nature portfolio

Peer Review File

Structure and mechanism of the Zorya anti-phage defense system

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 1:

Reviewer comments:

Referee #1

(Remarks to the Author)
This manuscript describes the structural characterization of the recently discovered Zorya phage-defense system in bacteria. This system comes in 3 types with a conserved core pair of genes, and other genes that vary across the types. The authors chose to focus on a type I EcZorya system that defends against a diverse set of coliphages. The authors argue that Zorya can defend against phage while maintaining cell viability, implying a direct defense against phage infection. The authors then resolve a cryoEM structure of the ZorA5B2 complex, which is an inner membrane complex with structural similarity to the motor subunit of flagellor stator unit MotA5B2. The authors suggest that the C-terminal region of ZorA may form a long cytoplasmic alpha-helical bundle stretching into the cytoplasm. The ZorB periplasmic domains form a homodimeric peptidoglycan binding domain that may be important in switching the complex to an open-channel state for protons to drive the rotor. The authors provide additional cryoEM structures of the ZorC, ZorD cytoplasmic genes and suggest that ZorC and ZorD are recruited to the ZorAB cytoplasmic helical tail during phage infection and play a role in phage defense by genome degradation.

Although the ZorAB structure presented is fascinating and suggestive of a unique defense mechanism, the paper ultimately does not elucidate the mechanistic basis of phage defense. The initial report on Zorya suggested that it functioned by causing membrane depolarization. Here, the authors suggest that ZorAB instead somehow use changes in ion/proton flow across the membrane to signal to the interior components, ZorCD, to drive a direct defense and phage DNA cleavage. While this could be the mechanism, there is little conclusive data to support it, as summarized below. There are also major issues with overinterpretation and speculation throughout the paper. Without more incisive and rigorous studies of the mechanism, the structure (and mutagenesis) reported here feels more appropriate for a specialty journal.

Major concerns:

Mechanism of defense::

Fig. 1e-g don't fully support the claims of direct defense. There's a clear MOI dependence in Fig. 1e with the OD dropping at even an MOI of 1, as classically seen with Abi systems. There may not be full collapse of the OD and clearing of the culture, but the patterns in Fig. 1e and Fig. 1g are not what one expects for a direct defense system. This warrants further investigation and clarification.

ZorAB as a possible rotary motor::

Page 9-10, lines 232-292: The authors point out the structural homology of the ZorA5B2 to the MotA5B2 and expect it to function as a proton motif force driven motor driving the rotation of the ZorA outer ring relative to the inner ZorB dimer in the membrane. Are there known mutations that arrest or break the rotation of the MotA5B2 complex (potentially by introducing structurally proximal paired Cys), that could be introduced into ZorA5B2? Specifically, this would be an interesting way to test the hypothesis that the ion permeation-driven motor is essential for phage defense. I understand that the authors test this idea by mutating the ion pathway, but arresting the rotation would be a more direct test of the rotation of ZorA.

lines 251-252 " which is consistent with freedom of ZorA to rotate around ZorB (through Brownian motion) in the mutant." But there is no evidence that rotation does occur. If it did, then it also seems like the cryo-EM would have revealed heterogeneity in the transmembrane sections of the complex, but it appears not to have. So additional data supporting rotational activity is needed. In short: the authors conclude that "ZorAB is a peptidoglycan-binding rotary motor" but the evidence to support this

claim are currently lacking.

Page 10, lines 282-291: Following the above point, the authors highlight the T147A mutation in the ion-permeation pathway and its effect on phage defense. Specifically, the authors claim that T147A “significantly decreases defense ability”. Based on Figure 3h and Extended Figure 6a, this is not a consistent phenotype across the various phage tested. For Bas24 and Bas58 it seems like T147A had almost no effect. Given the authors general model, such a mutation to break ion-permeation and motor function should dramatically affect defense against all phage.

Page 12, lines 316-323: The authors highlight the importance of the R108-D22 and H92-F228 electrostatic contacts for the integrity of the ZorA MPCD, and assembly of the dimer. But they infer this by looking at the ZroA(G223-T729) tail-deletion mutation. It seems prudent to test the double mutant directly in phage infectivity assays to confirm their importance in stabilizing the complex and maintaining phage defense.

ZorB and the possible role of peptidoglycan binding::

It's unclear to me whether the authors are proposing that ZorB senses changes in the peptidoglycan and then uses the possible rotary motor of the ZorAB complex to transmit a signal or whether ZorAB is sensing changes in the proton motif force known to be perturbed during phage infection, with ZorB binding to PG playing a more ancillary role. Whatever the case, there's insufficient evidence that ZorB binding to PG is important to sensing or transmission of a signal, and there's no direct evidence that ion flow through ZorAB occurs and is critical to phage sensing.

If ZorAB is simply detecting disruptions to the peptidoglycan and/or membrane, why doesn't Zorya provide broader protection against more phages? In Fig. 1b it seems many phages are not inhibited by Zorya at all. I.e. given the authors' model, how would one explain the phage specificity of the system?

Page 4: "...we propose that the dimeric ZorB peptidoglycan-binding domain anchors the ZorAB complex to the cell wall near the phage injection site and that the ion-driven motor rotates the ZorA tail within the cytosol." Unfortunately, no evidence is presented that ZorB switches from not bound to peptidoglycan to 'anchored' to it following phage infection. And what PG structure would ZorB be recognizing that is associated specifically with phage infection?

Following the point above, page 9, lines 216-230: With respect to the PG-binding domain of MotB, the authors hypothesize binding of the disrupted peptidoglycan layer to transmit information of phage invasion by driving motor rotation. Could the authors mutate the relevant PG-binding sites, or dimerization interface (and disulphide bonds) to show that this is important to phage defense?

ZorAB signaling to cytoplasm::

Page 12, lines 324-361: The authors test tail disruptions and their effect on phage defense by deleting sections of the ZorA C-terminus to reduce the presumed length of the helical bundle tail. The authors highlight that there are regular hydrophobic residues in ZorA (L250, L254, L258, L261, and further as you move down the bundle), that presumably form a core and stabilize the bundle structure. Could the authors disrupt the bundle formation without altering length by synthesizing a ZorA variant with these hydrophobic residues mutated, that could directly test the function of the bundle, versus the length of the C-terminus available. This would further add some data to justify the authors speculation that unraveling the ZorA tail might be important in activation, if these bundle de-stabilizing mutations lead to constitutively active Zorya (judged by microscopy, or potentially leading to cell toxicity).

Page 14, lines 395-407: While testing the function of various ZorC and ZorD mutants, the authors systematically confirm the importance of many structurally relevant residues and residue-networks. The authors highlight the DEAQ-motif in the ZorD C-terminus that is shown to be important for plasmid degradation in vitro (Figure 4i). It is important that the authors also include this double mutant in the phage defense assay in Figure 4h to connect the nuclease activity directly to the phage defense phenotype.

There is some evidence presented that ZorC binds DNA. Is there any sequence specificity? Additionally, to support a role for DNA binding in phage defense, the authors should demonstrate DNA binding during phage infection. The mutation of predicted DNA-binding residues ablates defense, but this is indirect and insufficient evidence for DNA binding occurring during an infection.
p. 14, line 405: "These results suggest that ZorD harbors a nuclease activity, and full-length ZorD is in an autoinhibited state, which is likely activated once the defense is triggered, presumably through a conformational change." This is an intriguing hypothesis but additional data are needed to substantiate it. Is there structural evidence that ZorD adopts two different conformations? How does ZorC binding influence ZorD activity? Is there evidence that ZorD cleaves phage DNA (and not host DNA) during an infection?

Cell biology and the localization of Zorya components::

Fig. 5 presents intriguing data that the localization of Zor components may change following phage infection. However, there's still quite a bit of localization of all components prior to infection so it's not clear how crucial the increases seen are to defense.

It's also unclear in Fig. 5 whether the foci corresponding to ZorB, ZorC, and ZorD, which are imaged separately, are co-

localizing. This should be addressed through co-localization studies. Co-IP or other experiments to demonstrate changes in interactions upon phage are also needed.

The authors should assess whether the Zor components are localizing to sites of infection or not to understand how Zorya ultimately provides defense. It could be that Zorya co-localizes to sites of phage genome injection, but it could also sense phage infection at a distance via changes in PG and/or the proton motif force and then somehow trigger ZorC/D to 'find' phage DNA.

The TIRF experiments need additional controls: (a) It is important to perform the experiment of mNG-tagged ZorC and ZorD, with phage infection but with ZorAB deleted. Phage infection causes dramatic and rapid changes to the cytoplasm and cellular metabolism, and it is important to confirm the puncta formed by ZorC and ZorD are not just due to phage infection. (b) To confirm that the tail plays a key role in recruiting the freely diffusing ZorC and ZorD, the authors should repeat the microscopy with the ZorAB complexes that have tail deletions which are compromised in phage defense. (c) The argument that T4 doesn't lead to a proportional increase in puncta during infection, different from a truly defended phage like Bas24 has a caveat. Specifically, phage like T4 have super-infection exclusion where the first infection blocks subsequent phage infections by inhibiting the lysozyme of the tail tip of the secondary phage particles (doi: 10.1038/s42003-020-01412-3). This would directly prevent the production of free PG-ends that your model requires to begin the downstream signaling. (d) Could the authors repeat the microscopy with E.coli expressing mNG-tagged Zorya exposed to lysozyme? This should generate ruptures in the peptidoglycan layer (analogous to most phage tails) and trigger the system per the author's model. (e) To confirm that it is not the ongoing phage lifecycle that leads to ZorC, ZorD puncta formation, the authors should UV- or EMS-mutagenize Bas24 until its MOI falls significantly. This is one of the classic experiments that generates functional phage particles but dramatically compromised phage genomes that cannot proceed in their transcriptional program. This can test the activation and puncta formation of Zorya proteins during phage infection, but without a proper phage lifecycle.

Additional comments on the proposed model::

In the introduction the authors argue that "A long rotating tail-spike located close to the site of phage DNA injection is strongly suggestive of a "bobbin" around which the invading DNA could be wound, thereby immobilizing it for subsequent degradation." and in the Discussion (page 19, lines 536-43) there are similar claims. This model is pure speculation - there are no data to support this model. And there are some data that argue against it, e.g. it seems the authors note the hydrophobic nature of the predicted ZorA cytoplasmic tail, which seems incompatible with DNA binding.

Why must the ZorA tail be so long, i.e. 70 nm? It's unclear how or why this extended conformation is needed.

Minor points::
1. S184 is mis-labelled as T184 in Figure 3g.
2. Page 19, lines 550-553: It is possible the nucleases are ineffective against the glucosylated genome of T4 (and its sisters), which is how T4 is resistant to many nuclease-based defense systems (RM, and CRISPR). The authors should test this speculation since it is easy enough to do.
3. Could the authors comment on the PG-domain of the ZorB homologs found in gram-positive bacteria? Is there a difference in how the PG domain must function in gram-positive bacteria with a cell-wall that has dramatically different architecture and depth compared the PG-layer of gram-negative bacteria between membranes. The authors show structural predictions (Extended Fig 11), but a sequence alignment of gram-positive versus gram-negative ZorB PG-binding domains may be further illustrative.
4. There is a possible lipid seen inside the ZorA tail. Any idea what lipid this might be and what its relevance is?

Referee #2

(Remarks to the Author)
Hu et al. present a significant contribution to the field with the high resolution structure of Zorya anti-phage system. This is significant because there are so few rotary ion-powered motors that have been structurally solved, and in particular this motor has a novel function coupled to rotation and as such offers insight into the function and origin of all such systems.

Overall the work is very clearly presented, well written, and it is easy to understand the insights arising from the work. I recommend acceptance with minor revisions which I detail in full below. I enjoyed the discussion and think the work is well stated with the appropriateness of the claims, abstract and conclusions.

Of my minor suggestions, I think the ones that perhaps require more attention surround the single molecule fluorescence results and the reporting of the change in diffusion (as well as the quality of the video data available in SI). In particular it would be nice to have more raw data for this as it is hard to discern changes in diffusion based on what is available. The authors could consider quantifying this further, but at a minimum making the raw imaging available would be preferrable to the GIFs presented in Supplementary Material.

Minor comments (* for more important ones):
L39 – not sure need comma after ZorD here.
L111 – I do not know what a bobbin is. I think I can work it out but perhaps a more natural word for wider clarity here?
L122 – not clear what ΔRM is here.

L125 – I had a hard time understanding this sentence: Phage adsorption was not affected by EcZorI, indicating that anti-phage defense occurs at a subsequent stage of infection (Fig. 1c). By L139-142 I understood it. But I think the underlying issue was that I didn't understand what was happening at 12 hr point in control, ie, what is the natural defence process (Fig 1e).
*Fig. 1 – does this mean ancestral state is with zorCD and this was subsequently lost in type II and type III? Can we assert this based on rooting of this tree? How was root established?
L152 – what is AAI? Not clear where this is in figure.
L205 – this is approached in discussion, but at this point I noted the shorter linker to the PGB domain vs MotB and was wondering how force sensing might occur (how does complex sense force via perturbation of PGB domain – please see comment below at L500)
*Fig. 2 – why is model of filament dead straight? EM micrographs appear supercoiled?
*L225 – it feels suitable to mention the shorter linker here to PGB domain.
L241 – what is MPDC? I found this in figure but probably worth mentioning here also to aid reader.
*L297 – I realise space is at premium but it's hard to tell immediately what is being probed with each of these deletions in the figure. Is it possible to group them at least to show ie tail deletion vs pore deletion. The info comes in later at L321 and L345 but I think would be nice if some indicator in figure also. For example use labels beginning, middle, tip, as per L345?
L297 – E.coli needs space.
L309 – dashed not bashed
L371 – we mapped…. Form an; should perhaps be we mapped… to form? I think perhaps a word missing here to make complete sentence.
L373 – individual substitution … abolish (should be individual substitution of E400 or H443… abolishes)
*L383 – how was non-specific dsDNA chosen and was there any effect ie with CG content or secondary DNA structure (ie hairpins or otherwise). Not sure I understand 'scrambled' in this context (L847-856) in DNA context this can be used to mean a deliberately non-complementary scrambled partner, but in methods only list single strand identity and refer to scrambled dsDNA substrate, please clarify, not clear what is scrambled relative to what.
*L451 – can we quantify diffusivity? It should be possible to do some particle tracking and then report change in diffusivity vs qualitatively. The extended data was a gif, and I couldn't understand raw intensity well, but also it appeared 200 ms intervals, would imagine hard to be sure about diffusion with a long interval, but not sure about relative diffusion speed of complex and frames available here. For example, L455 'appeared to become static', I think this should be quantifiable with a change in mean displacement.
*L500 – this appears first mention of length difference in linker. I think could be mentioned as result earlier at first observation. Also, for discussion could be options around knocking probe into cell (such as AFM, bead, micropipette or optical trap) and observing zorCD recruitment?
*L521 – how does tail recruit ZorCD. I think this is mentioned in discussion L530-543, but I was not sure what evidence was for DNA to be 'reeled in' vs just overwound or constrained in some way. Eg L201-202 Supplementary Material refers to this as 'almost inevitable' but that seems strong without some model or precedent showing (admittedly likely but this is quite novel, not much known regarding winding by molecular motor and at this length/timescale, so perhaps more caveat in text).
*L547 – what is min and max intensity of the images shown in Supp: Ext Data Fig 10? Asserting in text here increase in foci and intensity, but not sure we can interpret without knowing scale of image in SI.
L980 – no space between number and degrees centigrade, ie should be 30˚C
L991 – what is total laser flux for 14 frames, or flux in units /s?
L994 – what are settings in microbe, ie thresholds for maxima and particle detection, should be possible to do diffusion analysis as per above.
*L1008 – don't understand: 'randomly selected one system for each set of these flanking genes'. Can you clarify?
L1011 – how were representatives chosen?
L1013 – how were ZorA5ZorB2 multimers run as multimers in colabfold? As pentamer and dimer separately?

