nature portfolio

Peer Review File

Crystal structures reveal nucleotide-induced conformational changes in G motifs and distal regions in human guanylate-binding protein 2

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In this manuscript, the authors reveal structures of the GDP-bound G domain of GBP2 and nucleotide-free GBP2K51A in full length. They investigated GBP2 in different nucleotide-bound states and how these can contribute to GBP2 dimerization. They further illustrate the structural mechanism of how GDP-binding prevents GBP2 GD dimerization and analyse the interaction between the GD and the C-terminal helical domain of GBP2K51A. This paper is well-written and easy to follow. The work is interesting and would enhance our understanding of how GTP-hydrolysis regulates GBP protein conformational changes.

Comments:
• Given that GBP2 can bind to LPS, and the recombinant protein used in this study was purified from E. coli, it would be nice if the authors could comment on how LPS binding can affect GBP2 structure. It is possible that full-length WT GBP2 aggregates with E. coli LPS and thus leads to a low yield of this protein.
• The authors compared the structures of GBP2-GD and GBP1-GMPPNP. However, GBP1 is different to GBP2 as it can bind to both GTP and GDP to produce GMPI. It would be clearer if the author could comment on the structural difference between GBP1GD-GMP and GBP1-GMPPNP as a side-by-side comparison to current ones.
• The authors could provide a more detailed analysis comparing GBP2-GD and GBP2-full length (7E58) or the GBP2K51A structures in addition to that in supplementary figure 9.
• Introduction, paragraph 3 “Gbp” should be “GBP”?
• Figure 1c, it’s difficult to see the green curve for WT+GMPPNP, I suggest the author change a darker colour.
• This sentence needs to be revised: “The myriad of roles played by GBPs in mediating host defense range from forming assembly platforms for caspase-4 recruitment and activation 18,19, promoting the rupture of Salmonella containing vacuoles 20, disrupting the structural integrity of bacteria 21 to pyroptosis in cell 22.” Forming caspase-4 platforms, promoting the rupture of Salmonella-containing vacuoles and disrupting the structural integrity of bacteria all end up promoting pyroptosis, so they should not be used as parallel examples. Moreover, reference 20 is mainly about IRGB10-mediate pathogen clearance, PMID: 32510692 and PMID: 35906252 should be used instead.
• In the results section 2 “Crystal structure of GBP2GD in complex with GDP reveals a closed active site”, paragraph 1, residue T49, G50, Y53 and G68 are not labelled clearly in the figures.
• Analysis of the RMSD score is not described in the method.

Reviewer #2

(Remarks to the Author)
Guanylate-binding proteins (GBPs) comprise a family of interferon-inducible eukaryotic effector molecules of the innate immune response. They bind to the surface of internal membranes or to the membranes of invading bacteria, leading to membrane coating and eventually rupture, and activation of the inflammasome. GBPs recruitment to membranes is intimately linked to the GTP hydrolysis cycle, which has been extensively characterized by structural and biochemical experiments over the past 25 years. The study from Roy et al. now adds a high-resolution GDP-bound crystal structure of the GBP2 catalytic domain and a nucleotide-free full-length structure of a GBP2 mutant to the structural collection. In the GDP-

bound structure, the authors found Glu99 opposite the beta-phosphate of GDP to engage with the catalytic Lys51 in the P-loop. This interaction drives a central catalytic residue, Thr75, out of the catalytic site. In the transition state structure of GBP1, Thr75 positions the catalytic water molecule and coordinates a Mg2+ ion; accordingly, the absence of Thr75 from the catalytic center in the GDP-bound structure is consistent with the absence of the Mg2+ ion at this position. In addition to the GTPase domain construct, a full-length GBP2 construct containing a mutation of Lys51 shows some displacement of the helical bundle compared to the full-length GBP1. Based on these data and some GTP hydrolysis assays, the authors suggest that Glu99 acts as a glutamate finger after GTP hydrolysis to push Mg2+ and, consequently, GDP out of the nucleotide-binding pocket.

The submitted structural data (pdb and mtz files), including the structural statistics, appear overall sound. However, given the vast number of related structures of other GBPs and GBP2, a major concern is the novelty of the data. The identification and characterization of a catalytic glutamate finger in switch 2 pushing the Mg2+ out of the catalytic pocket and therefore facilitating GDP release could provide such novel mechanistic insights. However, the associated functional data are currently insufficient to support this claim.

Major

Fig. 1.: GBPs have low affinity for nucleotide (in the µM range) and they lose bound nucleotide during gelfiltration, with the notable exception of AlF3, having a higher nucleotide affinity. To make these experiments conclusive, the authors have to repeat the gelfiltration runs with >= 100 µM nucleotide in the running buffer.

Fig. 2.: Structure of the catalytic domain
Under physiological conditions, a negatively-charged glutamate residue is not expected to bind to the negatively-charged beta-phosphate of GDP due to charge repulsion. While some of the negative charge of Glu99 may be compensated by Lys51, a worry is that the observed conformation may be an artefact of the potentially acidic environment used during the crystallization (which contains GDP and 0.04 M KH_2PO_4, e.g. acidic substances), leading to protonation of Glu99. What is the pH value of the final crystallization solution, e.g. including GDP, KH_2PO_4, glycerol and protein buffer? Can crystals be prepared at pH 7.5 (or soaked with a corresponding buffer) and does the catalytic center look the same under these conditions?

Fig. 3: Biochemical data
The provided biochemical data are not sufficient to support the claim of a glutamate finger involved in the release of GDP. Mutating conserved residues in the catalytic center of a GTPase almost inevitably leads to a reduction of phosphate release in multiple-turnover GTPase assays, due to various defects in the catalytic cycle. A more detailed analysis has to be conducted to support the claim of Glu99 mediating nucleotide release.
Nucleotide-binding assays for GTPgammaS and GDP should be conducted for GBP2 and the Glu99 mutants. A Glu99A mutant should be included to clearly dissect the role of the acidic side chain. Is binding of nucleotide Mg2+ dependent, is it pH dependent (see also above)? Does full-length protein behave similarly to the catalytic domain construct? Do the mutants still dimerize in the presence of GDP-AlF3, e.g. is GTPase activity abolished since GDP cannot be released or since the mutants have a general defect in forming a catalytic transition state? Ideally, the authors should set up a single turnover GTPase assay for wt and the E99A mutant. Compared to the multiple turnover assay, the single turnover assay should show similar rates of GTP hydrolysis for both variants, since only nucleotide release should be affected by the E99A mutation.

