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Structural mechanism of CRL4-instructed STAT2 degradation via a novel cytomegaloviral DCAF receptor

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1st Editorial Decision **23rd Aug 2022**

Dear David,

Thank you for submitting your MS to The EMBO Journal. The manuscript was transferred from Review Commons where the review process was carried out.

I am sorry for the slight delay in getting back to you with a decision. I have now had a chance to take a careful look at the manuscript and at your revision plan. I appreciate the reported findings and the proposed additions. I would therefore like to invite you to submit a revised version should you be able to address the concerns raised as outlined in your response.

Let me know if it would be helpful to discuss the revisions further. I am available to do so via a zoom call.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Instructions for preparing your revised manuscript:

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We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (21st Nov 2022).

As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed.

Should you foresee a problem in meeting this three-month deadline, please let us know in advance as we can grant an extension.

Use the link below to submit your revision:

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Point-by-point reply

We thank all three reviewers for their insightful suggestions. We addressed all points raised by the reviewers. In addition to a thorough revision of the text and most figures, we provide more information regarding the quality control of structural data. Furthermore, we conducted an extensive series of new experiments. Briefly, we mutated the zinc-binding motif in the E27 context and showed that this abrogates the DDB1 interaction, we applied BAC-based mutagenesis to construct a novel MCMV mutant that expresses E27 instead of M27, which showed that E27 and M27 are interchangeable regarding STAT2 degradation in rat fibroblasts, we repeated all gel filtration experiments, in which full-length DDB1 had been applied, with $DDB1_{\Delta BPB}$ and obtained congruent results, and we experimentally tested if E27 displaces IRF9 from STAT2. We proceed with a point-by-point reply, in which the comments of the reviewers are written in italics.

Reviewer #1 (Evidence, reproducibility and clarity (Required)):

R#1: *Summary: Using proteome profiling of rat CMV infected cells, the authors of this study identify the E27 protein of rat cytomegalovirus as being crucial for proteasomal degradation of STAT2. Since E27 shares 56% sequence identity to the previously characterized STAT2 antagonist M27 of murine CMV the authors investigated association of E27 with the Cullin4-RING UbL CRL4. Using gel filtration chromatography they provide evidence that E27 forms a stable ternary complex with DDB1 and STAT2 suggesting that E27 bridges STAT2 to DDB1 which is further corroborated by data from cross-linking mass spectrometry. A cross-linked DDB1/DDA1/E27/STAT2 complex was then used for cryo-EM imaging experiments. The subsequent single particle analysis yielded a density map at 3.8 A resolution that was further used to generate an E27 molecular model. At this point it should be noted that resolution was not very high and data form AlphaFold2 prediction and CLMS experiments were necessary to build a model which was described as having "sufficient quality", however, no quality parameters are included for this model. In this model, a cryptic zinc-binding motif was identified that turned out to be well conserved in M27. At this point the study switches to a mutational analysis of M27: MCMV mutants either lacking M27 or bearing an AxAxxAA triple mutation were investigated both in cell culture and in animal models. Surprisingly, the M27-AxAxxA mutant while exhibiting attenuated IFN inhibition was still more active than an M27 deletion mutant. Later during the study it is postulated that this may be due to the fact that E27 binding to STAT2 abrogates the interaction with IRF9, however, this is only predicted from modeling and no experimental data are provided for this hypothesis. Furthermore, modeling approaches were used to predict how E27 replaces endogenous CRL4 substrate receptors and how E27 recruits STAT2 to mediate CRL4-catalysed ubiquitin transfer.*

Response: We thank R#1 for her/his insightful and constructive evaluation as well as for her/his suggestions, which helped us to strengthen our manuscript.

Major comments:

R#1: 1. *To my opinion the authors should perform mutational analysis in the context of E27 and RCMV. I accept that switching to M27 may be easier due to established procedures for MCMV mutagenesis and analysis, however, since all structural work is primarily done on E27 it would be consequent to confirm these structural predictions in the context of E27 before switching to a related protein.*

Response: As R#1 appreciated, there were several reasons for the switch from RCMV-E E27 to MCMV M27. Most importantly, the MCMV *in vivo* infection model in mice is very well established. Please also note that MCMV is applied far more often by virologists and immunologist as a standard model. Thus, the extension of our findings from RCMV to MCMV increases the relevance and outreach of the study. By performing the experiments in the MCMV context, we also aimed to emphasise that the function of the zinc-binding motif, which structurally organises the DDB1-binding domain, is functionally conserved among E27/M27-like proteins and affects the capacity of CMV replication *in vivo*. Obviously, R#1 could ask why we do not solve the structure of M27 parallel to E27. With the sole exception of E27, none of the rodent M27 homologues could be produced recombinantly in a soluble form, preventing the purification and structure analysis of M27.