Referee #3

(Remarks to the Author)
Hu et al. provide mechanistic insights into the Zorya anti-phage defense system discovered by the Sorek group in their seminar 2018 paper, where it was proposed that Zorya subunits ZorAB act as a MotAB-like channel to depolarize the host cell membrane in response to phage replication, resulting in abortive infection. In the current manuscript, Hu et al. use a sophisticated combination of cryo-EM, phage assays, biochemistry and in vivo imaging to provide the first detailed study of this system. They find that ZorAB is a rotary motor (with the typical 5:2 stoichiometry as previously discovered for MotAB by the Taylor group) with a peptidoglycan-binding periplasmic domain, and a long cytoplasmic tail. They demonstrate that ZorC is a dsDNA binding protein, and that ZorD is an autoinhibited nuclease. On this basis, they propose that ZorAB is the sensor module of the system, and upon phage DNA injection ZorC and ZorD are recruited to degrade phage DNA. This is a very strong piece of work that will undoubtedly be of broad interest. However, there are several concerns that should be addressed before the manuscript is accepted:

Major points:
We thank the authors for providing the maps and models. While the characterization of ZorAB is very thorough, it appears that much less consideration has been given to ZorC and ZorD. The structures of both are of sufficient quality (even given the preferred orientation of ZorC), but they provide little insights into the mechanism of Zorya. Additionally, the DNA binding assays in Fig 4d & e are a significant weakness of the manuscript.

Specific points to address:

1. In Fig 4d, at high concentrations, large aggregates of ZorC + DNA are stuck in the wells. Is this due to the size of the DNA substrate chosen? If they repeat this with a shorter dsDNA substrate, would this effect still be observed? If so, this might provide mechanistic insights - perhaps multiple copies of ZorC binding to a single phage genome upon recruitment by ZorAB is important for interference.

2. Do ZorC and ZorD form a complex? The authors should attempt this (ideally in the presence and absence of dsDNA substrate), together with AlphaFold2 multimer modeling the complex.

3. ZorD is autoinhibited, but the CTD appears to be an active nuclease. Have the authors attempted to use AlphaFold2 to predict alternative conformations that may be active in contrast to the closed, circular observation they observed in their DNA-free structure?

4. Where does ZorD bind DNA, and might this predict how their structure is autoinhibited? The authors should align other previously-determined structures bound to nucleic acids (as identified using Dali or FoldSeek) with their structure and analyse the results. This should also be performed for ZorC.

5. Perhaps the presence of both ZorC and ZorD together is required for ZorD nuclease activation. This should be tested experimentally. If this is the key to activating ZorD, perhaps this offers mechanistic insights i.e. ZorAB activation recruits ZorC and ZorD together, which is required for phage DNA degradation. If full-length ZorD is inactive in the absence of ZorC (as the authors demonstrate), then this may provide a mechanism for preventing aberrant activation in the absence of phage-induced ZorAB activation.

Minor points:

1. Since the authors already have TIRF data, would it be possible to perform foci tracking to determine rates of diffusion? It would be useful to quantify “diffusibility” in meaningful terms, especially since the single fluorescence micrographs in Fig 5 don’t look terribly different in the presence and absence of phage. In the exemplary images shown, the ZorB untreated vs +phage also appear to show a large increase in the number of foci akin to ZorC and ZorD, which contradicts the model provided by the authors, so diffusion rates etc would be very useful.

2. Could the authors speculate upon why certain clades of viruses are not targeted by Zorya? The Dhillonvirus, Demerecviridae and Straboviridae (amongst others) show complete immunity to Zorya - is there anything in particular about these viruses (putative anti-Zorya genes? Differences in DNA injection mechanism? DNA modification) that do not trigger Zorya?

3. Thank you again for providing the structures for review. They look good, and have been well-built. There is a minor issue, however - the hydrogen atoms have been left on the models (presumably from when they were refined in Isolde). These should be removed prior to pdb deposition, and the Phenix real-space refinement or comprehensive validation should be run again, since the clash score will likely change.

David Taylor and Jack Bravo

Version 2:

Reviewer comments:

Referee #1

(Remarks to the Author)
This revised paper has improved since the original submission with substantially less speculation than before. Some additional experiments have also been added and bolster certain aspects of the study. Notably, the notion that the PMF is used to drive a conformational change in ZorA to transmit a signal to ZorC/D is better supported and is an important aspect of how the system likely works. However, the other major facets of how Zorya functions remain incompletely defined. As with most defense systems, there are two general features - detection of a phage and activity of an effector. Each is discussed below.

Phage detection: For Zorya, the authors propose that ZorB plays the role of phage detector based on peptidoglycan (PG) binding. There are two primary models that could explain how ZorB works, as articulated in the response document from the authors. One is that ZorB senses a pinching of the cell envelope during phage infection, with the PG layer brought in closer proximity to the inner membrane and ZorB. The other is that ZorB recognizes some aspect of broken/perturbed PG created during infection. The model in Fig. 6 implies it's the latter, but the text favors the former - and neither is yet proven. In addition to reconciling the text and figures, the authors need to (i) clearly establish that ZorB binds PG and (ii) distinguish between these models (see detailed comments below as well). To me, the sensing of phage infection via a change in PG is the most novel and exciting aspect of the study at hand, so I think shoring up the conclusion that ZorB binds PG and discerning between the two possible models seems important, and feasible, to do.

Effector activity: it remains unclear how the rotation of ZorA that is likely triggered by ZorB and powered by the PMF leads to a change in ZorC/D within the cell. C and D don't seem to interact but are both required for defense and both colocalize to some extent with ZorA. Sorting out the details of how C and D contribute and how they interact with each other and ZorA

would take some work and isn't as exciting as the sensing mechanism, so I think punting here is not ideal but reasonable. But, I do think the authors at least need to address why they see colocalization via TIRF microscopy in only 20-30% of cells - see last point below.

Specific issues:

"Here, we reveal the molecular basis of Zorya defense" This is an overstatement in the abstract and should be rephrased. The Discussion has a more balanced and measured self-assessment, indicating that *some* aspects of Zorya defense are revealed here.

The authors' response makes a reasonable case that the mechanism of protection is direct and not an abortive infection. But how do ZorC/D avoid cutting the host chromosome? It's speculated that it could be localization-based, but there's no data as yet indicating that cleavage of the phage DNA occurs in vivo (only in vitro data).

line 166: it should read 'putative' or 'predicted' peptidoglycan binding domain. At this point, a biochemical activity has not been established, only predicted based on sequence.

The evidence that ZorB binds peptidoglycan needs to be bolstered. The only data presented are in Extended Data Fig. 4e and they show quite weak pulldown of ZorAB with PG, with no controls, e.g. a known PG binding protein and a mutant of ZorB thought to ablate binding in vivo. Without these sorts of controls, it's not rigorously established that ZorB binds PG in vitro, let alone in vivo.

It does not become clear until the Discussion that the authors are proposing a model in which ZorB does not initially bind PG and then PG deformation by the phage leads to binding. This aspect of the model remains murky and should be better substantiated, e.g. by assessing ZorB-PG binding before and during infection in vivo, and the model and data (with caveats) should be discussed within the manuscript. This is argued in the response document to be beyond the scope of the manuscript. While some aspects of the model can reasonably be argued to be beyond the scope, this facet seems central to the story at hand. There are several ways the authors could go about showing this or at least providing some additional evidence, from examining PG-protein association (as in https://doi.org/10.1128/jb.186.20.6728-6737.2004) to direct imaging.

Further to the point above, the authors write "These data confirm that ZorB PGBD integrity, dimerization and PG binding are required for Zorya defense." The data referred to here are simply a series of alanine mutations - again, the data to support the claim of PG binding are not adequate yet.

Finally, related to the model being proposed that it's about distance between the PG and the inner membrane/ZorB that is being 'sensed' by ZorB: the model in Fig. 6 is very misleading as it is drawn to imply that ZorB is binding/sensing frayed ends of the PG layer created by the phage. This is a very different model/idea than what the text says (and what is stated in the rebuttal document where both models are suggested to be possible).

When activated, does ZorA freely and continuously rotate around ZorB or simply change conformation? I know ZorAB is homologous to MotAB and there's free rotation for that system, but it's not clear to me that the same is necessarily the case here.

All mutations (e.g . Fig. 3h, 4c, 4i) are assumed to disrupt the function they're hypothesized to impact. But many could also be impacting protein levels/stability/folding - this needs to be explicitly acknowledged and ideally demonstrated not to be the case in key cases, e.g. by immunoblotting.

"in the presence of phage Bas24, at least one ZorC-mNG or ZorD-mNG focus was co-localized with a ZorB-HT focus in approximately 20% or 30% of cells, respectively (Fig. 5e, f). These data suggest that the cytosolic effector proteins ZorC and ZorD are recruited to ZorAB complexes that are activated by phage infection." What about the other 70-80% of cells?? It seems a little premature to conclude that ZorC/D are recruited to ZorAB if colocalization is only seen in a minority of infected cells. The authors could substantiate their model through co-IP studies before and during infection.

Referee #2

(Remarks to the Author)
Hu et al. have comprehensively answered my minor requests. In particular, I appreciate the consideration shown to rooting the Zorya tree and also accept the reasoning for not pursuing higher time resolution diffusion measurements (in response to R3.6). I also think the newer two-color co-localisation measurements are well suited to addressing the actual line of inquiry - which components come together in response to phage infection.

I also appreciate the addition of extra detail to the methods, which I think is required to make the work more accessible, and the restructuring of the paper to add supplementary discussion on more speculative claims.

In response to my query on L991 there is perhaps a minor thing, flux is usually in eg uW/s, but in my query I said 'total flux' over 14 frames, and then asked for flux in units/s so this was a somewhat confusing request from my end. I assume this is very minor and I am satisfied that you have measured the power here, which is ultimately what I was requesting.

There is a small typo however there also so maybe worth exploring:

"Samples were excited at 50 ms exposure for 14 frames using a 488 nm laser (flux of 550 µW with a sensor area 941 of 283.5 cm2) at 80% and emission was recovered via a quad TRIF filter cube (emission: 502-549 nm)."

should be TIRF not TRIF, and I estimate here this should be power not flux, ie laser power of 550uW delivered over an area of 283.5cm2? This does seem a little large tho, perhaps sensor area is the size of the chip on the camera? These are all minor points and I don't necessarily need to see again but I think just a small error here you are likely reporting power, not flux, and that is what I was requesting originally to get a feel for how intense the laser light was.

I thank the authors for improving the clarity on my end and accommodating my suggestions for extra words where required and appreciate the extended figures that really do aid my understanding of Fig. 3 and deletions.

Referee #3

(Remarks to the Author)
The authors have thoroughly addressed the concerns of this reviewer. The additional data strengthens the manuscript, and the explanations for why some experiments are either difficult or out of scope to perform are reasonable. I support publication in Nature.

David Taylor

Version 3:

Reviewer comments:

Referee #1

(Remarks to the Author)
The authors have further addressed my concerns and added a number of important controls and experiments. I think the paper has come a long way since the early, overly speculative version. I think it now provides a more thorough and rigorous set of insights in the Zorya system, with caveats and limitations more clearly stated. I support publication.

Point-by-point response to reviews' comment

"Structure and mechanism of the Zorya anti-phage defense system" – Nature-2023-07-12645A

We would like to thank the three reviewers for their detailed assessment of our manuscript and for their insightful comments that have been very helpful to improve the quality of this research article. We here present a response to all the queries and points raised by the reviewers.

Referee #1 (Remarks to the Author):

This manuscript describes the structural characterization of the recently discovered Zorya phage-defense system in bacteria. This system comes in 3 types with a conserved core pair of genes, and other genes that vary across the types. The authors chose to focus on a type I EcZorya system that defends against a diverse set of coliphages. The authors argue that Zorya can defend against phage while maintaining cell viability, implying a direct defense against phage infection. The authors then resolve a cryoEM structure of the ZorA5B2 complex, which is an inner membrane complex with structural similarity to the motor subunit of flagellor stator unit MotA5B2. The authors suggest that the C-terminal region of ZorA may form a long cytoplasmic alpha-helical bundle stretching into the cytoplasm. The ZorB periplasmic domains form a homodimeric peptidoglycan binding domain that may be important in switching the complex to an open-channel state for protons to drive the rotor. The authors provide additional cryoEM structures of the ZorC, ZorD cytoplasmic genes and suggest that ZorC and ZorD are recruited to the ZorAB cytoplasmic helical tail during phage infection and play a role in phage defense by genome degradation.

Although the ZorAB structure presented is fascinating and suggestive of a unique defense mechanism, the paper ultimately does not elucidate the mechanistic basis of phage defense. The initial report on Zorya suggested that it functioned by causing membrane depolarization. Here, the authors suggest that ZorAB instead somehow use changes in ion/proton flow across the membrane to signal to the interior components, ZorCD, to drive a direct defense and phage DNA cleavage. While this could be the mechanism, there is little conclusive data to support it, as summarized below. There are also major issues with overinterpretation and speculation throughout the paper. Without more incisive and rigorous studies of the mechanism, the structure (and mutagenesis) reported here feels more appropriate for a specialty journal.

We thank the reviewer for their overall comments, constructive critiques and several suggestions that we have used to improve the manuscript. However, there are some aspects where the reviewer appears to have misinterpreted our proposed model and/or the underlying data supporting this. For example, we do not claim that "…*ZorAB instead somehow use changes in ion/proton flow across the membrane*…". We propose that ZorAB is a proton-driven motor powered by the proton motif force (PMF), rather than 'use[ing] changes' in ion flow as a signal; we elaborate on this below and provide additional experimental support for this mechanism.