Figure 4: Full-length GBP2
Since the nucleotide-free GBP2 wt structure has already been published before and is very similar to the current nucleotide-free structure of the GBP2 mutant, these data currently do not contribute much to the story. Is there anything one can learn from this structure concerning the role of Glu99 in GBP2?

Figure 5: Model of GBP2 conformation changes.
The novelty of this story relates to the glutamate finger and its role in the GTPase cycle (if it turns out to be true). The model should therefore summarize details of the molecular changes in the catalytic center rather than repeat the overall GTPase cycle to which the current data contribute very little. For example, a movie showing the structural rearrangements of residues in the catalytic center during the entire GTPase cycle could be provided and further illustrated in the figure.

General implications:
How conserved is the glutamate in dynamin superfamily proteins, e.g. in the closely related atlastins and in other dynamin superfamily proteins? Could this be a general mechanism? If it was not conserved elsewhere, which residues are at the corresponding positions in other dynamin superfamily proteins?

Minor:
The GDP-bound structure should be carefully checked for the presence and absence of water molecules: Some placed water molecules have little density while some clearly defined electron densities in hydrogen bond distance to the protein are not filled with waters. There is a prominent, unfilled electron density at the carbonyl of the guanine base. Is it a phosphate? Some attempts should be performed to model this density.

Fig. S5B:
What is the motivation for these experiments? Has it been shown before that the isolated CHD can interact with the GTPase domain of GBP1? Without a reference or positive control, it is not conclusive to claim that the CHD does not regulate

GTPase domain enzymatic activity - it may do so in the context of the full-length protein.

L55: ‘GBP1 can bind both GTP and GDP with equimolar affinity. However, the physiological role of this nucleotide preference is still unknown.’
PMID 32433976 could be added in this regard.

L156: The here described structural rearrangements within the GTPase domain were also observed in GBP1. PMID 10970849, 16511497, 38267655 could be added.

L249: The observed salt bridge network between the GTPase domain and helix a12/13 has also been described for GBP1. PMID 32352209 and 20450919 could be added.

L338: ‘Mutants in full-length GBP2 and GBP2GD showed similar reduction in activity, suggesting the elongated C-terminal helical domain does not have any additional contribution to nucleotide binding or GTP hydrolysis.’
The presented biochemical data do not allow for this conclusion. Additional GTPase assays in a concentration-dependent setup and nucleotide binding assay comparing full-length GBP2 and GBP2GD would be necessary. Since both GBP1 and GBP2 show a salt bridge network between GD and CHD, one would envisage the CHD to have a regulative function in GTP hydrolysis.

L355: ‚Such elongated structures are necessary for coating bacterial membranes as has been shown for GBP1 (ref 15)’.
PMID 38267655 and the preprint 10.1101/2023.03.28.534355 could be included. These references might as well be added in the introduction as well as PMID 32510692.

L358: ‘We propose that in solution, α12 and α13 are capable of assuming any possible conformation in dimeric GBP2, from closely interacting with the GD of the other protomer to fully stretching out (Figure 5c).’
In regard to this conclusion, PMID 37314846 could be discussed.

Reviewer #3

(Remarks to the Author)
Roy et al attempted to elucidate the structural plasticity of human GTPase, GBP2 upon nucleotide binding through crystallographic analysis. They determined the structure of the free K51A mutant and the GBP2 G-domain in complex with GDP. They have also performed biochemical assays such as GTP hydrolysis and size-exclusion chromatography experiments.

The manuscript has several issues:
1. The main objective of the paper has not been clearly defined in the context of current literature available on biochemical function and structural elucidation (Febs J 2019, PNAS 2021, and Biochemistry 2023).
2. It is not clear how this study will provide knowledge on this protein as well as on its homologs.
3. The manuscript is not well written. Several information they have reported, are already available in the literature.
4. Several experimental issues:

The authors described in the text that they used analytical size-exclusion chromatography to determine the oligomeric state of the protein, but the elution volume presented in Fig 1b and the column material shown in Fig 1c legend together indicate that it is not the analytical size-exclusion chromatography set up. More importantly, why there is a huge difference in the elution volume of the full-length protein observed between Fig 1b and 1C? Although the oligomeric state has been mentioned, nowhere does it mention the estimated molecular mass. Without this, commenting on the oligomeric state does not make sense. In Fig 1b, what is the oligomeric state of CHD? Another important point is to be noted in Fig 1c: The elution volume of the WT+GDP.AlFx is lower than that of the protein marker (158 kDa). How do they conclude that the WT protein exists as a dimer in the presence of GDP.AlFx.

5. To elucidate the structural plasticity of GBP2, it is appropriate to compare the structure of this full-length protein in the free form and with GppNHp or GDP. However, they used the free K51A mutant instead of the WT whose structure is already available (PNAS 2021). For the comparison, they used the known structure of the GppNHp-bound hGBP1, which is not appropriate.
6. For the activity assay, the malachite green phosphate detection method was used, which is not very sensitive. Additionally, the substrate hydrolysis was measured instead of the production estimation. Authors can use more sensitive methods, such as HPLC or others.
7. Why did they use the S73A mutant as a control? Not even discussed. I think the study on the S73A mutant of GBP2 has already been reported.
8. In the text, inconsistency in the writing of GppNHp, the same is written somewhere as GMPPNP.
9. Page 3, confusing sentence, GBP1 can bind both GTP and GDP with equimolar affinity to produce guanosine-5’-monophosphate (GMP). GBP1 can bind both GTP and GDP with equimolar affinity, but cannot hydrolyze external GDP to GMP. This should be mentioned.

10. Helical domain does not contribute to GTP hydrolysis. This information has already been reported (2019).

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have addressed the concerns raised in the initial review. The manuscript is now clearer in the methodology and results sections.