Since we agree with R#1 regarding the necessity to validate the relevance of the zinc finger motif in the E27 context, we generated RCMV-E E27 mutants, in which either the three Cysteine residues ("AxAxxA") or the Histidine ("H332A") were changed to Alanine residues by site-directed mutagenesis. The intentionally introduced mutations were afterwards confirmed by sequencing. In accordance with our structural data and the relevance of the newly described zinc-binding motif, both **E27 mutants failed to precipitate DDB1** in Co-IP experiments (see **new data set in new Fig 7B**).
To interrogate the functional similarity between M27 and E27, we applied BAC-based mutagenesis to construct a novel MCMV mutant that lacks the *M27* gene but instead expresses an HA epitope-tagged E27. As others and we showed previously, rat fibroblasts are permissive for MCMV, whereas mouse cells are not permissive for RCMV (Bruggeman et al., 1982; Le-Trilling and Trilling, 2017; Sandford et al., 2001; Smith et al., 1986). Making use of MCMV permissiveness of rat cells, we infected rat fibroblasts with wt-MCMV (expressing M27), ΔM27-MCMV, ΔM27-MCMV-E27HA ("ΔM27-E27HA") or left the cells uninfected ("mock"). While STAT2 was detectable in mock and ΔM27-MCMV-infected cells, wt-MCMV and ΔM27-E27HA degraded STAT2, indicating that **M27 and E27 are interchangeable in rat fibroblasts regarding STAT2 degradation** (see **new Fig EV 5**).

R#1: *Moreover, data on the replication of the generated E27 deletion RCMV should be included in the manuscript (i.e. growth curves).*

Response: We compared the replication capacity of wt-RCMV-E and ΔE27 RCMV-E in rat fibroblasts (**see new Fig. 1B**). Briefly, these experiments demonstrated an impaired replication of the ΔE27 RCMV-E, indicating the relevance of E27.

R#1: 2. Resolution of the cryoEM structure is rather low and many predictions of the manuscript are based on modeling using AlphaFold2 prediction. The authors describe their model as of "sufficient quality", however, no quality measures are included in the manuscript. At least the discussion should address limitations of the used approach.

Response: While we apologize for not sufficiently describing our quality measures, we respectfully disagree regarding the argument. Our resolution (3.8 Å, map 1) lies well within the 3-4 Å resolution range of the majority of structures deposited to the Electron Microscopy Data Bank during the last five years (https://www.emdataresource.org/statistics.html). Nevertheless, *de novo* modelling in this resolution regime is challenging. This is why we sought additional guidance through cross-linking mass spectrometry (XL-MS) restraints and AlphaFold2. Please also note that modelling of E27 was not based solely on the AlphaFold2 prediction. Instead, a partial model corresponding to the α-domain was manually built in map 1, guided by XL-MS information (see Methods - "Model building and refinement" and new Fig EV1B, grey cartoon). This model proved to be in very good agreement with AlphaFold2 predictions (RMSD of 1.489 Å, 2764 aligned atoms). Only after this initial sanity check, the computational prediction was used for model completion, adjustment, and refinement.
We now added **new graphical overviews of model fits in Fig EV1 (map 1) and EV4 (map 2)**. Furthermore, we included detailed views of the fit of relevant side chains involved in intermolecular interaction to the experimental density (**new Fig EV2, EV3**). We also **calculated and listed quality indicators of the model-to-map fit in the new Table EV1** (correlation coefficients and model resolution based upon model-map FSC). To ensure the validity of our atomic model using an alternative method besides cryo-EM and XL-MS, we have performed site-directed mutagenesis of critical binding regions in E27, followed by in vitro reconstitution and analytical GF (**new Fig EV2B-E, EV3B-E**). The text was revised accordingly (see p9 [ll10] and p10 [ll24]).
In addition, we have now released the cryo-EM maps and atomic models for public access (see Data Availability statement):
map 1 - https://www.ebi.ac.uk/emdb/EMD-14802, https://www.rcsb.org/structure/7ZN7
map 2 - https://www.ebi.ac.uk/emdb/EMD-14812, https://www.rcsb.org/structure/7ZNN

R#1: 3. *The authors identify a cryptic zinc-binding motif in E27 that is conserved in homologous proteins. For this reviewer it is not clear: is there experimental evidence for zinc binding of E27 or can the presence of zinc reliably be detected in their structural data? If not, it would be worth to confirm zinc binding.*

Response: Our structural data show a tetragonal metal coordination geometry, involving three Cysteine side chains and one Histidine residue, with coordination bond lengths of 2.2 Å between the histidine nitrogen and the metal ion, and of 2.4 Å between the cysteine sulfurs and the metal ion. Another type of side chain interaction, e.g., a disulfide bond, cannot explain the density feature because this would lead to a steric clash with the remaining adjacent side chains. Based on the knowledge on metal-binding sites in proteins and metal-coordination chemistry, these characteristics indicate the presence of a structural zinc-binding site for the following reasons: (i) after magnesium, zinc is the second most prevalent metal in the Protein Data Bank (https://metalpdb.cerm.unifi.it/getSummary), however, magnesium is coordinated octahedrally by oxygen ligands (Tang and Yang, 2013); (ii) the most abundant zinc ligands are Cysteine and Histidine; (iii) the most abundant zinc coordination number is four ligands; (iv) the average coordination bond lengths are 2.12±0.19 Å and 2.33±0.12Å for nitrogen-zinc and sulfur-zinc interactions, respectively (Ireland and Martin, 2019; Laitaoja et al., 2013), which is in very good agreement with our structural observations. We **included this argumentation in the revised manuscript** (see p8 [ll8]), and added the **new Fig. EV1C for visualization**.

