid in the ADP-ribose binding pocket of Mac1 dramatically reduces ADP-ribose binding but largely maintains its enzyme activity. (A) Highly conserved aspartic acid (red) and asparagine (green) residues highlighted in alignments of coronavirus Mac1 and other related macrodomain proteins. **(B)** SARS-CoV-2 Mac1-WT and D-A proteins were incubated with increasing quantities of ADP-ribose and then assayed for thermal stability by differential scanning fluorimetry (DSF). **(C)** SARS-CoV-2 Mac1 WT and D-A proteins were tested for their ability to interact with an ADP-ribosylated peptide using an AlphaScreen assay. The results in B-C are representative of 2 independent experiments. **(D)** Isothermal titration calorimetry (ITC) of purified SARS-CoV-2 WT and D-A proteins in the presence of increasing concentrations of ADP-ribose. *SARS-CoV-2 WT protein was previously published (27). The results are the combined averages of 4 independent experiments. **(E)** WT and D-A proteins were incubated with MARylated GST-PARP10 CD for the indicated time and MARylation levels were measured by Western blotting for mono-ADP-ribose. GST antibody was used as a loading control. Results are from one experiment representative of 2 independent experiments.

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