Reviewer #2

(Remarks to the Author)
The new data, in particular the nucleotide-binding data, are very informative and contribute neatly to the story. However, they also point to an obvious problem of the structure: Despite a clear Mg2+-dependency in GDP binding and the presence of 5 mM Mg2+ in the crystallization solution, there is no Mg2+ visible in the catalytic center (one should see it at 2 Angstrom resolution). There is a clear deviation of the biochemical and the structural data!
As stated before, this could be consequence of the acidic pH (as it may be for GMP-bound GBP1 structure). In an appropriate microenvironment (e.g. when forming a salt bridge to Lys51), the local pKa of Glu99 can be quite different from the expected value of a glutamate in solution (see for example PMID 19324049). As also stated before, it does not make much sense that a negatively charged glutamate bonds with the beta-phosphate, e.g. in all likelihood, Glu99 is protonated in the crystal structure. Since the conformation of Glu99 is at the heart of the current study, the authors need to address this issue in more detail.
First, they should repeat the nucleotide-binding experiments at pH 6 (as previously requested) and include these data and the pH measurements of the crystallization solution, reported in the rebuttal letter, in the manuscript. Based on the outcome of these experiments, they should discuss why Mg2+-dependent GDP binding was observed only in the biochemical experiments, but no Mg2+ was seen in the structure (line 345 – 'The presence of GDP in the active site likely induces a low-energy conformation that precludes binding of magnesium ions' does not make much sense since the biochemical data show just the opposite). Finally, they should also discuss the differences of the wt protein and E99L in GDP-binding in the absence of Mg2+ – after all, the idea that E99 pushes GDP out of the catalytic pocket by charge repulsion when Mg2+ has left the catalytic pocket is supported by these data.

Some other minor issues:

Abstract
Binding of GDP induces a distinct closed conformation in the G motifs and changes in the distal regions, which are further transmitted to the C-terminal helical domain, leading to large-scale conformational rearrangements.
It has not been shown in this manuscript that an observed structural changes in the active site are further transmitted to the C-terminal helical domain, leading to large-scale conformational rearrangements. This statement is confusing for the current paper, please reformulate.

Figure 5: The new figure needs higher magnification into the catalytic site for each nucleotide-loading state, including some labeling of important elements and highlighting the molecular changes (for example, 'Glu99 in' vs 'Glu99 out' etc).

Figure S1 needs higher resolution.

Figure S2B-D: Please report the elution volume, not the retention time, for all three graphs to allow a direct comparison with Figure S2A.

Supplementary Video: In principle, this video could be neat and helpful if the transitions between the two states were more slowly, with short breaks and clear labeling of the individual nucleotide-loading states.

Line 251 GBP2GD activity remained the same in the presence of increasing concentration of GBP2CHD (Supplementary Figure 5f), suggesting it does not affect the GTPase activity in trans.
Maybe better: suggesting that the isolated GBP2CHD does not interact with the GBP2GD to affect the GTPase activity in trans.

Reviewer #3

(Remarks to the Author)
1. Line 47. Grammatical error
2. Line 34-35, may facilitate the development of targeted therapeutic strategies against intracellular pathogens. I think it is an overstatement.
3. Line 59, GBPs can homodimerize via their G domains. It is not mentioned that the intermediate region in the G domain of GBPs plays an important role in dimerization because, in the absence of the intermediate region, the catalytic domain cannot dimerize. It is already known in GBP1 and GBP2 and references should be provided.
4. Line 66, GBP1-mediated GMP production is also essential for anti-HCV activity. This is not stated and no reference is given.
5. Line 97, GBP2 is one of the two most highly induced GBPs 33 yet it is not well studied as GBP1 or GBP5. This statement

is not entirely true. There are four papers published on GBP2 including Febs Letter, 1996. However, two papers were published on GBP5.
6. Line 100, While GBP2 is able to produce GMP as the end product, the efficiency is much less. I think a possible molecular basis of lower GMP formation in this GTPase has been reported. This is not described and no reference is given.
7. Line 191, We then compared GBP2GD GDP and GBP1GD GMP structures to examine whether the product-bound structures of… This should have been done with the full-length protein with nucleotide. Otherwise, it may not give a complete picture.
8. Line 214, Together, conformational rearrangements in switch I and switch II regions dictate that once GTP is hydrolyzed to GDP, GBP2 can no longer form dimer. If so, why some amount of GMP is formed, because GMP formation is absolutely dependent on dimerization.
9. Line 227, We are particularly interested in the G3 motif residue E99 since its negative charge is positioned right next to the β-phosphate in the crystal structure (Figure 2d). This is based on the truncated protein structure with GDP. Thus, it may not give a clear picture in the absence of the full-length protein structure.
10. Line 330, On the opposite side, comparison of the two GBP2 structures reveals conformational changes in the α3-α3' linker and α4' region (Supplementary Figure 9e), which likely contribute to the movement of the C-terminal helical domain. This may be a correlation. Unless the study is done with a mutant which is defective in this process, one cannot make this conclusion.
11. Line 336, we reason that the C-terminal helical domain does not allosterically regulate GD activity. This has already been reported in GBP2, however, it is not discussed and no reference is provided.
12. Line 343, we propose that the captured crystal structure represents the second to last step in GTP hydrolysis – magnesium ion already released, but GDP remains binding (Figures 5a). This statement would be okay if the structure of full-length GBP2 with GDP had been done.
13. Line 385, it is likely that CHD has a regulatory role only in full-length GBP proteins. The CHD of GBP1 has a regulatory role in the GTP hydrolysis resulting in increased GMP formation, reported much earlier (I think JMB, 2009), yet not mentioned.
14. Line 466, Why the mobile phase does not have the nucleotide? In its absence, the complex is likely to be dissociated.
15. Was the activity assay performed with the SUMO tag protein? Should be clarified in the results section.
16. Can the authors discuss which residues are important for the first and second steps of GTP hydrolysis in the context of the present data since the protein has both GTPase and GDPase activity?
17. In the GTPase assay, shown in Fig 3 a-b, what are the concentrations of enzyme and GTP used?
18. GD WT and GBP2 FL WT, there is an 8-fold difference of Kd for the substrate binding affinity. Will it have any impact on the activity?
19. In SEC data Fig d, is it possible GBP2 GD can be completely converted into a dimer if the GDP.ALFx is used with a higher concentration? Also, the mobile phase would have the same concentration of GDP.ALFx.
20. In the response, there is something wrong in Fig 12b. The GDP elution matches with the standard, but GMP does not. Moreover, how GMP became significant in GBP2-mediated GTP hydrolysis.
21. In response to Q7, I checked literatures, S73A mutation has been studied in GBP1, GBP2 and GBP5. This mutation in these three proteins has no or little effect on GTP hydrolysis. Since the study has already been reported, appropriate references should be given and discussed accordingly.
22. Ideally, the structure of the K51 mutant of GBP2 should have been compared with the structure of GppNHp-bound GBP2 or GDP-bound GBP2. This would have been more informative in the context of the sequence of events during GTP hydrolysis.
23. The authors should discuss how the K51-E99 contact in the GDP-bound GBP2 structure affects the switch I loop containing the catalytic residue/machinery.