R#1: *4. The hypothesis that STAT2/E27 interaction is sterically incompatible with IRF9 binding is only based on structural prediction. It would help if the authors could present experimental evidence for such a mechanism.*

Response: We are indebted to R#1 for raising this crucial issue. She/he protected us from a misinterpretation. We challenged our hypothesis of IRF9 steric exclusion in an *in vitro* competition assay by analytical gel filtration, similar to the E27/DCAF1 DDB1-binding competition experiment shown in Fig 4E. These experiments clearly showed - contrary to our assumption - that E27 applied in equimolar concentrations does not disrupt STAT2/IRF9 complexes. We added the new data (**see new Appendix Fig S9**) and re-phrased the text accordingly (p15 [ll6]).

Reviewer #1 (Significance (Required)):

R#1: *This is an interesting and well written paper describing for the first time in molecular detail how a cytomegalovirus-encoded interferon antagonist degrades STAT2 by mimicking the molecular surface properties of cellular CRL4 substrate receptors.*
This study should be of broad interest for both virologists and structural biologists.

Response: We thank R#1 for the appreciation of our work.

Reviewer #2 (Evidence, reproducibility and clarity (Required)):

R#2: *The manuscript entitled "Structure and mechanism of a novel cytomegaloviral DCAF mediating interferon antagonism" by Dr. Schwefel and colleagues cleverly combines biochemistry, mass-spectrometry, Cryo-EM and cell biology to dissect how RCMV-E hijacks its hosts ubiquitylation machinery to mediate proteasomal degradation of STAT2, a key player driving the antiviral IFN response. They identify E27 as DDB1-binding element, which is able promote CRL4-dependent ubiquitylation of STAT2, and demonstrate its effect on STAT2 levels by knockout RCMV-E strains. These findings are supported by in vitro reconstitution of the DDB1/E27/STAT2 complex and analyses via XL-MS and Cryo-EM. The obtained data are then powerfully validated and analysed in mutational strains via infection of homologue in vivo models. The results collectively explain how E27 mimics endogenous CRL4 substrate receptors, thereby recruiting STAT2 to be targeted by CLR4 for ubiquitylation in a NEDD8-dependent manner.*
Overall this is an important study that provides convincing insights on how rodent CMVs antagonize their host interferon response by exploiting its ubiquitin-proteasome system.
The manuscript is well written and its introduction is extraordinarily comprehensive. There are a few minor points for the authors to consider below.

Response: We thank R#2 for this very positive overall assessment.

Minor points:

R#2: *Page 2, line 3. "Here," should be inserted before "Global proteome profiling..." to highlight the work of this manuscript.*

Response: Thank you very much for this suggestion. We changed the text accordingly.

R#2: *Page 3, line 21. "IFNs" instead of "IFN"*

Response: We changed the text accordingly.

R#2: *Page 4, lines 9,15,27. "Ubiquitin Ligases (UbL)" is not a common abbreviation and could be mistaken for Ubl (Ubiquitin-like proteins). Possible abbreviation is "E3s" for Ubiquitin E3 ligases*

Response: You are right. We have amended the respective abbreviations accordingly.

R#2: *Page 4 line 25. "RBX1" is the more common term for "ROC1"*

Response: This has been corrected throughout the manuscript.

R#2: *Page 5 lines 1-9. Citing of the first structure of DDB1 in complex with a viral protein is recommended. (Ti Li et al. Cell 2006).*

Response: Indeed, it should not have slipped our attention that we missed to cite this work. We thank R#2 for her/his suggestion. We cited this landmark publication in the revised version.

R#2: *Figure 1 a) STAT2 dot is cut off in second panel. I recommend highlighting STAT2 in both panels.*

Response: We amended the Figure accordingly. Additionally, we highlighted the “STAT2” text in both panels by increasing the font size and setting it in bold type.

R#2: *Page 7 line 17. "Cross-linking MS (CLMS)" is commonly abbreviated as (XL-MS)*

Response: We changed the text accordingly.

R#2: *Figure 2 a-c) These panels could benefit from thinner lines in order to increase visibility of chromatograms and cross-links.*

Response: The panels were revised according to the suggestion.