Furthermore, it is important to note that the membrane-depolarisation hypothesis presented in the original paper describing the Zorya system (Doron et al. 2018) was based on limited data (which are also compatible with our model) and in our opinion is relatively superficial conjecture that overlooks several important aspects of Zorya biology. For example, most known membrane-depolarisation defense systems rely on formation of membrane pores that rapidly dissipate the proton motif force (PMF) (e.g. RexAB), whereas a proton-driven motor seems an overly complex and inefficient mechanism for membrane depolarisation, by comparison. Furthermore, most membrane-depolarisation systems are abortive infection systems (inducing cell death or dormancy), and not direct defense mechanisms contrary to what we find for Zorya. Additionally, the long ZorA intracellular 'tail', which is evolutionarily conserved, would have no apparent role in a membrane-depolarisation mechanism. We posit that a proton-driven motor function for ZorAB makes more sense in view of all available data we presented in this research article and have now performed further experiments to support this. Below,

we provide point-by-point responses to the reviewer's comments and queries: we have numbered each main point for ease of reference.

Major concerns:

Mechanism of defense:

R1.1: *Fig. 1e-g don't fully support the claims of direct defense. There's a clear MOI dependence in Fig. 1e with the OD dropping at even an MOI of 1, as classically seen with Abi systems. There may not be full collapse of the OD and clearing of the culture, but the patterns in Fig. 1e and Fig. 1g are not what one expects for a direct defense system. This warrants further investigation and clarification.*

We agree that the data presented in the liquid phage infection time courses are not, on their own, sufficient evidence to support a direct defense mechanism. However, they do not disagree with a direct mechanism and in fact they do refute an abortive infection (Abi) type response. The reviewer's claims that the patterns in Fig 1e are not "*what one expects for a direct defense system*" overlooks several important considerations.

1) For a given defense system, the infection time course kinetics can vary substantially depending on the phage used. We tested three phages and two of them showed near-complete defense (no apparent reduction in growth kinetics) at MOIs up to 10 (**Extended Data Fig. 1g**), which strongly supports direct defense.

2) Whilst the growth kinetics for Bas24 (we included Bas24 in the main text figure because we have additional mechanistic data for this phage) are perturbed at high MOIs, the rate of culture collapse during the early timepoints (up to 2 h) in the presence of Zorya is slower than in the absence of Zorya (**Extended Data Fig. 1f**). This is not what is classically expected at high MOIs for abortive infection (Abi) responses that trigger cell death or dormancy upon infection.

3) It is becoming increasingly apparent in the field that some direct-acting defense systems can be overwhelmed at high phage infection pressures (e.g. due to limited enzyme capacity to clear infections in a timely fashion, or due to the rapid replication/host takeover of some phages leading to a point of no-return). For these reasons, relying on infection time course kinetics alone to conclude direct versus Abi-type responses is not prudent.

4) Our follow-up cell survival experiment (**Fig. 1g**), to directly probe whether the Zorya system invokes Abi-like cell death or dormancy, demonstrates cells are more likely to survive a single round of Bas24 infection in the presence of Zorya. These data refute an Abi mechanism, further supporting the alternate hypothesis of direct-acting defense.

5) Most importantly, to provide further experimental evidence supporting our statement that Zorya functions as a direct defense system and not an Abi-type defense system, we performed time-lapse microscopy experiments, which demonstrate that cell death or dormancy does not occur after phage exposure (Bas24, MOI 5) in the presence of Zorya. We have included these data (**Fig. 1h, i** and **Extended Data Video 1, 2**) and have updated the text to clarify the interpretation of these data and convey the most salient points in this section.

ZorAB as a possible rotary motor::

R1.2: *Page 9-10, lines 232-292: The authors point out the structural homology of the ZorA5B2 to the MotA5B2 and expect it to function as a proton motif force driven motor driving the rotation of the ZorA outer ring relative to the inner ZorB dimer in the membrane. Are there known mutations that arrest or break the rotation of the MotA5B2 complex (potentially by introducing structurally proximal paired Cys), that could be introduced into ZorA5B2? Specifically, this would be an interesting way to test the*

hypothesis that the ion permeation-driven motor is essential for phage defense. I understand that the authors test this idea by mutating the ion pathway, but arresting the rotation would be a more direct test of the rotation of ZorA.

As mentioned above, we propose that ion transport alone does not make mechanistic sense given the complexity and conservation of the ZorAB motor and tail. The rotary function is clearly established for other 5:2 ion-driven motors (e.g. MotAB/PomAB flagellar stator units) and several conserved mutations that impair ion permeation-coupled motor rotation in ZorAB, e.g. ZorB D24N, ZorA T147A and ZorA T147A/S184A (new data, see below), also resulted in attenuated or loss of phage defense. We therefore conclude that it is implausible to suppose that the effects (attenuated or loss of phage defense) observed from our mutagenesis can be attributed to loss of ion flow rather than loss of rotation.

Additionally, it has been long proposed that the rotation of 5:2 motor is directional (H. Hu et al. 2023; Santiveri et al. 2020; Deme et al. 2020; Chang et al. 2020), ensured by a hydrophobic residue from MotA/PomA (analogous to ZorA I144; **Extended Data Fig. 4g-k**) near the universally conserved residue D26. Instead of introducing structurally proximal paired Cys proposed by the reviewer, we designed and tested several mutants targeting the rotation directional checkpoint in ZorAB. We found that increasing the sidechain size and rigidity of the ZorA I144 ($ZorA^{I144F}$), which would block ZorA to rotate around ZorB, leads to non-functional Zorya (**Fig. 3h**). Again, These data support that arresting the rotation of ZorA around ZorB impacts Zorya defense.

It is worth noting that any mutation that arrests rotation will necessarily also perturb ion flow through the motor, so it is not practical to experimentally uncouple these processes. However, to investigate the importance of the PMF for Zorya phage defense function, we performed time-lapse microscopy experiments in the presence and absence of the protonophore carbonyl cyanide m-chlorophenyl hydrazone (CCCP). The protonophore CCCP has previously been used to demonstrate that rotation and protein secretion of the bacterial flagellum is dependent on the PMF (Paul et al. 2008; Gabel and Berg 2003). We first show (**Fig. 3i, j**) that addition of CCCP does not prevent infection or cell lysis after exposure to phage Bas24 in the absence of Zorya, demonstrating that DNA injection of Bas24 is not dependent on the PMF. We then demonstrate that dissipation of the PMF by addition of CCCP prevents Zorya-mediated phage defense in *E. coli* cells that express an otherwise function Zorya system. These data demonstrate that the PMF is necessary for Zorya-mediated phage defense and further support the idea that PMF-driven ZorAB motor rotation is essential for Zorya anti-phage function.

R1.3: *lines 251-252 " which is consistent with freedom of ZorA to rotate around ZorB (through Brownian motion) in the mutant." But there is no evidence that rotation does occur. If it did, then it also seems like the cryo-EM would have revealed heterogeneity in the transmembrane sections of the complex , but it appears not to have. So additional data supporting rotational activity is needed. In short: the authors conclude that "ZorAB is a peptidoglycan-binding rotary motor" but the evidence to support this claim are currently lacking.*

The mutant (ZorB 46-52>GGGSGGS) we designed, with the removal of the blockage (**Fig. 3d**; two pairs of salt bridges: ZorA(2) E164-ZorB(1) R49 and ZorA(5) E164-ZorB(2) R52 and several polar interactions in the vicinity of this region) observed in the ZorAB autoinhibited state, indeed revealed the heterogeneity in the transmembrane sections of the complex, as presented in the **Extended Data Fig. 4l-p** and described in the text. To make it clearer, we modified and stated in the text **line 228-234** '*In support of a rotation-suppressing role, when we replaced ZorB residues 46-52 with a GGGSGGS linker, we were able to generate a cryo-EM reconstruction that is similar to that of the WT complex. However, unlike the WT complex, where ZorB could be modelled from N- to C-terminus (**Fig. 3a, b, f**), in the mutant the densities for the ZorB TMs are unresolved and the ZorB PGBD density is poor, suggesting that, in the mutant, ZorA is free to rotate around ZorB (through Brownian motion) (**Extended Data Fig. 4l-p**).*'

R1.4: *Page 10, lines 282-291: Following the above point, the authors highlight the T147A mutation in the ion-permeation pathway and its effect on phage defense. Specifically, the authors claim that T147A*

"significantly decreases defense ability ". Based on Figure 3h and Extended Figure 6a, this is not a consistent phenotype across the various phage tested. For Bas24 and Bas58 it seems like T147A had almost no effect. Given the authors general model, such a mutation to break ion-permeation and motor function should dramatically affect defense against all phage.

We agree with the reviewer that our statement regarding the T147A mutant is inaccurate. We have revised this statement accordingly. In terms of the difference between phages, it is likely (and consistent with all other phage defense systems) that the strength/efficacy of Zorya defense against different phages varies for multiple reasons (e.g. the rate of phage genome injection, rate and nature of the host takeover mechanisms, superinfection exclusion mechanisms, rate of phage replication etc.). Upon closer examination of our data, we suspect the T147A does not completely abrogate ZorAB function and that this mutation results in an intermediate level of Zorya activity that is sufficient to protect against some phages but not others. In our view, inclusion of data for multiple phages is a strength of our work.

Furthermore, based on structural analysis and comparison (to MotAB and PomAB) and as we have stated in the text '*T147 and S184 resemble an ion selectivity filter that controls ion access from the periplasm to ZorB D26*'. We have now tested a double mutant ZorA$^{T147A/S184A}$, which abolishes Zorya activity, as expected (**Fig. 3h**), and have updated the text accordingly, **line 266-268**. Other 'stronger' mutations, such as ZorB D24N, disrupt Zorya activity against all phages tested. Please note, D24 is universally conserved in the 5:2 rotary motor family that is responsible for ion transportation during motor rotation.

R1.5: *Page 12, lines 316-323: The authors highlight the importance of the R108-E227 and H92-Y228 electrostatic contacts for the integrity of the ZorA MPCD, and assembly of the dimer. But they infer this by looking at the ZroA(G223-T729) tail-deletion mutation. It seems prudent to test the double mutant directly in phage infectivity assays to confirm their importance in stabilizing the complex and maintaining phage defense.*

We agree with the reviewer and have now included data for the R108-E227 and H92-Y228 mutants (**Fig. 3h; line 289-292**), which support our conclusions regarding the importance of these residues.

ZorB and the possible role of peptidoglycan binding::

R1.6: *It's unclear to me whether the authors are proposing that ZorB senses changes in the peptidoglycan and then uses the possible rotary motor of the ZorAB complex to transmit a signal or whether ZorAB is sensing changes in the proton motif force known to be perturbed during phage infection, with ZorB binding to PG playing a more ancillary role. Whatever the case, there's insufficient evidence that ZorB binding to PG is important to sensing or transmission of a signal, and there's no direct evidence that ion flow through ZorAB occurs and is critical to phage sensing.*

We apologise if this was not clear in the manuscript. As mentioned in the preface above, we propose the former, that "*…ZorB senses changes in the cell envelope and then uses the possible rotary motor of the ZorAB complex to transmit a signal…*". Our data for multiple point mutants known to impair ion flow coupled to motor rotation demonstrate the role for ion-driven ZorAB function in phage defense.

In addition, as mentioned above, we now show that uncoupling the PMF using CCCP impairs the Zorya defense response, without impairing phage infection (**Fig. 3i, j**), thereby providing direct evidence that the PMF is essential for driving ZorAB (consistent with MotAB-type proton motors). Again, these data do not support the alternative idea that 'ZorAB is sensing changes in the proton motif force' because disruption of the PMF by CCCP does not activate Zorya-mediated defense.

Furthermore, based on our cryo-EM structure, we have mapped the potential PG binding sites on ZorB PGBD (ZorB residue Y151, N152, L155, R159), residues that are conserved across the peptidoglycan binding domain family (**Extended Data Fig. 4d**). We generated the mutants ZorB^{Y151A}, ZorB^{N152A}, ZorB^{L155A} and ZorB^{R159A}, and tested these mutants and show that mutation of the proposed PG binding

site impairs Zorya defense (**Fig. 3h**). We have updated the text in the discussion to ensure the model is clearly stated.

R1.7: *If ZorAB is simply detecting disruptions to the peptidoglycan and/or membrane, why doesn't Zorya provide broader protection against more phages? In Fig. 1b it seems many phages are not inhibited by Zorya at all. I.e. given the authors' model, how would one explain the phage specificity of the system?*

This is indeed an interesting question and the subject of several discussions within our team throughout the project. Phage adsorption and the mechanisms of physical penetration of the cell envelope differ between phages, which might influence whether and how the IM-PG layer is disturbed. However, there are no clear patterns in our data that provide insight into this. Due to the many outstanding unknown aspects of phage biology, there are too many potential explanations for the Zorya activity profile that we deem further investigations to be outside the scope of our current study. For example, many phages encode inhibitors of defense systems (e.g. anti-CRISPRs, for which there are dozens of papers) and anti-Zorya proteins might be present in some phages but not others. In our view, the identification and characterisation of Zorya inhibitors warrants a stand-alone, follow-up study. Additionally, regardless of how Zorya is initially activated, phage-specific differences in replication, host takeover etc. (refer to **R1.4**) likely influence the effectiveness of Zorya defense (as with other defense systems). One aspect we have investigated is that the insensitivity of T4 to Zorya is not due to the glycosylation of T4 DNA; there was no difference in EOP for any phages between WT T4 (glycosylated DNA), hemi-methylated, or unmethylated T4 mutants. We deem that additional investigation into T4 or other phages resistant to Zorya is beyond the scope of this study.

R1.8: *Page 4: "...we propose that the dimeric ZorB peptidoglycan-binding domain anchors the ZorAB complex to the cell wall near the phage injection site and that the ion-driven motor rotates the ZorA tail within the cytosol." Unfortunately, no evidence is presented that ZorB switches from not bound to peptidoglycan to 'anchored' to it following phage infection. And what PG structure would ZorB be recognizing that is associated specifically with phage infection ?*

Due to the length of the ZorB ‘stalk’, it appears the PGBD cannot ‘reach’ the PG layer under ordinary conditions (based on the IM-PG distance and comparison to *E. coli* MotB) (**Fig. 3a** and **Extended Data Fig. 4f**). We have also demonstrated that mutation of the ZorB PG binding site impairs the Zorya defense (please refer to **R1.6**). We propose two potential activation mechanisms whereby the PGBD comes in contact with the PG layer to anchor ZorAB, summarised in **Fig. 6**.
1) There is evidence of ‘pinching’ of the IM-PG layer distance by some phage tail tubes which “pull up” the inner membrane closer towards the peptidoglycan (B. Hu et al. 2015) , which would allow the ZorB PGBD of any ZorAB in the localised vicinity to reach the PG layer and anchor/activate the complex.
2) ‘Frayed’ PG ends caused by muralytic activity of phage virion-associated lysins may enable binding and anchoring of ZorAB. It is very well established that *E. coli* phages depend on such virion-associated lyases in their infection process, e.g. T3 Gp16, T4 Gp5, T7 Gp16 (Latka et al. 2017).
We deem additional investigation of these hypotheses (1 & 2) require substantial experiments using completely different experimental approaches (for example, using cryo-ET) and are therefore out of scope of the current study. We have revised the text and only discuss these possibilities in our discussion section.