Version 2:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
The authors have adequately addressed my raised concerns and add new mechanistic insights which neatly contribute to the story. In my view, the manuscript is ready for publication.

Very minor:
One sentence could be added to the discussion why no Mg2+ was found in the crystal structure, e.g. something like 'Mg2+ only moderately increased affinity at acidic pH; the crystal lattice may have stabilized the Mg2+-free conformation, explaining the absence of Mg2+ in the active site in the crystal structure.'

Reviewer #3

(Remarks to the Author)
1. Abstract (Line 426): The abstract incorrectly states that the authors determined the crystal structure of nucleotide-free full-length GBP2. Instead, they determined the structure of the nucleotide-free GBP2 K51A mutant. However, they have considered it a nucleotide-free full-length GBP2 structure, which is misleading and should be corrected.
2. Literature Context (Line 104): The statement made in the manuscript is not accurate in the context of recent literature. This should be corrected and appropriately cited.

3. Beta Phosphate Coordination (Line 157): The manuscript states that the beta phosphate is coordinated by the side chain of E99. Given that both are negatively charged, this interaction is unlikely and needs clarification. The authors should provide a detailed explanation.
4. Heading Misrepresentation (Line 279): The heading inaccurately implies that the authors determined the crystal structure of the nucleotide-free full-length GBP2. This should be revised to reflect the actual structure studied (nucleotide-free GBP2 K51A mutant). In Fig 1b, can they clarify why the elution volume of CHD is lower than that of the GD because they have similar molecular masses ?
5. K51-E99 Contact: Based on the truncated GBP2 protein structure (devoid of the helical domain), the authors propose the presence of the K51-E99 contact. However, they did not measure the distance between their side chains in the full-length protein, which is critical to validating this claim. This is not described in the manuscript.
6. Choice of Comparison Structure: The authors used the GBP2 K51A mutant protein structure for comparison, citing low yields of the wild-type protein. However, the full-length WT GBP2 protein structure has already been reported by another group in PNAS 2021. This structure was neither acknowledged nor discussed. Since it is available, the distance between K51 and E99 should be estimated and described.
7. Figure 5a Legend: The legend is unclear and misleading. The nucleotide-free GBP2 structure should be explicitly used for comparison, with the appropriate PDB ID provided. Additionally, the subsequent use of GMPPNP-bound GBP1 should be clearly stated. The diagram shown in Figure 5a depicts the protein returning to the nucleotide-free state after step 4. If so, the authors need to explain how this reconciles with the lower amount of GMP observed in GBP2.
8. Homolog Comparison: GBP2 and GBP1 are close homologs. The authors used GMPPNP-bound GBP1 to explain their data on GBP2 and provided a model. However, using this model, can they explain why GBP1, a close homolog of GBP2, yields a higher amount of GMP?
9. Role of E99: The manuscript does not explain how E99 plays an important role in GTP hydrolysis. Is E99 a catalytic residue? If so, its role should be explicitly detailed.
10. There is an 8-fold difference in Kd for substrate binding affinity between GD WT and GBP2 FL WT. The authors should address whether this difference impacts enzymatic activity. This concern was raised in a previous review but has not been adequately addressed in the revised manuscript.

Referee expertise:

Referee #1: GBP enzymes

Referee #2: Structural studies of GTPases

Referee #3: Biochemistry and biophysics of GBP enzymes

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

In this manuscript, the authors reveal structures of the GDP-bound G domain of GBP2 and nucleotide-free GBP2K51A in full length. They investigated GBP2 in different nucleotide-bound states and how these can contribute to GBP2 dimerization. They further illustrate the structural mechanism of how GDP-binding prevents GBP2 GD dimerization and analyse the interaction between the GD and the C-terminal helical domain of GBP2K51A. This paper is well-written and easy to follow. The work is interesting and would enhance our understanding of how GTP-hydrolysis regulates GBP protein conformational changes.

We thank the reviewer's encouraging comments and constructive criticism.

Comments:
1. Given that GBP2 can bind to LPS, and the recombinant protein used in this study was purified from E. coli, it would be nice if the authors could comment on how LPS binding can affect GBP2 structure. It is possible that full-length WT GBP2 aggregates with E. coli LPS and thus leads to a low yield of this protein.

The reviewer raised a very interesting question. It is possible that full-length WT GBP2 aggregates with *E. coli* LPS during purification, leading to a low yield of WT GBP2. However, it might be difficult to dissect the effect of LPS binding from the intrinsic property of GBP2 that results in its low yield from *E. coli*. We have one line of evidence that argue for the intrinsic property of GBP2 playing a greater role: GBP1 binding to LPS is stronger than GBP2, however, we are able to produce GBP1 with much higher yield from *E. coli* than GBP2, suggesting LPS-induced aggregation is not a major contributor to protein yield.

In the revised Discussion, we added the comment that “The low yield of wild-type full-length GBP2 from *E. coli* may be the result of LPS-induced aggregation of GBP2, leading to its low solubility.” (lines 326-327)

2. The authors compared the structures of GBP2-GD and GBP1-GMPPNP. However, GBP1 is different to GBP2 as it can bind to both GTP and GDP to produce GMPI. It would be clearer if the author could comment on the structural difference between GBP1GD-GMP and GBP1-GMPPNP as a side-by-side comparison to current ones.

We understand the concern of the reviewer that GBP1 and GBP2 are different in their GTP hydrolysis end products. We conducted a structural comparison of GBP1GD·GMP (2D4H, cyan) and GBP1·GMPPNP (1F5N, gray) and included the comparison here but not in the manuscript as we feel a comparison of two GBP1 structures will distract the focus on GBP2.