R#2: *Figure 2 a-b) Could the authors elaborate on why STAT2 is stoichiometrically underrepresented in the SDS-PAGE of the E27/DDB1/STAT2 complex?*

Response: We applaud Reviewer #2 for their in-depth examination. Honestly, we were also puzzled by this. Based on the cryo-EM single particle analysis, we found an explanation: We separated a major contamination *in silico* during 2D classification (~12% of all particles). Out of curiosity, we reconstructed a

density map from these particles (**now shown in Appendix Fig S3**). The map was identical to a previous cryo-EM structure of the *E. coli* protein ArnA (Yang et al., 2019), a notorious contaminant in *E. coli* Ni-NTA protein purifications (Andersen et al., 2013). ArnA migrates similar to E27 on the SDS-PAGE, the band runs just a little bit faster (compare fraction 6 [ArnA] and fractions 8/9 [E27] from the SDS-PAGE of the analytical GF run of E27 in isolation, Fig 2A, green trace). However, in analytical GF, ArnA elutes at higher molecular weight fractions, since it forms hexamers (Ve~10.2 ml). Incidentally, this elution volume of the ArnA hexamer almost equals the one of DDB1 or DDB1$_{\Delta BPB}$/DDA1/E27/STAT2 complexes. This leads to a superposition of ArnA and E27 bands in the respective SDS-PAGE lanes corresponding to GF fraction 6. Accordingly, we concluded that it is actually not STAT2, which is underrepresented, but rather E27 seems overrepresented due to SDS-PAGE band overlap with the ArnA contaminant. We have **now indicated the contaminant in all Figures** showing SDS-PAGEs of E27-containing GF runs, amended the legend, and extended Appendix Fig S3 to indicate at which point of the cryo-EM analysis the contaminating ArnA particles were separated, and to show the ArnA model to map fit.

R#2: *Paragraph "The E27 structure.." page 9. Placing this paragraph after the overall structure is recommended.*

Response: Thank you very much for this excellent suggestion. Accordingly, we have now moved this section to the **end of the results section** (p12 [ll31], new Fig 7).

R#2: *Figure 3 a) The grey mesh being laid over the ribbon structures is not contributing to the overall visibility. Adding a panel of the cryo-EM structure alone in cost of alphafold models is recommended. Figure 4a) same issue with grey mesh*

Response: Thank you very much for this smart suggestion. We have removed the mesh representation and included panels just showing the segmented cryo-EM map, now in the **revised Fig 3A**.

R#2: *c) panels could benefit from fewer amino acids being labeled/shown*

Response: We understand and appreciate the train of thoughts of R#2. However, we would prefer to depict all relevant side chain interactions in these panels. However, the new rearrangement of the figure, i.e. showing the overview of the interacting regions before the detailed panels, should make them more accessible to the reader (**new Fig 3B**).

R#2: *d) may want to avoid red-green coloring to improve for colorblindness*

Response: We apologize for our ignorance. We changed the colors accordingly (**see new Fig 3B, C**).

R#2: Figure 6a) s.a grey mesh

Response: We removed the mesh representations and included panels just showing the segmented cryo-EM density in the **new Fig 5C**.

CROSS-CONSULTATION COMMENTS

R#2: *A 3.8 A overall resolution map and the approach to fitting may be suitable, but it is unclear from the authors' figures whether the side-chains shown in the figures are clearly visible in the map or if they are modeled by some other approach. Side chains should ideally be visible in the maps if shown in figures, and if not, close-ups of the corresponding regions of the maps should be shown with sufficient depthcue to allow the reader to gauge how the map corresponds to the model.*

Response: This is a relevant point. We have now included very detailed views of the fit of relevant side chains involved in intermolecular interaction to the experimental density (**new Fig EV2, EV3**). For more details, please also see our response to R#1 above ("major point 2").

R#2: *Along these lines, the figures with the mesh maps do not clearly show how well the model fits the map. This needs to be clearly visible in figures, and ideally maps and models provided to reviewers in order for the reviewers to gauge the level of accuracy of the fit.*

Response: We now included graphical overviews of model fits in **new Figs EV1 and EV4**. We also calculated and listed quality indicators of the model-to-map fit in the **new Table EV1** (correlation coefficients and model resolution based upon model-map FSC). To ensure the validity of our atomic model using an alternative method besides cryo-EM and XL-MS, we have performed site-directed mutagenesis of critical binding regions in E27, followed by in vitro reconstitution and analytical GF (**new Fig EV2B-E, EV3B-E**). The text was revised accordingly (see p9 [ll10] and p10 [ll24]). Please also see our response to R#1 above (section "major point 2").
In addition, we have now released the cryo-EM maps and atomic models for public access (see Data Availability statement):
map 1 - https://www.ebi.ac.uk/emdb/EMD-14802, https://www.rcsb.org/structure/7ZN7
map 2 - https://www.ebi.ac.uk/emdb/EMD-14812, https://www.rcsb.org/structure/7ZNN

R#2: *At minimum, the authors have nicely assembled proteomics and cell biological data indicating that E27 hijacks CRL4 to turn over Stat2 in rat cells in a manner paralagous to M27 hijacking in mouse cells, biophysical/structural data for a model of a CUL4-DDB1-E27-Stat2 complex, and mutagenesis of a putative zinc binding site in M27.*
I feel most of the issues raised by all 3 reviewers could be addressed in the text, with more clarity about the structural models, and better explanation for why the construct with proteins from various organisms were used for structural studies (the authors had made human DDB1 before, and it expressed well, and perhaps didn't consider to make from rat? Or this mixture expressed, purified best? Gave best quality EM data?). Also, the presentation of the zinc binding site should come after the overall structure.