R1.9: *Following the point above, page 9, lines 216-230: With respect to the PG-binding domain of MotB, the authors hypothesize binding of the disrupted peptidoglycan layer to transmit information of phage invasion by driving motor rotation. Could the authors mutate the relevant PG-binding sites, or dimerization interface (and disulphide bonds) to show that this is important to phage defense?*

Please see **R1.6** where we now include the PG-binding site mutational data. We also mutated the ZorB PGBD dimerization interface and broke the disulfide bond, demonstrating their importance in Zorya defense (**Fig. 3h** and **Extended Data Fig. 2k, 6a**).

ZorAB signaling to cytoplasm::

R1.10: *Page 12, lines 324-361: The authors test tail disruptions and their effect on phage defense by deleting sections of the ZorA C-terminus to reduce the presumed length of the helical bundle tail. The authors highlight that there are regular hydrophobic residues in ZorA (L250, L254, L258, L261, and further as you move down the bundle), that presumably form a core and stabilize the bundle structure. Could the authors disrupt the bundle formation without altering length by synthesizing a ZorA variant with these hydrophobic residues mutated, that could directly test the function of the bundle, versus the length of the C-terminus available. This would further add some data to justify the authors speculation that unraveling the ZorA tail might be important in activation, if these bundle de-stabilizing mutations lead to constitutively active Zorya (judged by microscopy, or potentially leading to cell toxicity).*

As we presented in the text, and shown in **Extended Data Fig. 3d, e**, there are many hydrophobic residues at the interior surface of the tail, and the hydrophobic interactions inside the tail could be a primary driving force for tail assembly. Given the large number of residues, it is impractical to systematically mutate all the hydrophobic residues that line the internal surface of the tail. However, as suggested by the reviewer, we generated three mutants targeting the hydrophobic motif at the beginning of the tail (ZorA, L250, L254, L258, L261): 1) ZorA$^{L250A/L254A/L258A/L261A}$, 2) ZorA$^{L250G/L254G/L258G/L261G}$, and 3) ZorA$^{L250N/L254N/L258N/L261N}$. All mutants resulted in loss of phage defence (**Fig. 3h**).
Additionally, we expressed and purified the ZorA$^{L250G/L254G/L258G/L261G}$ZorB and ZorA$^{L250N/L254N/L258N/L261N}$ZorB and collected cryo-EM datasets on this two mutants. We found that L to N or L to G mutation completely destroy ZorAB TMD motor assembly; however, we still observe the tail bundle assembly from the two mutants we purified (**Extended Data Fig. 5g, h**).
This data indicates that these hydrophobic residues at the beginning of the tail (and potentially the lipid molecule that we observed from cryo-EM map) are critical for the correct assembly of the ZorAB TMD motor. We have included these data in the manuscript. However, like stated above, there are a lot of residues that line the internal surface of the tail, and mutants targeting ZorA, L250, L254, L258 and L261 are not sufficient to de-stabilize the bundle. For the speculations on unravelling of the ZorA tail, we have now moved the text into a **Supplementary Discussion** file and focus on in the discussion the most substantial advances of understanding in the structure and function provided by our study.

R1.11: *Page 14, lines 395-407: While testing the function of various ZorC and ZorD mutants, the authors systematically confirm the importance of many structurally relevant residues and residue-networks. The authors highlight the DEAQ-motif in the ZorD C-terminus that is shown to be important for plasmid degradation in vitro (Figure 4i). It is important that the authors also include this double mutant in the phage defense assay in Figure 4h to connect the nuclease activity directly to the phage defense phenotype.*

We agree with the importance of this double mutant ZorD$^{D730A/E731A}$ and have now included the data in the manuscript (**Fig 4i**). As predicted, the double mutant inactivates phage defense.

R1.12: *There is some evidence presented that ZorC binds DNA. Is there any sequence specificity? Additionally, to support a role for DNA binding in phage defense, the authors should demonstrate DNA binding during phage infection. The mutation of predicted DNA-binding residues ablates defense, but this is indirect and insufficient evidence for DNA binding occurring during an infection.*

In our original submission, we used EMSAs to test whether ZorC can bind DNA. These experiments used a single 55bp dsDNA substrate (5' FAM-labelled dsDNA stored in our lab for another project). To investigate sequence specificity (and to address related queries from Reviewer 3), we have now tested four additional dsDNA substrates with different sequences, lengths and GC content (**Extended Data Fig. 7h**). In all cases, ZorC binds the dsDNA oligos. In further support of direct DNA binding, we have now also obtained a cryo-EM dataset of a ZorC-DNA complex (**Fig. 4f**), which directly shows that the EH signature motif containing domain is responsible for DNA binding. Most importantly, our mutagenesis confirms key residues from the EH signature motif (ZorC E400 and H443) are required

for the ZorC-DNA interaction (**Fig. 4e)** , and introducing E400A or H443A mutations into the Zorya operon abolishes Zorya defense, indicating that ZorC DNA binding is indispensable for Zorya defense (**Fig. 4c**).

R1.13: *p. 14, line 405: "These results suggest that ZorD harbors a nuclease activity, and full-length ZorD is in an autoinhibited state, which is likely activated once the defense is triggered, presumably through a conformational change." This is an intriguing hypothesis but additional data are needed to substantiate it. Is there structural evidence that ZorD adopts two different conformations? How does ZorC binding influence ZorD activity? Is there evidence that ZorD cleaves phage DNA (and not host DNA) during an infection?*

This is indeed a good point, as also queried by **Reviewer 3**. In the cryo-EM data of WT ZorD, we did not observe any additional conformation other than the circular, closed one, indicating that ZorD nuclease activity is tightly inhibited (consistent with mitigating autoimmunity in the absence of activation). We have now investigated whether ZorD nuclease activity is influenced by ZorC *in vitro* (included in **Extended Data Fig. 8f**) but do not find evidence for any activation effects for WT ZorD. There data are consistent with the findings we presented in the manuscript that ZorC and ZorD alone (without ZorAB) are not sufficient to provide defense, and they need the activation signal from the ZorAB motor.

To query possible alternative conformations of ZorD, we predicted the ZorD-DNA-ATP structure using AlphaFold3 (also see response to **R3.3**), which would represent the activated, DNA-bound nuclease state (ipTM 0.79; **Extended Data Fig. 8h**). In the predicted ZorD-ATP-DNA structure, we observed a linearized ZorD conformation, in which the N-terminal domain is freed from the interaction with ZorD C-terminal nuclease domain, which would be consistent with a transition from an autoinhibited ring-like conformation to an active linearized conformation. Furthermore, the predicted DNA-bound C-terminal ZorD domain structure closely matches that of the SNF-family chromatin remodelling domain PDB 7X3T (**Extended Data Fig. 8l;** Z-score = 26.6). As discussed above, we do not think that this activated ZorD state can occur in the absence of ZorAB signalling, but it might represent the activated DNA-bound state. A plausible ZorC-ZorD-ATP-DNA structure was also predicted (**Extended Data Fig. 8m**), suggesting a ZorC-ZorD complex might be required (or an intermediate step) for ZorD-DNA binding. However, the ipTM score for this complex is below 0.6 (ipTM 0.48). In addition, we did not detect this complex in vitro (again supporting a requirement for ZorAB activation) and we deem further investigations to be outside the scope of our already very comprehensive study.

As we have shown in our live-cell fluorescent microscopy data, ZorC and ZorD are recruited to activated ZorAB, and the recruitment and subsequent or concurrent activation of ZorD activity is probably restricted to occur only in the proximity of the cell membrane (where ZorAB is), which might constitute a mechanism to prevent damage to host DNA. Since our data (including new time lapse microscopy) show Zorya does not act via an abortive infection mechanism, host DNA is not degraded by ZorD, consistent with our proposed model of a localised immune response for Zorya (in the proximity of the cell membrane).

R1.14: *Fig. 5 presents intriguing data that the localization of Zor components may change following phage infection. However, there's still quite a bit of localization of all components prior to infection so it's not clear how crucial the increases seen are to defense.*

Please see our response to the related **R3.6** query, where we provide more detailed insight into the single cell microscopy and interpretation.

R1.15: *It's also unclear in Fig. 5 whether the foci corresponding to ZorB, ZorC, and ZorD, which are imaged separately, are co-localizing. This should be addressed through co-localization studies. Co-IP or other experiments to demonstrate changes in interactions upon phage are also needed.*

Please see our response to the related **R3.6** query, where we provide more detailed insight into the single cell microscopy and interpretation.

R1.16: *The authors should assess whether the Zor components are localizing to sites of infection or not to understand how Zorya ultimately provides defense. It could be that Zorya co-localizes to sites of phage genome injection, but it could also sense phage infection at a distance via changes in PG and/or the proton motif force and then somehow trigger ZorC/D to 'find' phage DNA.*

We refer again to our response to **R3.6** where we detail our investigation into co-localization of Zorya components using live-cell TIRF microscopy in the presence and absence of phage Bas24, which provides strong evidence of recruitment/activation of the soluble Zorya components only in the presence of a functional ZorAB motor and in the presence of phage. Our CCCP data rule out the proposed PMF 'sensing' alternative model, and our genetic complementation data demonstrate a requirement for a direct interaction between ZorAB and ZorC/D (again consistent with our recruitment model supported by new data). We investigated several potential approaches to measure co-localisation of phage infection sites and ZorAB activation and subsequent ZorC/D recruitment. However, due to the very rapid early stages of phage infection, it was not possible to visualise this with our TIRF microscopy setup. Future work requiring specialised approaches, for example cryo-EM tomography time series of individual cells at short increments post-infection, are likely required. However, our present study provides a solid foundation for future specialised studies of the more granular mechanistic detail of Zorya activity. We have updated the Discussion to focus more on the substantial insights our present data provide (all consistent with our overall model) and to highlight where future studies might focus.

R1.17: *The TIRF experiments need additional controls:*

(a) It is important to perform the experiment of mNG-tagged ZorC and ZorD, with phage infection but with ZorAB deleted. Phage infection causes dramatic and rapid changes to the cytoplasm and cellular metabolism, and it is important to confirm the puncta formed by ZorC and ZorD are not just due to phage infection.

We have investigated co-localization of ZorD with a non-rotating ZorB^{D26N} mutant using live-cell TIRF microscopy (**Fig. 5f**). We do not observe ZorD-ZorB co-localization in the presence of phage, in contrast to functional ZorB, demonstrating that the observed increase in cluster formation of the soluble Zorya effectors ZorC and ZorD are not an artefact of phage infection and are dependent on the (functional, rotation-capable) membrane-embedded motor complex ZorAB.

(b) To confirm that the tail plays a key role in recruiting the freely diffusing ZorC and ZorD, the authors should repeat the microscopy with the ZorAB complexes that have tail deletions which are compromised in phage defense.

In addition to the phage-induced colocalization of WT ZorD-ZorB, we show that phage-induced ZorD-ZorB co-localisation does not occur for a ZorA tail tip truncation mutant (ZorA$^{\Delta 483\text{-}739}$; **Fig. 5f**). These data demonstrate a crucial role of the ZorA tail in recruiting soluble Zorya effectors upon phage infection.

(c) The argument that T4 doesn't lead to a proportional increase in puncta during infection, different from a truly defended phage like Bas24 has a caveat. Specifically, phage like T4 have super-infection exclusion where the first infection blocks subsequent phage infections by inhibiting the lysozyme of the tail tip of the secondary phage particles (doi: 10.1038/s42003-020-01412-3). This would directly prevent the production of free PG-ends that your model requires to begin the downstream signaling.

We agree that the superinfection exclusion mechanisms of T4 complicate interpretation of these data. After further investigations, we suspect the spackle/Imm superinfection factors, and possibly phage defense inhibitor(s), may be having confounding effects of the microscopy analyses. Therefore, we discontinued work with T4 and focused on the Zorya susceptible phage Bas24. As discussed in **R1.7**

and **R3.7**, we deem that further investigation as to why some phages are not susceptible to Zorya is beyond the scope of the present study.

(d) Could the authors repeat the microscopy with E.coli expressing mNG-tagged Zorya exposed to lysozyme? This should generate ruptures in the peptidoglycan layer (analogous to most phage tails) and trigger the system per the author's model.

Exogenous addition of lysozyme would be physiologically very different from the early stages of phage infection. Contractile phage genome injection (e.g. via the tail spike penetrating the cell envelope) is typically very localised and although it can (but not always does) involve virion-associated lyase activity to penetrate the PG layer, this is unlikely to be mimicked by exogenous lysozyme activity (which, in Gram-negative bacteria, also requires disruption of the LPS layer (by addition of EDTA) to allow access to the PG). As such, we elected not to investigate exogenous lysozyme addition as it would be unlikely to yield biologically relevant insight.

(e) To confirm that it is not the ongoing phage lifecycle that leads to ZorC, ZorD puncta formation, the authors should UV- or EMS-mutagenize Bas24 until its MOI falls significantly. This is one of the classic experiments that generates functional phage particles but dramatically compromised phage genomes that cannot proceed in their transcriptional program. This can test the activation and puncta formation of Zorya proteins during phage infection, but without a proper phage lifecycle.

Whilst this is conceptually an interesting experiment, our data presented above showing the PMF is required for Zorya function demonstrates ZorAB motor function initiates ZorC/D foci formation (see above), not simply a generic phage factor. Therefore, we do not think the proposed experiments are necessary or would add substantially more insight.

R1.18: *Additional comments on the proposed model::*
In the introduction the authors argue that "A long rotating tail-spike located close to the site of phage DNA injection is strongly suggestive of a "bobbin" around which the invading DNA could be wound, thereby immobilizing it for subsequent degradation." and in the Discussion (page 19, lines 536-43) there are similar claims. This model is pure speculation - there are no data to support this model. And there are some data that argue against it, e.g. it seems the authors note the hydrophobic nature of the predicted ZorA cytoplasmic tail, which seems incompatible with DNA binding.