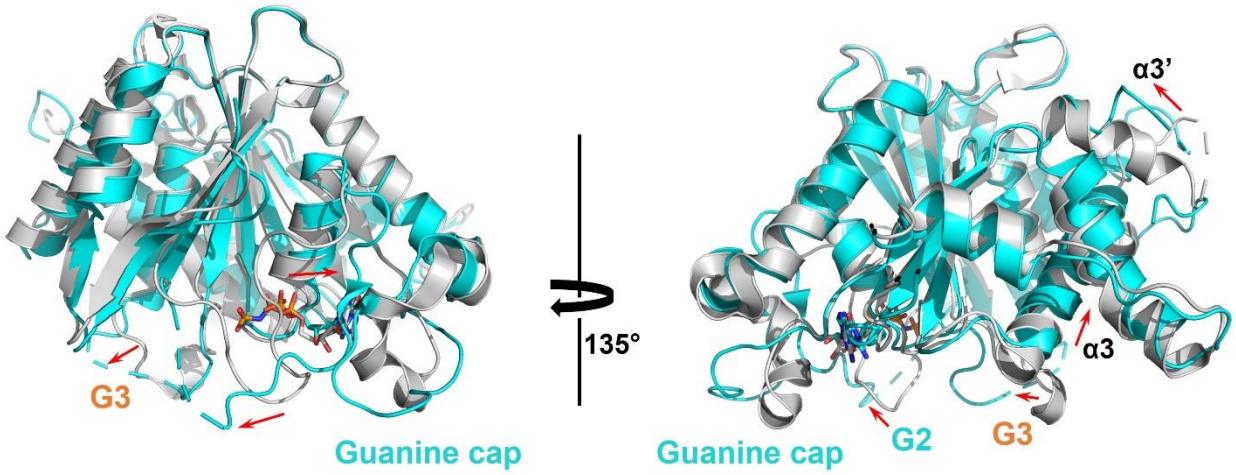


Figure 1 Structure comparison of GBP1GD·GMP (cyan, PDB ID 2D4H) and GBP1FL·GMPPNP (gray, PDB ID 1F5N) in two views.

The two structures contrast in both the guanine nucleotide binding pocket and distal regions at the back of the molecules. In the GBP1GD·GMP structure, the G2/switch I region moves outward, away from the GMP molecule. The movement of the G2/switch I region also pushes the guanine cap outward, so it no longer interacts with the guanine base. The G3/switch II region also shows conformational change. At the backside of the molecule, α3 in the GBP1GD·GMP structure moves upward compared to the GMPPNP structure. α3’ region is less ordered, but it clearly swings upward from

the α3’ region in the GMPPNP structure. The α4 helices in the two structures superpose well with each other.

3. The authors could provide a more detailed analysis comparing GBP2-GD and GBP2-full length (7E58) or the GBP2K51A structures in addition to that in supplementary figure 9.

We have expanded the analysis of the two GBP2 structures, GBP2GD·GDP and GBP2^{K51A}. We selected GBP2^{K51A} for comparison because all G motif residues are available, therefore providing more structural information, while the nucleotide free GBP2 structure (7E58) lacks important G1 and G2 motif residues. The expanded Supplementary Figure 9 is shown below, and the revised description now reads “Superposing the G domain structure in GBP2^{K51A} to the GBP2GD·GDP structure reveals conformational changes in the nucleotide binding pocket, in particular the G2/switch I region, the loop following the G3 motif, and the guanine cap (Supplementary Figure 9a). In the GBP2^{K51A} structure, the N-terminal portion of the G2 motif moves toward the guanine cap, pushing it outward. The G1 motifs in the two structures adopt very similar mainchain conformations, though the sidechains of Y47 and R48 point outward in the GBP2^{K51A} structure (Supplementary Figure 9b). The G2/switch I region, however, displays drastic rearrangement. In the nucleotide free GBP2^{K51A} structure, the G2 motif moves outward, no longer able to support GDP binding (Supplementary Figure 9c). Similarly, the G3 motif also slightly moves away from the nucleotide binding pocket. (Supplementary Figure 9d). Collectively, the nucleotide binding pocket in the GBP2^{K51A} structure is more open than in the GBP2GD·GDP structure. On the opposite side, comparison of the two GBP2 structures reveals conformational changes in the α3-α3’ linker and α4’ region (Supplementary Figure 9e), which likely contribute to the movement of the C-terminal helical domain.” (lines 308-319)