Response: We thank R#2 for her/his positive overall assessment. As mentioned in the two cross-consultation comments before, and in the response to R#1 (see section "major point 2"), in the revised manuscript, we now provide adequate measures allowing the reader to judge the quality of our structural models. As indicated in the response to R#3 below (see section "major point 2"), we have also added Appendix Fig S10 and extended the Discussion to explain and justify the use of different protein constructs.

R#2: *As for the use of MCMV to assess the role of the zinc binding site, placing this last in the text might allow this to flow better.*

Response: Thank you very much for this suggestion. According to the excellent suggestions of all three reviewers, the manuscript has been restructured. Briefly, details of the zinc-binding motif and the MCMV assays are now shown in Fig 7 and described in the text just before the Discussion.

R#2: *This reviewer agrees that at least testing mutants in the E27 in some assays would be appropriate.*

As detailed in the response to R#1 (section "major point 1"), we generated two different RCMV-E E27 mutants either targeting the zinc-binding motif by site-directed mutagenesis of the three Cystine residues or the Histidine residue. In clear contrast to WT E27, both **mutated E27 lacking a functional zinc-binding motif failed to pull down DDB1**. The results are now included in the **new Fig 7B**, and in the revised text (p11 [ll29]). Furthermore, we document an interchangeability of E27 and M27 regarding STAT2 degradation in rat fibroblasts justifying our approach for the elucidation of structure and function of rodent CMV-

encoded CRL4 binders and STAT2 antagonists. These results are now included as Fig EV5 and on p12 [ll26].

Reviewer #2 (Significance (Required)):

R#2: *The work of Schwefel and colleagues combines several powerful state-of-the art techniques to dissect the mechanism of the viral protein E27 and, for the first time, provides a rational for its ability to act as STAT2 antagonist. They performed outstanding structure-function analyses of the ubiquitin system, including the first global proteomic profiling of RCMV-infected cells, setting the standard for its human counterpart as rodent CMVs are commonly used as infection models. The manuscript is highly suitable for publication in any of the journals associated with the review commons platform.*

Response: We thank R#2 for her/his kind words and the appreciation of our work.

Reviewer #3 (Evidence, reproducibility and clarity (Required)):

R#3: *Le-Trilling et al. present the first proteomic analysis of RCMV-infected cells, where they identified STAT2 as one of the most heavily downregulated (and degraded) proteins. This analysis showed that RCMV mediated degradation of STAT2 is conserved in closely related species used as animal models (rat and mouse) and human, despite the intra-host adaptation of each CMV. They also identify E27 as the RCMV factor that targets STAT2 for degradation, that exhibits ~50% homology with MCMV pM27. This study also identifies a Zinc binding motif in E27 using Cryo-EM which is conserved in other CMV species and is potentially involved in antagonising Type I and III responses.*

Response: We thank the reviewer for the accurate summary of our work.

Major and minor concerns to be addressed:

Major points

R#3: *1. The authors claim the identification of a Zinc-binding motif in the protein E27 (RCMV) using Cryo-EM, then validation of the phenotype with MCMV WT, delM27 and M27 AxAxxA. To justify the change to MCMV to perform the functional validation, they stated "MCMV M27, the closest E27 homologue, exhibits 56% and 76% amino acid sequence identity and similarity, respectively (Fig. S4B). E27 and M27 AlphaFold2 structure predictions are almost indistinguishable (RMSD of 1.195 Å, 6652 aligned atoms) (Figs. 3B, S4A), and structural alignment of these predictions demonstrated conservation of side chain positions involved in zinc-binding (Fig. 3C). Thus, M27 represents a valid model to study functional consequences of interference with the zinc coordination motif through site-directed mutagenesis, and to test the predictive power of our E27/M27 model". Although they rationalise the change to MCMV to validate the functional outcomes of the newly identified zinc binding motif with alignments and Cryo-EM data, it falls within the DDB1 binding region that is less conserved (Fig S4B). The addition of a mouse model here provides a solid result but given the aim of the paper is to provide a proper characterisation of RCMV and elucidate some inter-species adaptations, I strongly recommend the validation with E27 here given the potential impact of this motif. Rather than having to repeat this in a rat model (which would clearly be a large amount of work), this could simply be achieved by constructing the relevant deletion / mutant viruses and assessing in vitro in a relevant cell line (readout - either virus titre or luciferase assay as shown in Figure 3G/H).*

Response: As suggested by R#3, we applied site-directed mutagenesis of E27 to either mutate the CxCxxC motif or the Histidine. We demonstrate that these **mutants lacking a functional zinc-binding motif indeed fail to co-immunoprecipitate DDB1**. The results are now included in in **the new Fig 7B**, and in the revised text (p11 [ll29]).

Moreover, we now show that upon infection of rat fibroblasts, an E27-expressing ΔM27-MCMV variant is able to degrade STAT, **indicating a functional exchangeability of E27 and M27** regarding STAT2 degradation. These results are now included as **new Fig EV5** and on p12 [ll26].