As we discuss above, there remain some interesting outstanding questions that are beyond the scope of our present study. We propose several potential interesting hypotheses that require future, focused investigation using highly specialised techniques (possible DNA winding around the ZorA tail being one of these). Nonetheless, we have now removed the more speculative future-focused ideas (e.g. DNA reeling) into a **Supplementary Discussion** and focus in the Discussion on the most substantial advances of understanding in the structure and function provided by our study.

We additionally clarify here (in response the reviewer query regarding ZorA tail hydrophobicity) that, as we stated in the text, based on the cryo-EM structure and our predicted model, that there are many hydrophobic residues at the interior surface of the tail, and the hydrophobic interactions inside the tail could be a primary driving force for tail assembly. We have additionally shown in the updated **Extended Data Fig. 3d**, the outer surface of the tail is predominantly hydrophilic (consistent also with the tail protruding into the cytosol).

R1.19: *Why must the ZorA tail be so long, i.e. 70 nm? It's unclear how or why this extended conformation is needed.*

This is indeed an interesting question. We know the tail length is evolutionarily conserved (**Extended Data Fig. 6b**), implying that the length is important to the mechanism. We now show in the manuscript that the tip of the ZorA tail (residues 483-729) is required for Zorya defense and for ZorD recruitment. We also show that the total length (retaining the tip; mutant Δ359-592) is important for phage defence

(**Fig. 3h**). We confirm in **Extended Data Fig. 5j** that a shorter tail still forms in these two tail truncation mutants. In the **Supplementary Discussion**, we speculate that the ZorA tail length is reminiscent of chemotaxis signal transduction proteins which use similar long intracellular bundles for signalling (and where conformational rearrangements are important). We speculate that upon ZorAB activation, due to the rotation of ZorA around ZorB in the direction counter to twist of the ZorA tail, rotation induces conformational rearrangement of the tail. It is conceivable (as observed in chemotaxis signalling) (Riechmann and Zhang 2023) that a long tail is required for sufficient 'signal' generation, possibly due to a long tail having more rotation inertia and 'friction' within the viscous cellular environment than would occur for a short tail. However, similar to our comments above, we deem that further detailed investigation is required, likely involving biophysics measurements of rotation torque with different tail length mutants.

Minor points:

R1.20: *S184 is mis-labelled as T184 in Figure 3g.*

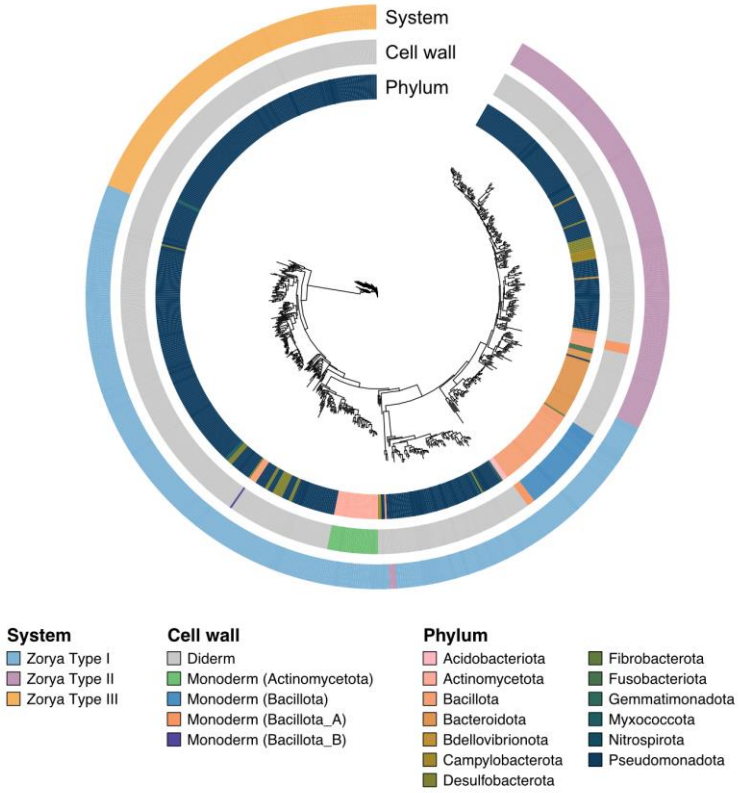
Corrected.

R1.21: *Page 19, lines 550-553: It is possible the nucleases are ineffective against the glucosylated genome of T4 (and its sisters), which is how T4 is resistant to many nuclease-based defense systems (RM, and CRISPR). The authors should test this speculation since it is easy enough to do.*

We tested this hypothesis using T4 mutants with WT (glycosylated), 5hmC, and unmodified DNA but in all cases the phages were not susceptible to Zorya defense (if required, we could include the data in the manuscript but opted to present a more streamlined Results and Discussion). T4 is known to encode multiple inhibitors of anti-phage defense systems (e.g. anti-DSR, anti-CBASS and anti-RM). Therefore, as discussed above, another possibility is that T4 also encodes a Zorya inhibitor but this is outside the scope of the current study.

R1.22: *Could the authors comment on the PG-domain of the ZorB homologs found in gram-positive bacteria? Is there a difference in how the PG domain must function in gram-positive bacteria with a cell-wall that has dramatically different architecture and depth compared the PG-layer of gram-negative bacteria between membranes. The authors show structural predictions (Extended Fig 11), but a sequence alignment of gram-positive versus gram-negative ZorB PG-binding domains may be further illustrative.*

We now include phylogenetic analyses of Zorya systems in different phyla with known single membrane (monoderm, typically Gram-positive) or double membrane (diderm) envelopes (**Extended Data Fig. 1c**). We additionally investigated whether the PGBD has specialised in the monoderm vs diderm Zorya systems and found some evidence for this occurring but the dominant effects are between Zorya types rather than monoderm vs diderm (see **Review Figure 1**, below). However, the apparent specialisation is not necessarily related directly to the cell wall structure, as we see similar phylogenetic clustering within other phyla. The inner surface of the PG layer (where ZorB would attach) is also not substantially different in molecular structure between monoderms and diderms (and this is further supported by many Gram-negative endolysins having activity against purified Gram-positive PG). Therefore, we opted not to include these analyses in the manuscript (as they do not add insight). We did also observe diversification of the ZorB "stalk" length, which would be consistent with adaptation to different cell envelopes in different phyla (**Extended Data Fig. 10**). In all cases, the ZorB linkers are structured (alpha helical) versus the longer and flexible linker observed for MotB. An in depth structural phylogenetic evaluation of Zorya function is beyond the scope and topic of put study but our structural findings will greatly support these efforts and evolutionary insights in future works.



Review Figure 1: Phylogenetic analysis of ZorB proteins, revealing that Zorya found in monoderm bacteria (typically Gram-positive) are no more phylogenetically distinct than ZorB found in many other phyla.

R1.23: *There is a possible lipid seen inside the ZorA tail. Any idea what lipid this might be and what its relevance is?*

Based on the density we observed from the cryo-EM map (**Extended Data Fig. 5d**), we speculate it is a palmitic acid molecule, the most common fatty acid found in microorganisms, rather than the detergent LMNG we used to purify the ZorAB complex, which has two longer alkyl chains attached to two maltose headgroups or other lipids. Regarding its possible function, as suggested by reviewer, we mutated those hydrophobic residues (ZorA, L250, L254, L258, L261) surrounding this putative fatty

acid. We found these residues are critical for ZorAB motor assembly and subsequently affect the Zorya defence, please also see **R1.10**.

Referee #2 (Remarks to the Author):

Hu et al. present a significant contribution to the field with the high resolution structure of Zorya anti-phage system. This is significant because there are so few rotary ion-powered motors that have been structurally solved, and in particular this motor has a novel function coupled to rotation and as such offers insight into the function and origin of all such systems.

Overall the work is very clearly presented, well written, and it is easy to understand the insights arising from the work. I recommend acceptance with minor revisions which I detail in full below. I enjoyed the discussion and think the work is well stated with the appropriateness of the claims, abstract and conclusions.

Of my minor suggestions, I think the ones that perhaps require more attention surround the single molecule fluorescence results and the reporting of the change in diffusion (as well as the quality of the video data available in SI). In particular it would be nice to have more raw data for this as it is hard to discern changes in diffusion based on what is available. The authors could consider quantifying this further, but at a minimum making the raw imaging available would be preferrable to the GIFs presented in Supplementary Material.

We thank the reviewer for their enthusiasm, detailed assessment, and positive feedback. In additional to addressing the minor comments below, we have provided additional live-cell TIRF microscopy data showing the (co-)localization of Zorya components *in vivo* in the updated Fig. 5.

Minor comments (* for more important ones):

L39 – not sure need comma after ZorD here.

Corrected.

L111 – I do not know what a bobbin is. I think I can work it out but perhaps a more natural word for wider clarity here?

We agreed that this word might not be the most natural or widely known, so we have removed this term. Additionally, we have deleted this sentence from the introduction and now only discuss this hypothesis in the **Supplementary Discussion** section on future investigations.

L122 – not clear what ΔRM is here.

ΔRM strain is an *E. coli* strain used to isolate the Basel phage collection, as described in the paper (Maffei et al. 2021). The strain is a derivative of *E. coli* MG1655 in which all native restriction modification systems have been deleted and is widely used for studies of phage defence system function. We have more clearly described this in the Methods and for clarity we now only refer to "*E. coli*" in the main text.

L125 – I had a hard time understanding this sentence: Phage adsorption was not affected by EcZorI, indicating that anti-phage defense occurs at a subsequent stage of infection (Fig. 1c). By L139-142 I understood it. But I think the underlying issue was that I didn't understand what was happening at 12 hr point in control, ie, what is the natural defense process (Fig 1e).

For clarity, we now simply state the observation first (for the adsorption experiment **Fig. 1c**), then later summarise all findings. "*EcZorI did not impair the adsorption of phages to host cells (**Fig. 1c**)…*" In terms of the bacterial growth in the population time course for the control (**Fig. 1e**), the uptick in growth after 12 h is commonly observed for these types of experiments and is usually due to selection for naturally occurring phage resistant mutants in the population (e.g. within the starting cell populations there may be naturally occurring mutants in the LPS or LamB surface receptors used by phage Bas24).

These later timepoints (e.g. beyond the first ~8 h) are not relevant to the findings of our study, so we could remove these data from the main text and include in the supplement instead, if required.

**Fig. 1 – does this mean ancestral state is with zorCD and this was subsequently lost in type II and type III? Can we assert this based on rooting of this tree? How was root established?*

We have now regenerated the ZorA phylogenetic tree using an *E. coli* MotA homolog as a root. To enable direct comparison with MotA, we built the tree using only the 'motor' part of ZorA (excluding the tail, which is not found in MotA). Please see the updated Methods for further details. Additionally, we removed some duplication present in the original dataset (based on sequence similarity between closely related strains) and now also provide an extended analyses of Zorya prevalence in diverse phyla. The phyla-level analyses (**Extended data Fig. 1b**) include a breakdown of predicted cell wall type (monoderm versus diderm; typically referred to as Gram-positive and Gram-negative but this is not always the case). In terms of evolution of Zoyra types (I, II and III), we do not have sufficient data to speculate on the ancestor state and composition of effector subunits, e.g. ZorC and ZorD (type I) versus ZorE (type II) or ZorF and ZorG (type III).

L152 – what is AAI? Not clear where this is in figure.

AAI represents the average amino acid identity between the phage proteins shown in **Fig. 1b**; in our experience, AAI provides more robust comparisons between phages than ANI (average nucleotide identity). We now state in the legend: "*AAI: Average amino acid identity between phage proteins encoded by each phage (providing an estimate of the relatedness between phages).*"

L205 – this is approached in discussion, but at this point I noted the shorter linker to the PGB domain vs MotB and was wondering how force sensing might occur (how does complex sense force via perturbation of PGB domain – please see comment below at L500)

The reviewer is correct. The ZorB PGB domain connects to the ZorB TM through helix α1 rather than a long linker observed in the stator unit MotB (**Extended data Fig. 4f**) . This difference is an important aspect for our model of Zorya function. We propose that due to the length of the ZorB 'stalk' (**Fig. 3a**) the PGBD cannot 'reach' the PG layer under ordinary conditions. Our model does not involve sensing of 'force', rather we propose that a reduction in the IM-PG distance, as previously demonstrated to occur during breaching of the cell envelope by phages (B. Hu et al. 2015), allows the ZorB PGBD to 'reach' and anchor to the PG layer. We have updated the manuscript to more clearly present and discuss the implications of this finding. Please also see response to **R1.8.**

**Fig. 2 – why is model of filament dead straight? EM micrographs appear supercoiled?*

From negative stain images and mass spectrometry, we confirmed that the purified ZorAB contains the intact ZorA C-terminal region (filament). Secondary structure prediction revealed a preference for the tail to adopt α-helical structures, suggesting that the rest of the ZorA tail is likely to be a continuation of the experimentally determined structure, which folds into a coiled coil with a right-handed super-helical twist towards cytoplasm. We agree with the reviewer that the tail appears flexible in the EM girds (**Fig. 2b**). The model we presented in **Fig. 2g** is an idealized full-length ZorAB model. Therefore, we have included explicit mention in the text and in the methods that the tail is likely flexible to some degree.

**L225 – it feels suitable to mention the shorter linker here to PGB domain.*

We thank the reviewer for the suggestion. As above, we have now included in the text that this is indeed a short linker between ZorB TM and PGBD compared to that one observed in the stator unit MotB (**Extended data Fig. 4f; line 199-204**).

L241 – what is MPDC? I found this in figure but probably worth mentioning here also to aid reader.

Agreed, we now define this in the main text: "*…membrane-proximal cytoplasmic domain (MPCD; spanning ZorA residues G48-L127 and K207-S222)….*". **Line 167-168**.

**L297 – I realise space is at premium but it's hard to tell immediately what is being probed with each of these deletions in the figure. Is it possible to group them at least to show ie tail deletion vs pore deletion. The info comes in later at L321 and L345 but I think would be nice if some indicator in figure also. For example use labels beginning, middle, tip, as per L345?*

We agree and have modified **Fig. 3h** to incorporate this. We also have annotated the tail deletion mutants in the **Extended Data Fig. 5a**.

L297 – E.coli needs space.

Corrected.

L309 – dashed not bashed

Corrected.

L371 – we mapped…. Form an; should perhaps be we mapped… to form? I think perhaps a word missing here to make complete sentence.

Corrected.

L373 – individual substitution … abolish (should be individual substitution of E400 or H443… abolishes)

Corrected.