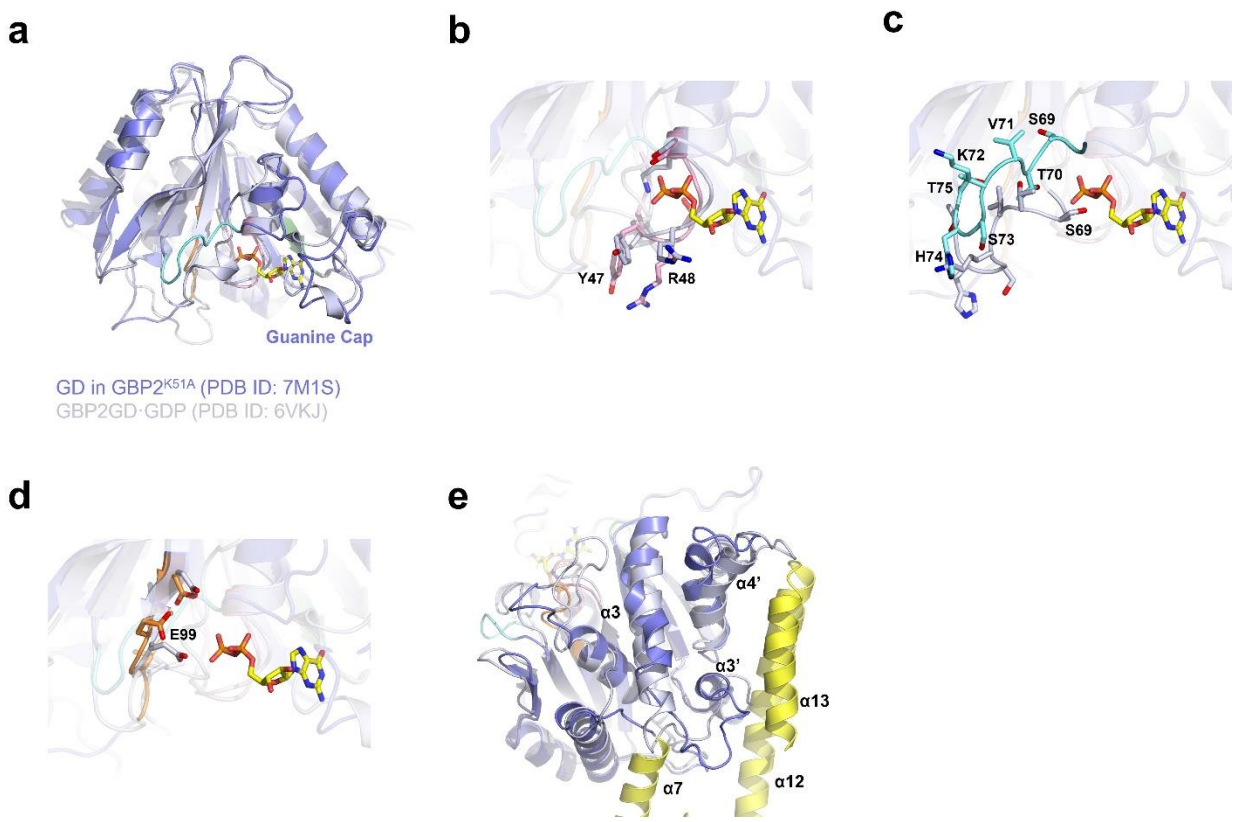


Figure 2 (Figure S9) Comparison of G domain conformations in full-length GBP2^{K51A} and GBP2GD·GDP structures. (a) GD conformation in GBP2^{K51A} structure (blue) superposed with GD domain in GBP2GD·GDP structure (light blue, PDB ID: 6VKJ). The G1-G4 motifs in full-length GBP2^{K51A} structure are colored pink, cyan, orange, and deep green, as in Figure 2. GDP is shown as sticks. (b-d) Comparison of G1 (b), G2 (c), and G3 (d) motif conformations. The G motif residues are shown as sticks. Residues with conformational changes are labeled. (e) Conformational differences in the CHD-contacting region between GBP2^{K51A} structure and GBP2GD·GDP structure.

4. Introduction, paragraph 3 “Gbp” should be “GBP”?

We thank the reviewer for pointing this out. The research article cited in paragraph 3, ref 14, tested the antibacterial functions of the mouse Gbp family, therefore we used “Gbp” instead of “GBP” to follow the gene nomenclature convention. To clarify the nomenclature, we changed “Gbp” to “mouse *Gbp*”. (line 69)

5. Figure 1c, it’s difficult to see the green curve for WT+GMPPNP, I suggest the author change a darker colour.

We appreciate the suggestions and have changed the WT+GMPPNP curve color to black to increase the contrast with other curves. (lines 722, 730-733)

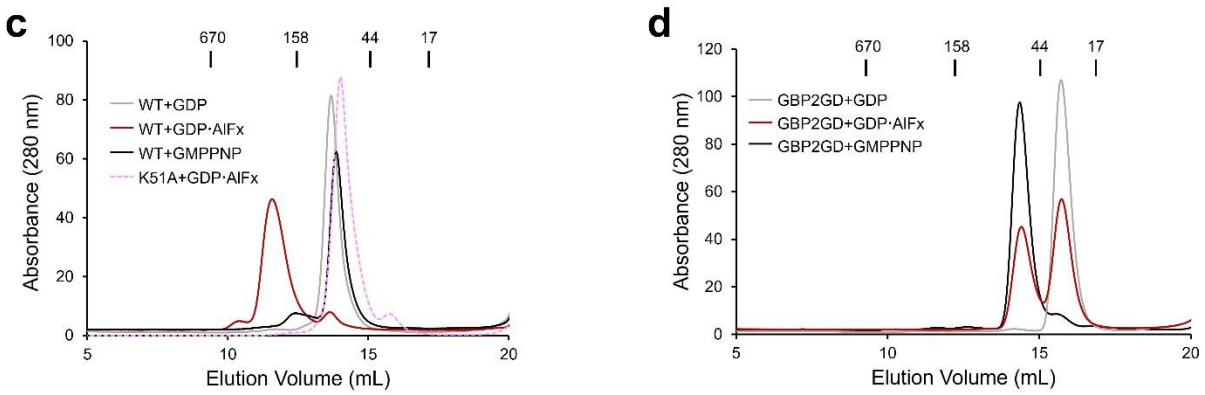


Figure 3 (Revised Figure 1c and 1d) GBP2 forms dimer upon GTP hydrolysis in solution. Curve colors were changed in 1c and 1d to increase contrast.

6. This sentence needs to be revised: “The myriad of roles played by GBPs in mediating host defense range from forming assembly platforms for caspase-4 recruitment and activation 18,19, promoting the rupture of Salmonella containing vacuoles 20, disrupting the structural integrity of bacteria 21 to pyroptosis in cell 22.” Forming caspase-4 platforms, promoting the rupture of Salmonella-containing vacuoles and disrupting the structural integrity of bacteria all end up promoting pyroptosis, so they should not be used as parallel examples. Moreover, reference 20 is mainly about IRGB10-mediate pathogen clearance, PMID: 32510692 and PMID: 35906252 should be used instead.

We thank the reviewer for pointing out the inconsistency. We have revised the sentence to “GBPs mediate host defense by initiating the assembly of platforms for caspase-4 recruitment and activation [23,24], promoting the rupture of *Salmonella*-containing vacuoles [25], and disrupting the structural integrity of bacteria, either alone or together with other interferon-inducible GTPases [17,26,27], all of which lead to pyroptosis within the cell [28].” The two citations suggested by the reviewer are included as refs 17 and 27. (lines 82-85)

7. In the results section 2 “Crystal structure of GBP2GD in complex with GDP reveals a closed active site”, paragraph 1, residue T49, G50, Y53 and G68 are not labelled clearly in the figures.