In this context, we would like to highlight that almost two thirds of E27 residues in direct contact with DDB1 are at least type-conserved in M27, and the zinc-coordinating side chains are totally conserved (Fig 3C). Taking the structural organization of the respective binding regions (Appendix Fig S8) and the experimental results obtained with aforementioned E27 and M27 construct as well as the virus mutagenesis results (Fig 7, EV5) into account, the most parsimonious explanation is that the DDB1-binding mode of E27 and M27 are structurally and functionally conserved. We discussed this point in the revised Discussion section (p14 [ll11]).

Please also see our responses to R#1 (section “major point 1”) and R#2 (section “cross-consultation comments”) regarding this point.

R#3: *2. Given that previous data in mice showed that the E27 homologue pM27 binds a component of host Cullin4-RING UbLs (CRL4), to induce the poly-ubiquitination of STAT2, the current study also addressed if this mechanism was preserved in RCMV. Yet, they seemed to do this with E27, rnSTAT2 and hsDDB1 - Page 7 lines 1 to 3: "These results prompted us to explore the association of E27 with Rattus norvegicus (rn) STAT2 and Homo sapiens (hs) DDB1 in vitro. Importantly, 1128 of 1140 amino acids are identical between hsDDB1 and rnDDB1 (...)". They identify the residues and regions where the DDB1 is different between both species, but should provide a structure/alignment with this highlighted. In addition, DDB1 is a DNA damage*

protein that is annotated in the Rattus norvegicus genome. The authors should justify the assays between rnSTAT2-hsDDB1 instead of using the both proteins from rn, and present the equivalent data for rnDDB1 in the paper.

Response: Rat and human DDB1 have 1128 out of 1140 amino acids in common. Among the very few alterations (12), 4 are type-conserved (Appendix Fig S10A). Thus, >99% of amino acids of this huge protein are identical or similar. As suggested, we mapped all exchanges on a model of full length human DDB1 bound to E27 and the rat STAT2 CCD. None are involved in intermolecular contacts (**new Appendix Fig S10B, C**). Please note that due to the high conservation of DDB1 across eukaryotes, this inter-species approach has been used by others and us to study DDB1-containing complexes (e.g., the SV5V, WHX, SIV Vpx and Vpr, zebrafish DDB2, and chicken CRBN proteins have been *in vitro* reconstituted with human DDB1 for structural characterisation) and valid biological conclusions have been drawn from these studies (Angers et al., 2006; Banchenko et al., 2021; Fischer et al., 2014; Fischer et al., 2011; Li et al., 2006; Li et al., 2010; Schwefel et al., 2015; Schwefel et al., 2014; Wu et al., 2015). As suggested, we discussed this point in the revised manuscript (p16 [ll13]).

R#3: *Furthermore, in Figure 2, the GF assay was performed using full-length DDB1, however CLMS was performed using DDB1 delBPB (interchange between these two proteins continues in the remainder of the paper). This should be at least justified, and preferably one or other of wt DDB1 and DDB1 delBPB used in the GF or CLMS assay where this has not yet been performed. Later on in the results section (Fig 5E), the authors use wt DDB1 while in fig 4 they used the delBPB to describe the interaction with E27 - would be relevant to have consistency across the paper and some supplementary data that could support using one or the other in each assay.*

Response: To address the very valid concern of R#3, we **repeated all GF experiments in which full length DDB1 had been used with DDB1$_{\Delta BPB}$ and obtained congruent results**. These data are now included in the **new Fig 2A, EV2C, EV3D**.
Protein complex preparations including full length DDB1 did not yield cryo-EM reconstructions at appropriate resolution for model building, almost certainly due to the known flexibility of the DDB1 BPB, impeding proper alignment of the cryo-EM particle images. This is why we applied DDB1$_{\Delta BPB}$. Importantly, the structure model including full length DDB1 (Appendix Fig S10B) clearly demonstrates that the BPB is located on the opposite side of the E27 binding interface on DDB1, where it is situated to flexibly connect to the CUL4 scaffold to create the ubiquitination zone around immobilised substrates (Fig 6). This orientation rules out an involvement of DDB1 BPB in E27- and/or STAT2-binding processes. Several previous studies have employed DDB1$_{\Delta BPB}$ to facilitate structure determination, and have successfully applied the resulting structural models for functional follow-up experiments in the context of complete CRL4 assemblies (Bussiere et al., 2020; Petzold et al., 2016; Slabicki et al., 2020). **We now mention this in the Discussion** (p16 [ll27]).

Minor points:

R#3: *1. In fig 5D, the authors present the H-box alignment, where it is clear that this motif is not conserved. The lack of H-box conservation should be discussed in the results and discussion, to provide an explanation for the competition/binding observed.*

Response: Please note, that the H-box is far from being our invention but rather represents a well-accepted motif known in the field, see e.g., (Li *et al.*, 2010). There is conservation of amino acid side chains, regarding their physicochemical properties, observable in the H-box motif. Furthermore, the secondary structure is conserved. We **extended the Discussion to cover this point** more appropriately (p15 [ll22]).