**L383 – how was non-specific dsDNA chosen and was there any effect ie with CG content or secondary DNA structure (ie hairpins or otherwise). Not sure I understand 'scrambled' in this context (L847-856) in DNA context this can be used to mean a deliberately non-complementary scrambled partner, but in methods only list single strand identity and refer to scrambled dsDNA substrate, please clarify, not clear what is scrambled relative to what.*

We thank the reviewer pointing this out and agree this was not clear. Please also see response to **R3.1**. As stated in the text, based on the analysis of the cryo-EM structure of the ZorC apo form, we predicted that ZorC is a DNA binding protein. We tested this idea through EMSA with a arbitrary 55bp dsDNA (5' FAM-labelled dsDNA available in our lab). In agreement with our prediction, we show that ZorC indeed binds to DNA. To explore the DNA binding specificity of ZorC, we tested four DNA oligos of different lengths, sequences and GC content, and we observed no significant difference in binding between each of these (**Extended Data Fig. 7h**). We have now also obtained a cryoEM dataset of a ZorC-DNA complex. We show that the EH signature-containing domain of ZorC is responsible for DNA binding (**Fig. 4f**). Most importantly, our mutagenesis confirms key residues from the EH signature motif (ZorC E400 and H443) are required for ZorC-DNA interaction and in Zorya defense against phage invasion (**Fig. 4c**). We have included this in the manuscript (**line 357-365**) and updated figures and methods.

**L451 – can we quantify diffusivity? It should be possible to do some particle tracking and then report change in diffusivity vs qualitatively. The extended data was a gif, and I couldn't understand raw intensity well, but also it appeared 200 ms intervals, would imagine hard to be sure about diffusion with a long interval, but not sure about relative diffusion speed of complex and frames available here. For example, L455 'appeared to become static', I think this should be quantifiable with a change in mean displacement.*

Please see our detailed response to **R3.6** below.

**L500 – this appears first mention of length difference in linker. I think could be mentioned as result earlier at first observation. Also, for discussion could be options around knocking probe into cell (such as AFM, bead, micropipette or optical trap) and observing zorCD recruitment?*

We agree with introducing this earlier (please see related responses above) and now include additional analyses of the striking differences between the ZorB and MotB linkers (**Extended Data Fig. 4f**). Future experimental investigations to physically mimic cell envelope perturbations occurring during phage infection (e.g. via AFM, as suggested) are certainly interesting but are beyond the scope of our study. We looked at including these ideas in the Discussion but opted to streamline the text.

**L521 – how does tail recruit ZorCD. I think this is mentioned in discussion L530-543, but I was not sure what evidence was for DNA to be 'reeled in' vs just overwound or constrained in some way. Eg L201-202 Supplementary Material refers to this as 'almost inevitable' but that seems strong without some model or precedent showing (admittedly likely but this is quite novel, not much known regarding winding by molecular motor and at this length/timescale, so perhaps more caveat in text).*

We agree with the reviewer. We have reduced speculation to focus on presenting our core advancements and novel mechanistic findings. Nonetheless, we have now removed the more speculative future-focused ideas (e.g. tail conformational change, DNA reeling) into a **Supplementary Discussion** file.

**L547 – what is min and max intensity of the images shown in Supp: Ext Data Fig 10? Asserting in text here increase in foci and intensity, but not sure we can interpret without knowing scale of image in SI.*

We thank the reviewer for pointing this out. We did not want to imply an increase of grey values (fluorescence intensity) when referring to "intensity" in this context, but rather that fluorescent foci corresponding to clusters of ZorC/D were more readily detected. With the revisions to the Discussion, this text is no longer included.

L980 – no space between number and degrees centigrade, ie should be 30˚C

Corrected here and throughout the manuscript.

L991 – what is total laser flux for 14 frames, or flux in units /s?

At 80% laser intensity and a critical TIRF angle we determined a flux of 550 µW for 488nm and 930 µW for 561nm, respectively. We added this information to the methods section. We note that the laser flux at critical TIRF angle is about 330-fold less compared to non-angled laser mode.

L994 – what are settings in microbe, ie thresholds for maxima and particle detection, should be possible to do diffusion analysis as per above.

For ZorB the maxima detection was set to 50 tolerance and for ZorB/C to 120 in MicrobeJ. Although we agree with the reviewer that determining the diffusion of Zorya components would be an exciting experiment, we refer to our response to **R3.6** discussing why this is not feasible.

**L1008 – don't understand: 'randomly selected one system for each set of these flanking genes'. Can you clarify? And L1011 – how were representatives chosen?*

In short, we look at the three gene either side of each Zorya system and if there are systems with the same sets of adjacent genes, we class these as "redundant" observations (usually due to very similar genomes being sequenced). We then pick one representative example of each "redundant" observation (Zorya system identified). Because the redundancy primarily originates due to similar genomes from the same species but our analyses are at the phylum level, we randomly select the representatives for

each group of redundant observations. We use this standardised redundancy reduction approach in all our bioinformatic analyses of phage defence system abundance and phylogenetic analyses. We have updated the text to clarify: "*To reduce redundancy due to highly related genome sequences in the RefSeq database, we then selected representative Zorya systems by first clustering the sequences (using MMseqs2 v14.7e28431 with options: --min-seq-id 0.3 --coverage 0.8) of the proteins encoded by the three adjacent open reading frames on either side of each Zorya system, then randomly selecting one representative system for each unique genetic context observed.*"

L1013 – how were ZorA5ZorB2 multimers run as multimers in colabfold? As pentamer and dimer separately?

The $ZorA_5B_2$ multimer predictions were run as full complexes (heteroheptamers). We obtained similar domain-level predictions for $ZorA_5$ or $ZorB_2$ run independently. We now state in the Methods: "*Structures were predicted for each MMseqs2 cluster representative of each ZorA and ZorB family using ColabFold v1.5.2 with options: --num-recycle 3 --num-models 1 --model-type auto --amber --use-gpu-relax*".

Referee #3 (Remarks to the Author):

Hu et al. provide mechanistic insights into the Zorya anti-phage defense system discovered by the Sorek group in their seminar 2018 paper, where it was proposed that Zorya subunits ZorAB act as a MotAB-like channel to depolarize the host cell membrane in response to phage replication, resulting in abortive infection. In the current manuscript, Hu et al. use a sophisticated combination of cryo-EM, phage assays, biochemistry and in vivo imaging to provide the first detailed study of this system. They find that ZorAB is a rotary motor (with the typical 5:2 stoichiometry as previously discovered for MotAB by the Taylor group) with a peptidoglycan-binding periplasmic domain, and a long cytoplasmic tail. They demonstrate that ZorC is a dsDNA binding protein, and that ZorD is an autoinhibited nuclease. On this basis, they propose that ZorAB is the sensor module of the system, and upon phage DNA injection ZorC and ZorD are recruited to degrade phage DNA. This is a very strong piece of work that will undoubtedly be of broad interest. However, there are several concerns that should be addressed before the manuscript is accepted:

Major points:

We thank the authors for providing the maps and models. While the characterization of ZorAB is very thorough, it appears that much less consideration has been given to ZorC and ZorD. The structures of both are of sufficient quality (even given the preferred orientation of ZorC), but they provide little insights into the mechanism of Zorya. Additionally, the DNA binding assays in Fig 4d & e are a significant weakness of the manuscript.

We thank the reviewers for the comments, suggestions, and appreciation of the thoroughness of our work describing the core $ZorA_5B_2$ complex. For ZorC and ZorD, in addition to the structural insights that our work provides for these proteins, our mutagenesis confirms key active site residues and the requirement for these enzymatic domains in Zorya defense. Additionally, our updated TIRF microscopy results (**Fig. 5e, f**) now show that both ZorC and ZorD co-localize with ZorAB during phage invasion and that this recruitment is dependent on a functional ZorAB motor. We have also optimized and improved our ZorC-DNA EMSAs (updated **Fig. 4d, e** and **Extended Data Fig. 7h**) and we further confirmed the ZorC-DNA interaction by obtaining a cryo-EM dataset of a ZorC-DNA complex. We show that it is indeed the EH signature-containing domain of the ZorC that is responsible for DNA binding (**Fig. 4f**). We have also explored several aspects of ZorC and ZorD as queried by the reviewer, e.g., the interaction of ZorC with ZorD, ZorC DNA binding specificity, as detailed below.

Specific points to address:

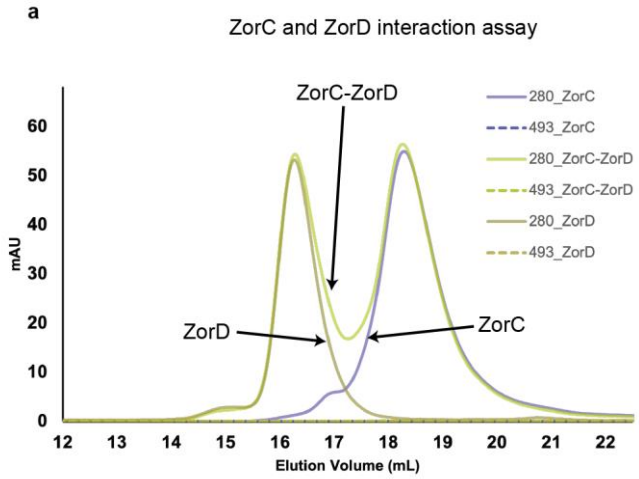
R3.1: *In Fig 4d, at high concentrations, large aggregates of ZorC + DNA are stuck in the wells. Is this due to the size of the DNA substrate chosen? If they repeat this with a shorter dsDNA substrate, would this effect still be observed? If so, this might provide mechanistic insights - perhaps multiple copies of ZorC binding to a single phage genome upon recruitment by ZorAB is important for interference.*

We thank the review for the suggestions. For the ZorC-DNA aggregates in the wells, we discovered that the running buffer used for this assay was sub-optimal and by switching to a phosphate running buffer (instead of the previous TBE buffer), we were able to observe clear migration of the ZorC-DNA complex within the gel (**Fig. 4d, e** and **Extended Data Fig. 7h**). These data fully support our earlier claims for ZorC DNA binding. We also explored the DNA binding specificity of ZorC using DNA oligos of different lengths, sequences and GC content (**Extended Data Fig. 7h)**. In all cases, ZorC-DNA binding was observed, suggesting that ZorC binds DNA in a sequence-independent manner. Our mutagenesis results (phage EOP and EMSAs) show the EH signature-containing domain is essential for ZorC DNA binding. We have now also collected a cryo-EM dataset of ZorC in complex with dsDNA (plasmid DNA) and although we did not achieve a high-resolution reconstruction of this complex, the 2D classes clearly show that the EH signature-containing domain of ZorC indeed interacts with DNA. We have included this in the manuscript (**Fig. 4f**). Given the size of the ZorC EH signature-containing

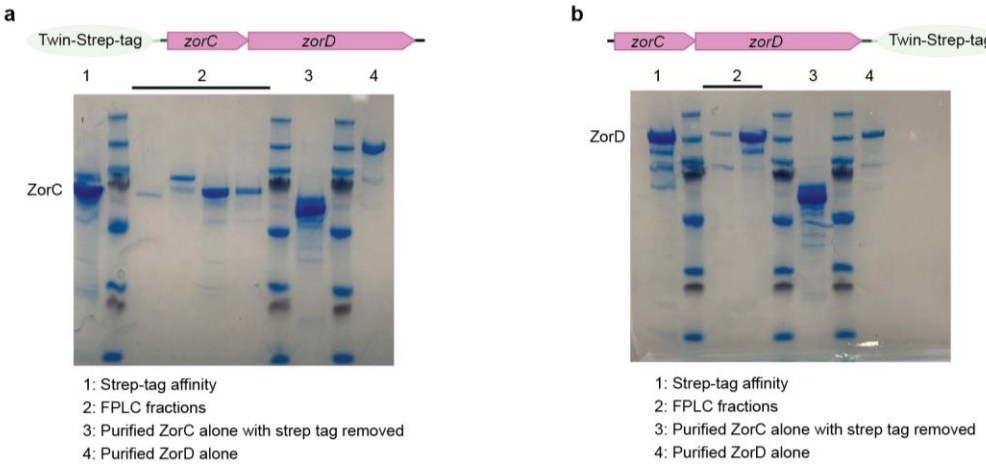
domain, it is roughly estimated that this domain binds to a 12bp dsDNA. Additionally, our updated TIRF microscopy results (**Fig. 5**) show that both ZorC and ZorD are co-localized with ZorAB during phage invasion and that recruitment is dependent on a functional ZorAB motor as rotation-impaired ZorAB or a ZorA tail truncation mutant do not show recruitment. We have now updated the relevant text and figures, including the method section with these new data.

R3.2: *Do ZorC and ZorD form a complex? The authors should attempt this (ideally in the presence and absence of dsDNA substrate), together with AlphaFold2 multimer modeling the complex.*

We investigated this *in vitro* and *in silico* using several approaches:
In vitro, we performed multiple interaction studies, but none revealed a detectable ZorC–ZorD interaction:
1. We incubated separately purified ZorC and ZorD and ran the mixture over the FPLC but did not observe complex formation (see below figure).



2. We co-expressed ZorC and ZorD from an operon, with either an N-terminal tag on ZorC or a C-terminal tag on ZorD, as informed by functional phage EOP data for fluorescent protein tagged constructs shown in **Extended Data Fig. 9c**, but did not observe any interaction using pull-downs (see gel below). These experiments rule out a complex forming by ZorC/D formed upon co-expression of ZorC and ZorD



3. We also tested for an interaction between ZorC and ZorD in the presence of DNA *in vitro* but again, there was no apparent evidence showing that ZorD affect ZorC DNA binding or they form a complex in presence of DNA (**Extended Data Fig. 8g**).
Overall, these negative results are consistent with our model that ZorAB is required to activate ZorC/D. It is likely that the autoinhibited form of the ZorD prevents binding with ZorC or a ZorC-DNA complex.