We have revised Figure 2d to include the labeling for residues T49, G50, Y53, and G68. (line 737)

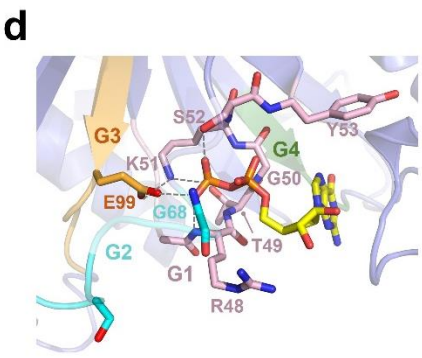


Figure 4 (Figure 2d) GBP2GD adopts a closed active site conformation when bound to GDP. (d) Close-up view of diphosphate-binding pocket.

8. Analysis of the RMSD score is not described in the method.
The RMSD score of aligned α carbon atoms (Cαs) were calculated by The PyMOL Molecular Graphics System, Version 2.5.0, Schrödinger, LLC. We have included the description in the Materials and Methods section. (lines 491-493)

Reviewer #2 (Remarks to the Author):

Guanylate-binding proteins (GBPs) comprise a family of interferon-inducible eukaryotic effector molecules of the innate immune response. They bind to the surface of internal membranes or to the membranes of invading bacteria, leading to membrane coating and eventually rupture, and activation of the inflammasome. GBPs recruitment to membranes is intimately linked to the GTP hydrolysis cycle, which has been extensively characterized by structural and biochemical experiments over the past 25 years. The study from Roy et al. now adds a high-resolution GDP-bound crystal structure of the GBP2 catalytic domain and a nucleotide-free full-length structure of a GBP2 mutant to the structural collection. In the GDP-bound structure, the authors found Glu99 opposite the beta-phosphate of GDP to engage with the catalytic Lys51 in the P-loop. This interaction drives a central catalytic residue, Thr75, out of the catalytic site. In the transition state structure of GBP1, Thr75 positions the catalytic water molecule and coordinates a Mg2+ ion; accordingly, the absence of Thr75 from the catalytic center in the GDP-bound structure is consistent with the absence of the Mg2+ ion at this position. In addition to the GTPase domain construct, a full-length GBP2 construct containing a mutation of Lys51 shows some displacement of the helical bundle compared to the full-length GBP1. Based on these data and some GTP hydrolysis assays, the authors suggest that Glu99 acts as a glutamate finger after GTP hydrolysis to push Mg2+ and,

consequently, GDP out of the nucleotide-binding pocket.

The submitted structural data (pdb and mtz files), including the structural statistics, appear overall sound. However, given the vast number of related structures of other GBPs and GBP2, a major concern is the novelty of the data. The identification and characterization of a catalytic glutamate finger in switch 2 pushing the Mg2+ out of the catalytic pocket and therefore facilitating GDP release could provide such novel mechanistic insights. However, the associated functional data are currently insufficient to support this claim.

We thank the reviewer's insightful comments and constructive criticism.

Major

1. Fig. 1.: GBPs have low affinity for nucleotide (in the µM range) and they lose bound nucleotide during gel filtration, with the notable exception of AlF3, having a higher nucleotide affinity. To make these experiments conclusive, the authors have to repeat the gelfiltration runs with >= 100 µM nucleotide in the running buffer.

We agree with the reviewer's comments that GBPs have low affinity for nucleotides and the bound nucleotide may be lost during gel filtration. To investigate whether full-length GBP2 pre-incubated with GMPPNP loses the bound GMPPNP during gel filtration, we repeated the experiment with gel filtration buffer containing 100 µM of GMPPNP (below and Supplementary Figure 2a). Even with 100 µM GMPPNP in the gel filtration buffer, only a small portion of GBP2 (pre-incubated with GMPPNP) eluted at an earlier, presumably dimer position, mirroring our observation when no GMPPNP is included in the gel filtration buffer (Figure 1c). This is in sharp contrast to the dimer formation induced by GDP·AlFx, or the dimer formation of GBP2GD induced by GMPPNP (Figure 1c, 1d). Indeed, GBP2GD forms a dimer with GMPPNP, and the dimer remains intact in a gel filtration buffer lacking GMPPNP (Figure 1d), which argues against GBPs losing bound nucleotides during gel filtration. Combining all results, our conclusion remains that full-length GBP2 is monomeric when bound to GMPPNP.

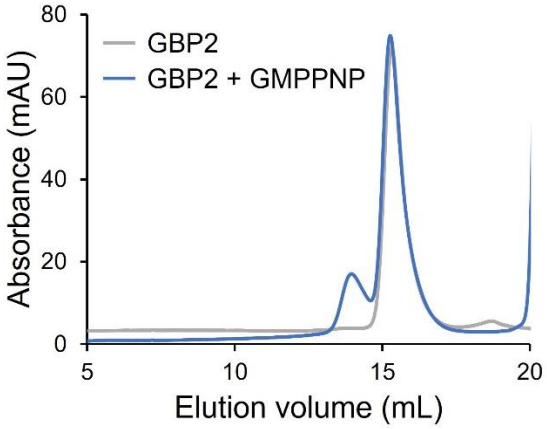


Figure 5 (Figure S2a) Size exclusion chromatography profiles of nucleotide free GBP2 (gray) and GBP2 preincubated with GMPPNP (blue). The running buffer for GBP2+GMPPNP contains 100 μM of GMPPNP to minimize GMPPNP dissociation.

2. Fig. 2.: Structure of the catalytic domain

Under physiological conditions, a negatively-charged glutamate residue is not expected to bind to the negatively-charged beta-phosphate of GDP due to charge repulsion. While some of the negative charge of Glu99 may be compensated by Lys51, a worry is that the observed conformation may be an artefact of the potentially acidic environment used during the crystallization (which contains GDP and 0.04 M KH2PO4, e.g. acidic substances), leading to protonation of Glu99. What is the pH value of the final crystallization solution, e.g. including GDP, KH2PO4, glycerol and protein buffer? Can crystals be prepared at pH 7.5 (or soaked with a corresponding buffer) and does the catalytic center look the same under these conditions?

We examined the pH of the crystallization solution using pH test strips (the figure below). The pH value is ~ 6.0, higher than 4.25, the sidechain pKa value of glutamate residue. At pH 6, the sidechain of Glu99 should be deprotonated. We could not produce any crystal at pH 7.5.