R#3: *2. Although they present sufficient detail in the methods, further details in the text should be given as to the number of repeats performed in each case, and whether the data shown is representative or based on an average of repeats (preferably the latter; if representative, the data for other repeats should be shown in supplementary information).*

Response: Thank you very much for this important remark. This information regarding repeats has now been added to all respective Figure legends.

3. *The authors commence their abstract justifying the study on the grounds of the usefulness of rodent HCMV counterparts as common infection models for HCMV. They should return to this theme in the discussion - what is the usefulness of their findings with regards to HCMV (particularly given the relatively low homology between E27 and HCMV pUL27, and the alternative mechanism for STAT2 antagonism encoded by HCMV UL145)?*

Response: We **extended the Discussion** in this regard (p14 [ll31], p17 [ll11]). Briefly, our data, to our knowledge for the first time, reveal that RCMV E27 exploits the CRL4 E3 ligase to induce ubiquitylation and proteasomal degradation of STAT2. The structural results, together with sequence alignments, site-directed mutagenesis, and previous studies of MCMV M27 strongly suggest that this mechanism is conserved among rodent CMVs. Mediated by UL145, HCMV also hijacks host CRL4 to induce STAT2 degradation. The exact molecular mechanisms of UL145-mediated STAT2 recruitment to CRL4, however, remain to be determined, since UL145 is evolutionary unrelated to E27/M27.
In clear contrast to HCMV, rat and mouse CMV are both amenable to in vivo experiments in small animal models. Over 40 years ago, HCMV has been called the troll of transplantation due to its grim impact on immunosuppressed individuals after transplantation surgery (Balfour, 1979). Despite tremendous efforts, HCMV still harms and kills graft recipients. While MCMV allows various experiments regarding general principles of cytomegaloviral pathogenesis and antiviral immunity, one shortcoming is that the mouse obviously is a rather small animal, preventing various chirurgical and solid organ transplantation (SOT) procedures. In clear contrast, SOT procedures that are indispensable for human medicine can be recapitulated in rat models. Thus, according to our opinion, our work lays the molecular, structural, and functional foundation for future studies addressing the relevance of STAT2 and CMV-induced STAT2 degradation in rat SOT models.

Reviewer #3 (Significance (Required)):

R#3: *The present work provides the first proteomics analysis of RCMV infection in rat cells, comparing infected vs non-infected rat fibroblasts to access potential RCMV targets. Then, it focuses on the characterisation of RCMV E27 and its role targeting and interacting with STAT2 (plus recruiting the Cul4 complex for STAT2 degradation). Finally, it provides the Cryo-EM structure of E27 and its CMV homologues, and the structure of the complex of E27 with elements of the CUL4 complex and STAT2. This is the first time that E27 function and structure are characterised. These are all novel findings - although the mouse homologue M27 has previously been found to interact with and degrade STAT2 (published by some of the same authors in Plos pathogens in 2011, (https://doi.org/10.1371/journal.ppat.1002069). Therefore the chief novel information is the structural studies.*
The manuscript will be of interest to researchers working with human and animal herpesviruses.
My field of expertise is in Virology, Innate Immunity and host-virus interactions from an evolutionary perspective. I do not have expertise in Cryo-EM, so I could not evaluate the methods used in the section.

Authors Response: We are very grateful for the positive evaluation of our work and its significance.

References

Andersen, K.R., Leksa, N.C., and Schwartz, T.U. (2013). Optimized E. coli expression strain LOBSTR eliminates common contaminants from His-tag purification. Proteins *81*, 1857-1861. 10.1002/prot.24364.

Angers, S., Li, T., Yi, X., MacCoss, M.J., Moon, R.T., and Zheng, N. (2006). Molecular architecture and assembly of the DDB1-CUL4A ubiquitin ligase machinery. Nature *443*, 590-593. 10.1038/nature05175.

Balfour, H.H., Jr. (1979). Cytomegalovirus: the troll of transplantation. Arch Intern Med *139*, 279-280. 10.1001/archinte.139.3.279.

Banchenko, S., Krupp, F., Gotthold, C., Burger, J., Graziadei, A., O'Reilly, F.J., Sinn, L., Ruda, O., Rappsilber, J., Spahn, C.M.T., et al. (2021). Structural insights into Cullin4-RING ubiquitin ligase remodelling by Vpr from simian immunodeficiency viruses. PLoS pathogens *17*, e1009775. 10.1371/journal.ppat.1009775.

Bruggeman, C.A., Meijer, H., Dormans, P.H., Debie, W.M., Grauls, G.E., and van Boven, C.P. (1982). Isolation of a cytomegalovirus-like agent from wild rats. Arch Virol *73*, 231-241. 10.1007/BF01318077.

Bussiere, D.E., Xie, L., Srinivas, H., Shu, W., Burke, A., Be, C., Zhao, J., Godbole, A., King, D., Karki, R.G., et al. (2020). Structural basis of indisulam-mediated RBM39 recruitment to DCAF15 E3 ligase complex. Nat Chem Biol *16*, 15-23. 10.1038/s41589-019-0411-6.