In silico, we used AlphaFold3 to predict multiple complexes:
1. ZorC alone: generally agreed with the core domains resolved in our CryoEM ZorC structure (RMSD = 3.83 Å over 213 aligned Cα atoms in the core domain).
2. ZorC + DNA (18 bp dsDNA we used in the manuscript, **Extended Data Fig. 7h, i**): successfully predicted our experimentally observed ZorC-DNA interaction, including the binding domain and orientation (EH signature motif containing domain, positively charged surface patch).
3. ZorD + ATP +DNA: A reasonably confident prediction for the interaction between the ZorD-CTD with DNA (ipTM 0.79, pTM = 0.64). Interestingly, ZorD adopts a linearized conformation, distinct from the closed, circular from we obtained through cryo-EM. A similar linearized conformation was also observed from AlphaFold3 predicted ZorD + ATP model (ipTM 0.97, pTM = 0.61), suggesting the AlphaFold3 prediction might represent the activated ZorD state (**Extended Data Fig. 8h, i**).
4. ZorC + ZorD + DNA + ATP + Mg^{2+}: We obtained a plausible model in which the ZorC EH domain and the ZorD C-terminal Snf2-related domain both directly bind to dsDNA. Additionally, in the presence of ZorD, the ZorC C-terminal domain (which was absent from our cryo-EM reconstruction) is also interacting directly with the DNA. However, the ipTM score for this ZorC-ZorD-DNA complex is below 0.5 (ipTM 0.44, pTM = 0.50), which has been noted as a potential significance threshold for AlphaFold3 complexes, although there is a lack of empirical data to support this cutoff at present (**Extended Data Fig. 8m**).
Overall, the AlphaFold3-predicted ZorC-ZorD-DNA complex provides a potential model for ZorC–ZorD–DNA interactions but requires experimental validation in follow-up work (our attempts to generate this complex experimentally in the absence of ZorAB demonstrate that further experimental investigation of the structure will not be trivial and we deem this beyond the scope of our current study). We have included these models in the **Extended Data Fig. 8** to stimulate future investigations.

R3.3: *ZorD is autoinhibited, but the CTD appears to be an active nuclease. Have the authors attempted to use AlphaFold2 to predict alternative conformations that may be active in contrast to the closed, circular observation they observed in their DNA-free structure?*

Please see R3.2 above.

R3.4: *Where does ZorD bind DNA, and might this predict how their structure is autoinhibited? The authors should align other previously-determined structures bound to nucleic acids (as identified using Dali or FoldSeek) with their structure and analyse the results. This should also be performed for ZorC.*

For ZorD, we have shown in vitro, through EMSA, that the purified full-length ZorD does not interact with DNA, nor does it affect ZorC DNA binding (**Extended Data Fig. 8g**). The AlphaFold-predicted ZorD-DNA interaction is consistent with experimental structures for other Snf2-family domains, including the Chromatin remodeler ISW1a (PDB: 7X3T; Z-score = 26.6); now shown in **Extended Data Fig. 8l**. Multiple similar structures were identified using both DALI and FoldSeek.
For ZorC, HHpred (sequence alignment), DALI, and Foldseek provide minimal insights, supporting the novelty of our structural and functional characterisation of ZorC, including demonstration that ZorC is a DNA binding protein (facilitated by the EH signature motif-containing domain) and that the ZorC DNA binding activity is essential for Zorya anti-phage defense.

R3.5: *Perhaps the presence of both ZorC and ZorD together is required for ZorD nuclease activation. This should be tested experimentally. If this is the key to activating ZorD, perhaps this offers mechanistic insights i.e. ZorAB activation recruits ZorC and ZorD together, which is required for phage DNA degradation. If full-length ZorD is inactive in the absence of ZorC (as the authors demonstrate), then this may provide a mechanism for preventing aberrant activation in the absence of phage-induced ZorAB activation.*

Indeed, our data support that ZorC is required for ZorD activation, because functional Zorya requires all the components, but that ZorC and ZorD together are not sufficient for ZorD nuclease activation without an active ZorAB motor (**Fig. 3h, 4c, 4i** and **Extended Data Fig. 8f**). In our model, the activated

ZorAB motor recruits ZorC and ZorD during phage invasion and Zorya defense, the process of which will likely trigger ZorD activation (**Fig. 6**). The inability of ZorC and ZorD to function independent of ZorAB activation (keeping in mind that ZorAB is a membrane-embedded ion-driven rotary motor), likely necessitates alternative *in vivo* approaches to further understand the Zorya mechanism (such as cryo-ET), which we deem beyond the scope of the current work.

Minor points:

R3.6: *Since the authors already have TIRF data, would it be possible to perform foci tracking to determine rates of diffusion? It would be useful to quantify "diffusibility" in meaningful terms, especially since the single fluorescence micrographs in Fig 5 don't look terribly different in the presence and absence of phage. In the exemplary images shown, the ZorB untreated vs +phage also appear to show a large increase in the number of foci akin to ZorC and ZorD, which contradicts the model provided by the authors, so diffusion rates etc would be very useful.*

We agree that quantifying the diffusivity of single Zorya molecules will be an exciting experiment. However, our current experimental setup is not optimized for such analyses. Specifically, we believe that capturing the complete phage infection cycle from phage attachment and cell envelope penetration to DNA injection will be crucial to accurately assess diffusivity changes of Zorya components. Achieving this requires a sophisticated microfluidics setup that we hope to establish in the future. At the moment, our TIRF microscopy observations are limited to a few selected time points post-phage exposure. Additionally, our current mNeonGreen (mNG) and HaloTag (HT) fusions to Zorya components are prone to photobleaching, which restricts us to using longer time intervals that are unsuitable for accurate diffusivity measurements.

Instead, we focused on examining the co-localization of membrane-bound ZorB with the soluble, cytoplasmic effectors ZorC and ZorD. Using new dual-labelled Zorya constructs (mNG and HT), we found that both ZorC and ZorD co-localize with wild-type ZorB at the cell membrane only in the presence of phage (**Fig 5e, f**). Importantly, in the non-rotating ZorB^{D26N} mutant or in a ZorA truncated tail mutant, ZorD does not co-localize with ZorB even in the presence of phage Bas24 (**Fig. 5f**). This demonstrates that the activation of the Zorya system, exemplified by ZorD recruitment and foci formation, is dependent on both Zorya motor rotation and the ZorA cytoplasmic tail.

The reviewer further correctly notes the formation of ZorB foci independent of the presence of phage, albeit there is a small yet significant increase in the presence of phage. We confirmed this finding using a functional HaloTag fusion to ZorB and with the non-rotating ZorB_D26N mutant (**Fig 5a, b**). Our working theory (consistent with our overall Zorya activation model) is that $ZorA_5B_2$ complexes continuously 'screen' the cell envelope for perturbations. We propose that the ZorB foci observed in our microscopy data represent stationary ZorAB complexes that become anchored due to the pinching of the IM-PG layers by phage, which allows the ZorB PGBDs to reach, and anchor to, the PG layer (fluorescence from non-stationary ZorB would be diffuse and not observed as a discrete foci). For ZorB foci observed in the absence of phage infection, we suspect ZorAB mobility can transiently become impaired/blocked by obstructions in the IM/periplasm, such as collision with other large membrane-bound complexes. In these cases, the ZorB foci remain inactive (since the ZorB PGBD is unable to reach the PG layer) and do not recruit/activate ZorC/D, as supported by our data (**Fig. 5d,f**). Overall, our new dual-tagged data strongly supports our model where the membrane complex ZorAB senses phage infection and transmits this information to the cytoplasm for recruitment of the Zorya effectors ZorC and ZorD.

R3.7: *Could the authors speculate upon why certain clades of viruses are not targeted by Zorya? The Dhillonvirus, Demerecviridae and Straboviridae (amongst others) show complete immunity to Zorya - is there anything in particular about these viruses (putative anti-Zorya genes? Differences in DNA injection mechanism? DNA modification) that do not trigger Zorya?*

As above for **R1.7**, this is an interesting question that we have briefly explored (e.g. ruling out DNA modification for T4) but warrants more investigation in follow up studies. Our overall Zorya model reveals multiple potential targets/critical points where phage-encoded inhibitors could suppress or avoid Zorya defense and provides a solid foundation for testing candidate anti-Zorya factors.

R3.8: *Thank you again for providing the structures for review. They look good, and have been well-built. There is a minor issue, however - the hydrogen atoms have been left on the models (presumably from when they were refined in Isolde). These should be removed prior to pdb deposition, and the Phenix real-space refinement or comprehensive validation should be run again, since the clash score will likely change.*

We thank the reviewer pointing this out. This issue comes from the model of ZorD in complex with the ATP-γ-S. The model was indeed refined first using Isolde and then Phenix. We have deleted the hydrogen atoms and refined this model against the map, and validated the model. We also have updated the **Extended Data Table 1** and deposited the updated ZorD ATP-γ-S model into the PDB, under the same accession code.

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Point-by-point response to reviews' comment

"Structure and mechanism of the Zorya anti-phage defense system" – Nature-2023-07-12645B
Referees' comments:
Referee #1 (Remarks to the Author):

This revised paper has improved since the original submission with substantially less speculation than before. Some additional experiments have also been added and bolster certain aspects of the study. Notably, the notion that the PMF is used to drive a conformational change in ZorA to transmit a signal to ZorC/D is better supported and is an important aspect of how the system likely works. However, the other major facets of how Zorya functions remain incompletely defined. As with most defense systems, there are two general features - detection of a phage and activity of an effector. Each is discussed below.

Phage detection: For Zorya, the authors propose that ZorB plays the role of phage detector based on peptidoglycan (PG) binding. There are two primary models that could explain how ZorB works, as articulated in the response document from the authors. One is that ZorB senses a pinching of the cell envelope during phage infection, with the PG layer brought in closer proximity to the inner membrane and ZorB. The other is that ZorB recognizes some aspect of broken/perturbed PG created during infection. The model in Fig. 6 implies it's the latter, but the text favors the former - and neither is yet proven. In addition to reconciling the text and figures, the authors need to (i) clearly establish that ZorB binds PG and (ii) distinguish between these models (see detailed comments below as well). To me, the sensing of phage infection via a change in PG is the most novel and exciting aspect of the study at hand, so I think shoring up the conclusion that ZorB binds PG and discerning between the two possible models seems important, and feasible, to do.

Effector activity: it remains unclear how the rotation of ZorA that is likely triggered by ZorB and powered by the PMF leads to a change in ZorC/D within the cell. C and D don't seem to interact but are both required for defense and both colocalize to some extent with ZorA. Sorting out the details of how C and D contribute and how they interact with each other and ZorA would take some work and isn't as exciting as the sensing mechanism, so I think punting here is not ideal but reasonable. But, I do think the authors at least need to address why they see colocalization via TIRF microscopy in only 20-30% of cells - see last point below.

RE: We thank the reviewer for their positive feedback on the revised paper, as well as the additional queries to clarify some aspects of the work.

Concerning ZorAB binding to peptidoglycan (PG), our data suggests that ZorAB functions as a PMF-dependent rotary motor analogous to flagellar stator unit MotAB. In order to function as a rotary motor, MotB/ZorB must anchor the membrane-embedded motor by binding to the peptidoglycan via its PG binding domain (PGBD), the domain architecture of which is evolutionally conserved, including those residues responsible for PG binding, as shown in **Extended Data Fig. 4b, d**. Only upon binding to PG, proton flow through the ZorAB TMD channel can then power rotation of ZorA around ZorB. Importantly, our mutagenesis data confirm that those key residues involved in PG binding are critical for Zorya defense.

To further address the reviewer's concerns and to strengthen our conclusions, we performed additional peptidoglycan pulldown experiments using purified wildtype ZorAB complex, ZorAB complex with a mutated ZorB PGBD, as well as purified ZorB PGBD (new **Extended Data Fig. 4g, h**). We further purified *E.coli* MotB PGBD and use it as a positive control to examine the interaction between ZorB PGBD and peptidoglycan (see our response to R1.4 below).

Our structural data indicates that the PGBDs of ZorB are too far away to bind to PG in the absence of phage, because the distance between inner-membrane and PG is too large, hence preventing activation of ZorAB motor function. Our general model is that ZorAB detects a decrease in the distance between

PG and inner membrane. As the reviewer points out, we highlight two ways that could be done, both of which occur in phage infection (the breaking and fraying of the PG and the pulling up of the inner membrane to be closer to the PG). We however do not claim we can determine their relative importance, indeed it is not unlikely that both could be involved. We agree with the reviewer that this could have been pointed out more clearly in the text, which we have edited for clarity. Furthermore, based on the reviewer's comment we have also rearranged **Figure 6** for increased clarity as to the order of events.

In terms of effector activity and DNA degradation activity by Zorya, our previous *in vivo* (mutagenesis of conserved active site residues leading to loss of phage defense) and *in vitro* (nuclease activity assays showing ZorD-mediated phage DNA degradation and loss of this nuclease activity upon mutation of conserved active site residues) data support the nuclease activity of ZorD. To further demonstrate the role of DNA degradation by Zorya during phage defense, we have now performed time-lapse microscopy to directly visualise Zorya-mediated degradation of injected phage DNA *in vivo* (see response to R 1.2 below). All of the new data provide additional evidence for our foundational model of the key mechanisms of Zorya-mediated phage defense, which are supported by multiple lines of structural, in vitro and in vivo evidence.

Below, we provide additional point-by-point responses to the reviewer's comments and queries.

Specific issues:

R1.1: "Here, we reveal the molecular basis of Zorya defense" This is an overstatement in the abstract and should be rephrased. The Discussion has a more balanced and measured self-assessment, indicating that *some* aspects of Zorya defense are revealed here.

RE: We have toned down the statement by changing it to '*Here, we investigate the molecular basis of Zorya defense…*'

R1.2: The authors' response makes a reasonable case that the mechanism of protection is direct and not an abortive infection. But how do ZorC/D avoid cutting the host chromosome? It's speculated that it could be localization-based, but there's no data as yet indicating that cleavage of the phage DNA occurs in vivo (only in vitro data).

RE: As the reviewer mentioned, our data demonstrated that Zorya defense is not via an 'abortive' mechanism targeting host DNA (since infected cells continue growing unaffected by Zorya activity) and our previous revision provided multiple lines of evidence for ZorD nuclease activity both *in vitro* and *in vivo*. We do not have data investigating the precise mechanism how the host chromosome is protected, since probing this aspect of Zorya activity will not be trivial and, as such, is beyond the scope of our current study which already provides substantial advances in understanding multiple aspects of Zorya-mediated defense. However, to address the comment about cleavage of phage DNA in vivo, we performed new time-lapse microscopy to directly visualize the fate of incoming phage DNA in the presence and absence of Zorya (new **Fig. 4l-n**).

To this end, we engineered phages to harbor a *parS* site inside their genome to directly visualize phage DNA within cells using the *parS*-ParB system. When used to infect cells expressing ParB-mScarlett, the ParB-mScarlett binds the *parS* loci and oligomerises, resulting in fluorescent foci indicating the presence of the *parS*-containing phage DNA(Owen et al. 2021). However, for unknown reasons and despite success in other phages, we were unable to get this *parS*-ParB system working in Bas24 (in the absence of Zorya). However, we were able to get the system working the Bas54, which is also protected against by Zorya. Using this system, we used time-lapse microscopy to track the presence of phage DNA during Bas54 infection in the absence or presence of Zorya. Consistent with our model for Zorya-mediated phage DNA degradation, we observed Bas54::*parS* loci within infected cells (detected as spots of ParB-mScarlett fluorescence) in the absence but not presence of Zorya (new **Extended Data Fig. 7j**). The timing of the appearance of these foci is consistent with earlier studies of the *parS*-ParB system and the longer replication rate of Bas54 relative to Bas24(Owen et al. 2021; Mulla et al. 2024).

Whilst supporting our DNA-degrading model for Zorya, the absence of *parS*-ParB foci could theoretically also be explained by a mechanism where the Zorya system prevents injection of phage DNA into the cytoplasm of host cells. To exclude this possibility, we directly visualised Bas24 DNA injection and subsequent degradation by Zorya (new **Fig. 4l-n**). To do this, we labelled Bas24 phage DNA with a fluorescent dye (SYTOX Orange) and used time-lapse imaging to track phage adsorption, DNA injection, and subsequent fate of the infected cells and injected DNA. In the absence of Zorya, we observed an increase in cytoplasmic SYTOX Orange fluorescence, suggesting injection and accumulation of labelled phage DNA, followed by later cell lysis (at the completion of the infection cycle). Remarkably, we were also able to observe and follow the fate of individual phage particles from adsorption (occurring during the timelapse), allowing us to directly observe DNA injection (apparent as transfer of fluorescence from the phage capsid to intracellular fluorescence). In contrast, in the presence of Zorya, DNA injection still occurred (since the fluorescence from the adsorbed phage particles decreased over time, consistent with the rate of DNA injection in the absence of Zorya) but we detected no intracellular SYTOX Orange fluorescence and the cells were protected from lysis. These results directly show i) that the Zorya system does not interfere with DNA injection and ii) that the incoming phage DNA is rapidly degraded by Zorya. Additionally, the apparent lack of any, even minor amount, of intracellular injected DNA in the presence of Zorya supports our proposal for localized and immediate degradation of incoming phage DNA as a mechanism to avoid cutting the host chromosome by an activated Zorya system.

R1.3: line 166: it should read 'putative' or 'predicted' peptidoglycan binding domain. At this point, a biochemical activity has not been established, only predicted based on sequence.

RE: We agree with the reviewer that until this point in the text, we did not formally demonstrate PG binding by ZorB. While we have performed additional pulldown experiments demonstrating ZorAB binding to PG, we now state at this point in the text '*…a predicted peptidoglycan binding domain…*'.

R1.4: The evidence that ZorB binds peptidoglycan needs to be bolstered. The only data presented are in Extended Data Fig. 4e and they show quite weak pulldown of ZorAB with PG, with no controls, e.g. a known PG binding protein and a mutant of ZorB thought to ablate binding in vivo. Without these sorts of controls, it's not rigorously established that ZorB binds PG in vitro, let alone in vivo.

It does not become clear until the Discussion that the authors are proposing a model in which ZorB does not initially bind PG and then PG deformation by the phage leads to binding. This aspect of the model remains murky and should be better substantiated, e.g. by assessing ZorB-PG binding before and during infection in vivo, and the model and data (with caveats) should be discussed within the manuscript. This is argued in the response document to be beyond the scope of the manuscript. While some aspects of the model can reasonably be argued to be beyond the scope, this facet seems central to the story at hand. There are several ways the authors could go about showing this or at least providing some additional evidence, from examining PG-protein association (as in https://doi.org/10.1128/jb.186.20.6728-6737.2004) to direct imaging.

RE: The PGBD of ZorB consist of an evolutionarily conserved core structure that has a common peptidoglycan binding function. However, in order to further support that the ZorB PGBD binds to PG, we performed additional PG pulldown experiments using purified ZorAB complex and purified ZorB PGBD. We also purified the *E.coli* MotAB PGBD domain and used it as a positive control and the ZorAB PG-binding site mutant to use as a negative control (new **Extended Data Fig. 4g, h**). Negative stained electron microscopy of purified ZorAB PG-binding site mutant demonstrated fully assembled particles, suggesting correct folding of the ZorB PGBD point mutant (new **Extended Data Fig. 4q**). The pulldown data show that binding to peptidoglycan is more than 3-fold reduced in the ZorAB PG-binding site mutant compared to wildtype ZorAB complex and the *E.coli* MotAB PGBD domain. Purified mutant ZorB PGBD also showed a substantially decreased binding to PG compared to purified wildtype ZorB PGBD, while a purified negative control protein, ZorE, did not bind to PG (new **Extended Data Fig. 4h**). As the expected PG binding interface is rather large, a complete abolishment in PG binding was not expected for this four residues point mutant of the ZorB PGBD. Further, we note

that full PG binding likely occurs only under torque (Nord et al. 2017) i.e. activation of the ZorAB motor, which explains why the phenotype of the ZorAB PG-binding site mutant is stronger *in vivo* under phage infection conditions.

In summary, our additional results further support that the ZorB PGBDs bind to PG as do the flagellar stator unit MotB PGBDs. Additionally, our EOP data clearly show that the PG-binding site mutation impairs Zorya defense. Similar to previous studies on MotAB, the data presented here support that PG binding is critical for Zorya defense. We acknowledge that the precise mechanism of phage-induced ZorAB anchoring and activation remains to be uncovered, as stated in the Discussion, page 15. Investigation of this requires, for example, high-resolution cryo-ET to capture the snapshots from phage attachment and cell envelope penetration to DNA injection and Zorya activation. We believe that these studies will require a sophisticated experimental setup, will not be trivial and will be supported by our current study, yet are clearly beyond the scope of this initial foundational study.

R1.5: Further to the point above, the authors write "These data confirm that ZorB PGBD integrity, dimerization and PG binding are required for Zorya defense." The data referred to here are simply a series of alanine mutations - again, the data to support the claim of PG binding are not adequate yet.

RE: Please see our response to R1.4 above. We now provide additional experiments to support our claim that ZorB PGBD binds to PG similar to the binding of MotB PGBD to PG.
In addition, we would like to emphasize that whilst mutagenesis and *in vivo* functional testing provides only indirect evidence for the function of specific residues and protein domains, it is important to note that many of the mutated residues and domains are conserved with the well-studied MotAB stator unit of the flagellar motor, where function has been established via multiple lines of evidence over several decades of study. Out structural data provides very high confidence that the mutations made are directly analogous to those studied in the MotAB system, and that the conserved domains (e.g. PGBD) are most likely to function similarly in ZorAB as for MotAB (there are no plausible alternate hypotheses to test). As well as providing additional data for the role of PG binding (as above), we have amended the statement to '*These data support that ZorB PGBD integrity, dimerization and PG binding are required for Zorya defense.*'

R1.6: Finally, related to the model being proposed that it's about distance between the PG and the inner membrane/ZorB that is being 'sensed' by ZorB: the model in Fig. 6 is very misleading as it is drawn to imply that ZorB is binding/sensing frayed ends of the PG layer created by the phage. This is a very different model/idea than what the text says (and what is stated in the rebuttal document where both models are suggested to be possible).

RE: We apologise if this was not clear in the manuscript. Due to the length of the ZorB 'stalk', it appears the PGBD cannot 'reach' the PG layer under ordinary conditions (based on the IM-PG distance and comparison to *E. coli* MotB) (**Fig. 3a** and **Extended Data Fig. 4e**). As we stated in the first rebuttal document that during the phage infection, the PGBDs of ZorB could contact PG layers through two possible mechanisms, both of which would reduce the IM-PG distance:

1) 'Pinching' of the IM-PG layer distance by some phage tail tubes which "pull up" the inner membrane closer towards the peptidoglycan (B. Hu et al. 2015) , which would allow the ZorB PGBD of any ZorAB in the localised vicinity to reach the PG layer and anchor/activate the complex.
2) 'Frayed' PG ends caused by muralytic activity of phage virion-associated lysins may enable the binding and anchoring of ZorAB. It is well established that *E. coli* phages depend on such virion-associated lyases in their infection process, e.g. T3 Gp16, T4 Gp5, T7 Gp16 (Latka et al. 2017).

We depicted these possibilities in **Fig. 6b**. Again, in both scenarios, phage infection would reduce the distance between the IM and PG, allowing ZorB PGBDs bind and anchor to the PG, which is required for an activated ZorAB motor as mentioned above. We have revised the text (page 14) to ensure these two possibilities are clearly explained in the paper. Elucidating the details of the presented molecular sensing mechanism will be a focus of our future studies.

R1.7: When activated, does ZorA freely and continuously rotate around ZorB or simply change conformation? I know ZorAB is homologous to MotAB and there's free rotation for that system, but it's not clear to me that the same is necessarily the case here.

RE: It has long been proposed that 5:2 motors (such as flagellar stator unit MotAB and bacteria Ton motor subcomplex ExbBD) function as directional rotary machineries (Hu et al. 2023; Rieu et al. 2022; Hu et al. 2021; Martin et al. 2024). In the motor active state, ions permeate through the channel connecting the periplasmic space to the cytosol, via a universally conserved aspartate residue (in the case of ZorB, D26), enabling the A subunit to rotate around the B subunit. in this process, the B subunit's PGBDs anchor to the PG, and the complex's stoichiometry and structure allow the A subunit to rotate directionally around the B subunit.
For ZorAB, comparison between the WT ZorAB and the putatively freely rotating mutant (with ZorB residues 46-52 replaced by a GGGSGGS linker) shows no significant difference in ZorA EM density(**Extended Data Fig. 4s**), arguing against the idea that ZorA undergoes a simple conformational change upon rotation around ZorB. Additionally, our EOP data and microscopy data show that neutralizing the charge of ZorB D26, which is a key residue responsible for ion transportation and motor rotation, abolishes Zorya defense. Further, disrupting the proton motive force by addition of the uncoupler CCCP, which has previously been used as a mean to stop rotation of the bacterial flagellum, prevented Zorya anti-phage defense function. These data strongly suggest that, similar to MotAB, ZorAB motor and its PMF-dependent rotation is necessary for Zorya defense. We cannot rule out that there is no conformational change at any time, and we think it is even likely to happen to allow recruitment of ZorC and ZorD (see **Supplementary Discussion**), nor can we rule out that rotation does not stop towards the end of the Zorya mechanism, but these questions are out of scope of the present manuscript and will need to be addressed in a following study.

R1.8: All mutations (e.g . Fig. 3h, 4c, 4i) are assumed to disrupt the function they're hypothesized to impact. But many could also be impacting protein levels/stability/folding - this needs to be explicitly acknowledged and ideally demonstrated not to be the case in key cases, e.g. by immunoblotting.

RE: We agree with the reviewer that mutations, which we introduced to probe key residue function, may impact protein expression level, stability and folding. However, as above, many of the mutations are analogous to those in MotAB, which have been extensively validated in that system. Additionally, we provide in vitro protein assay data (with standardised protein amounts) for the key ZorC and ZorD mutants. Nonetheless, we have now performed quantitative western blot analysis of wt ZorB and the crucial ZorB^{D26N} mutant protein (used for many of the microscopy experiments), demonstrating that ZorB steady-state protein levels are stable for both wt ZorB and ZorB^{D26N} (new **Extended Data Fig. 6e**).

R1.9: "in the presence of phage Bas24, at least one ZorC-mNG or ZorD-mNG focus was co-localized with a ZorB-HT focus in approximately 20% or 30% of cells, respectively (Fig. 5e, f). These data suggest that the cytosolic effector proteins ZorC and ZorD are recruited to ZorAB complexes that are activated by phage infection." What about the other 70-80% of cells?? It seems a little premature to conclude that ZorC/D are recruited to ZorAB if colocalization is only seen in a minority of infected cells. The authors could substantiate their model through co-IP studies before and during infection.

RE: We believe that the observed colocalization in 20–30% of cells is an inherent limitation of the used imaging technique. Total Internal Reflection Fluorescence (TIRF) microscopy is intrinsically limited to visualizing fluorophores within ~100–200 nm of the glass coverslip. This means that only the basal membrane of the bacterial cell—approximately one-quarter to one-third of the total cell volume—is illuminated and imaged at any given time. As a result, any colocalization events occurring in other regions of the cell are not captured in our TIRF microscopy images. While phage infection can occur at any position of the cell, our imaging approach restricts us to detecting only those events occurring near the cell/cover slip interface. Given this limitation, the 20–30% colocalization we observed is likely an underrepresentation of the actual frequency of ZorC/D recruitment to ZorAB complexes during

phage infection. Further, we cannot exclude that some cells have not been infected by phages at the time point of imaging. Finally, we note that we employed a conservative fluorescent signal threshold to count a given ZorC/D foci colocalized with a ZorB as true co-localization. To clarify the likely under-estimation in the text, we have added the statement "…*since TIRF microscopy has a limited depth of view (we estimate that approximately half the cell depth is visible), the observed co-localization is likely an under-representation of the actual ZorC/D recruitment frequency*".

Referee #2 (Remarks to the Author):

Hu et al. have comprehensively answered my minor requests. In particular, I appreciate the consideration shown to rooting the Zorya tree and also accept the reasoning for not pursuing higher time resolution diffusion measurements (in response to R3.6). I also think the newer two-color co-localisation measurements are well suited to addressing the actual line of inquiry - which components come together in response to phage infection.

I also appreciate the addition of extra detail to the methods, which I think is required to make the work more accessible, and the restructuring of the paper to add supplementary discussion on more speculative claims.

RE: We are grateful to the reviewer for their feedback and positive assessment of our manuscript.

R2.1: In response to my query on L991 there is perhaps a minor thing, flux is usually in eg uW/s, but in my query I said 'total flux' over 14 frames, and then asked for flux in units/s so this was a somewhat confusing request from my end. I assume this is very minor and I am satisfied that you have measured the power here, which is ultimately what I was requesting.
There is a small typo however there also so maybe worth exploring:
"Samples were excited at 50 ms exposure for 14 frames using a 488 nm laser (flux of 550 µW with a sensor area 941 of 283.5 cm2) at 80% and emission was recovered via a quad TRIF filter cube (emission: 502-549 nm)." should be TIRF not TRIF, and I estimate here this should be power not flux, ie laser power of 550uW delivered over an area of 283.5cm2? This does seem a little large tho, perhaps sensor area is the size of the chip on the camera? These are all minor points and I don't necessarily need to see again but I think just a small error here you are likely reporting power, not flux, and that is what I was requesting originally to get a feel for how intense the laser light was.

RE: We thank reviewer two for highlighting this and we have corrected the materials and methods accordingly.

I thank the authors for improving the clarity on my end and accommodating my suggestions for extra words where required and appreciate the extended figures that really do aid my understanding of Fig. 3 and deletions.
Referee #3 (Remarks to the Author):
The authors have thoroughly addressed the concerns of this reviewer. The additional data strengthens the manuscript, and the explanations for why some experiments are either difficult or out of scope to perform are reasonable. I support publication in Nature.
David Taylor

RE: Thank you for your positive evaluation and constructive feedback on our manuscript.

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