Fischer, E.S., Bohm, K., Lydeard, J.R., Yang, H., Stadler, M.B., Cavadini, S., Nagel, J., Serluca, F., Acker, V., Lingaraju, G.M., et al. (2014). Structure of the DDB1-CRBN E3 ubiquitin ligase in complex with thalidomide. Nature *512*, 49-53. 10.1038/nature13527.

Fischer, E.S., Scrima, A., Bohm, K., Matsumoto, S., Lingaraju, G.M., Faty, M., Yasuda, T., Cavadini, S., Wakasugi, M., Hanaoka, F., et al. (2011). The molecular basis of CRL4DDB2/CSA ubiquitin ligase architecture, targeting, and activation. Cell *147*, 1024-1039. 10.1016/j.cell.2011.10.035.

Ireland, S.M., and Martin, A.C.R. (2019). ZincBind-the database of zinc binding sites. Database (Oxford) *2019*. 10.1093/database/baz006.

Laitaoja, M., Valjakka, J., and Janis, J. (2013). Zinc coordination spheres in protein structures. Inorg Chem *52*, 10983-10991. 10.1021/ic401072d.

Le-Trilling, V.T., and Trilling, M. (2017). Mouse newborn cells allow highly productive mouse cytomegalovirus replication, constituting a novel convenient primary cell culture system. PloS one *12*, e0174695. 10.1371/journal.pone.0174695.

Li, T., Chen, X., Garbutt, K.C., Zhou, P., and Zheng, N. (2006). Structure of DDB1 in complex with a paramyxovirus V protein: viral hijack of a propeller cluster in ubiquitin ligase. Cell *124*, 105-117. 10.1016/j.cell.2005.10.033.

Li, T., Robert, E.I., van Breugel, P.C., Strubin, M., and Zheng, N. (2010). A promiscuous alpha-helical motif anchors viral hijackers and substrate receptors to the CUL4-DDB1 ubiquitin ligase machinery. Nature structural & molecular biology *17*, 105-111. 10.1038/nsmb.1719.

Petzold, G., Fischer, E.S., and Thoma, N.H. (2016). Structural basis of lenalidomide-induced CK1alpha degradation by the CRL4(CRBN) ubiquitin ligase. Nature *532*, 127-130. 10.1038/nature16979.

Sandford, G.R., Brock, L.E., Voigt, S., Forester, C.M., and Burns, W.H. (2001). Rat cytomegalovirus major immediate-early enhancer switching results in altered growth characteristics. Journal of virology *75*, 5076-5083. 10.1128/JVI.75.11.5076-5083.2001.

Schwefel, D., Boucherit, V.C., Christodoulou, E., Walker, P.A., Stoye, J.P., Bishop, K.N., and Taylor, I.A. (2015). Molecular Determinants for Recognition of Divergent SAMHD1 Proteins by the Lentiviral Accessory Protein Vpx. Cell host & microbe *17*, 489-499. 10.1016/j.chom.2015.03.004.

Schwefel, D., Groom, H.C., Boucherit, V.C., Christodoulou, E., Walker, P.A., Stoye, J.P., Bishop, K.N., and Taylor, I.A. (2014). Structural basis of lentiviral subversion of a cellular protein degradation pathway. Nature *505*, 234-238. 10.1038/nature12815.

Slabicki, M., Kozicka, Z., Petzold, G., Li, Y.D., Manojkumar, M., Bunker, R.D., Donovan, K.A., Sievers, Q.L., Koeppel, J., Suchyta, D., et al. (2020). The CDK inhibitor CR8 acts as a molecular glue degrader that depletes cyclin K. Nature *585*, 293-297. 10.1038/s41586-020-2374-x.

Smith, C.B., Wei, L.S., and Griffiths, M. (1986). Mouse cytomegalovirus is infectious for rats and alters lymphocyte subsets and spleen cell proliferation. Arch Virol *90*, 313-323. 10.1007/BF01317379.

Tang, S., and Yang, J.J. (2013). Magnesium Binding Sites in Proteins. In Encyclopedia of Metalloproteins, R.H. Kretsinger, V.N. Uversky, and E.A. Permyakov, eds. (Springer New York), pp. 1243-1250. 10.1007/978-1-4614-1533-6_257.

Wu, Y., Koharudin, L.M., Mehrens, J., DeLucia, M., Byeon, C.H., Byeon, I.J., Calero, G., Ahn, J., and Gronenborn, A.M. (2015). Structural Basis of Clade-specific Engagement of SAMHD1 (Sterile alpha Motif and Histidine/Aspartate-containing Protein 1) Restriction Factors by Lentiviral Viral Protein X (Vpx) Virulence Factors. The Journal of biological chemistry *290*, 17935-17945. 10.1074/jbc.M115.665513.

Yang, M., Chen, Y.S., Ichikawa, M., Calles-Garcia, D., Basu, K., Fakih, R., Bui, K.H., and Gehring, K. (2019). Cryo-electron microscopy structures of ArnA, a key enzyme for polymyxin resistance, revealed unexpected oligomerizations and domain movements. J Struct Biol *208*, 43-50. 10.1016/j.jsb.2019.07.009.

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