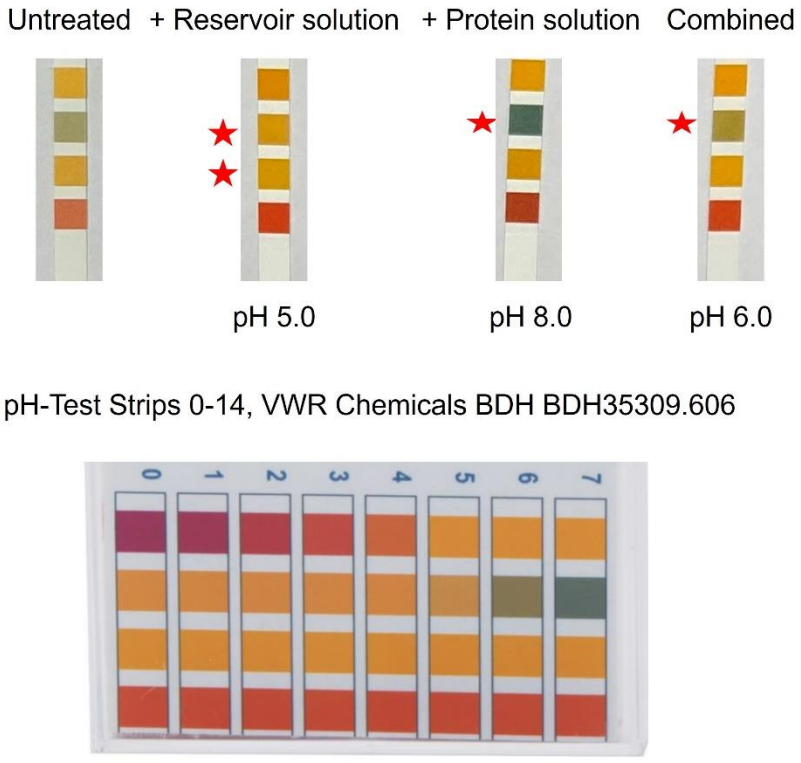


Figure 6 pH assessment of crystallization conditions using pH strips. Top panels: pH strip color change without any solution (first), with crystallization reservoir solution (second), with protein solution (third), and with reservoir solution and protein solution at 1:1 ratio (fourth); Lower panel: pH color palette at different pH values as provided by the manufacturer.

In addition, we examined the structure of GBP1 GD with GMP (PDB ID 2D4H). In this structure, GBP1 E99 occupies a similar conformation that points towards the α phosphate. Notably, the β phosphate of GDP in our GBP2GD·GDP structure occupies the same space as the α phosphate of GMP in GBP1GD·GMP (Figure 2f). The crystallization condition GBP1GD·GMP contains 130 mM of NH_4NO_3, which has a pH of about 5.5 (acidic).

Combining the observations, we believe the E99 conformation we observed in the GBP2GD·GDP structure is not an artefact, and it may represent the end-product conformation in both GBP1 and GBP2.

3. Fig. 3: Biochemical data

The provided biochemical data are not sufficient to support the claim of a glutamate finger involved in the release of GDP. Mutating conserved residues in the catalytic center of a GTPase almost inevitably leads to a reduction of phosphate release in multiple-turnover GTPase assays, due to various defects in the catalytic cycle. A more detailed analysis has to be conducted to support the claim of Glu99 mediating

nucleotide release.
Nucleotide-binding assays for GTPgammaS and GDP should be conducted for GBP2 and the Glu99 mutants. A Glu99A mutant should be included to clearly dissect the role of the acidic side chain. Is binding of nucleotide Mg2+ dependent, is it pH dependent (see also above)? Does full-length protein behave similarly to the catalytic domain construct? Do the mutants still dimerize in the presence of GDP-AlF3, e.g. is GTPase activity abolished since GDP cannot be released or since the mutants have a general defect in forming a catalytic transition state? Ideally, the authors should set up a single turnover GTPase assay for wt and the E99A mutant. Compared to the multiple turnover assay, the single turnover assay should show similar rates of GTP hydrolysis for both variants, since only nucleotide release should be affected by the E99A mutation.

We appreciate the comments and suggestions from the reviewer and carried out nucleotide binding assays and oligomerization assays.

We used the mutant E99L for the reason of consistency. We also think E99L causes less disruption to the catalytic core than E99A. We used GBP2GD E99L mutant because it expressed at decent level.

Compared to GBP2GD WT, the GBP2GD E99L bound to GTPγS and GDP with reduced affinities (Figure below, Figure 3c, and Supplementary Figure 5b). Guanine nucleotide binding for GBP2GD WT is Mg^{2+} dependent. Interesting, the absence of Mg^{2+} weakened, but not completed abrogated GBP2GD E99L binding to GTPγS or GDP (Supplementary Figure 5c and 5d). In addition, the GBP2GD E99L mutant did not dimerize in the presence of GMPPNP or GDP·AlF_3 (Figure 3d), suggesting the mutation has a general defect in forming a catalytic transition state. We have incorporated the results into the manuscript and revised the manuscript accordingly.

“We further investigated the GBP2GD E99L mutant to probe whether the mutation affects nucleotide binding. Using fluorescently labeled GTP and GDP analogs, we found that the E99L mutation reduced both GTP and GDP binding by 4-5 folds (Figure 3c, Supplementary Figure 5b). Interestingly, in the absence of Mg2+, the WT protein bound to GTP or GDP very weakly, yet the E99L mutant maintained a low-level affinity to both GTP and GDP, stronger than the WT (Supplementary Figure 5c and 5d), indicating that E99 is important in the proper positioning of Mg2+ for high affinity binding of the guanine nucleotides. The E99L mutant did not dimerize in the presence of GMPPNP or the transition state mimic GDP·AlFx (Figure 3d), suggesting deficiency in catalysis as well. Collectively, our results demonstrate that E99 is important in GTP binding and formation of the catalytic center.” (lines 236-246).

We also revised the Discussion “It is tempting to postulate that the conserved glutamate residue in GBP proteins assumes the ‘glutamate finger’ role usually provided

by GEFs *in trans*. The presence of R48 and E99 will then explain the intrinsically high GTPase activity of GBPs in the absence of GAPs or GEFs. However, our data showed that mutation of E99 affected both guanine nucleotide binding and formation of the transition state. More detailed experiments are needed to further clarify its role in GTP hydrolysis." (lines 376-381)

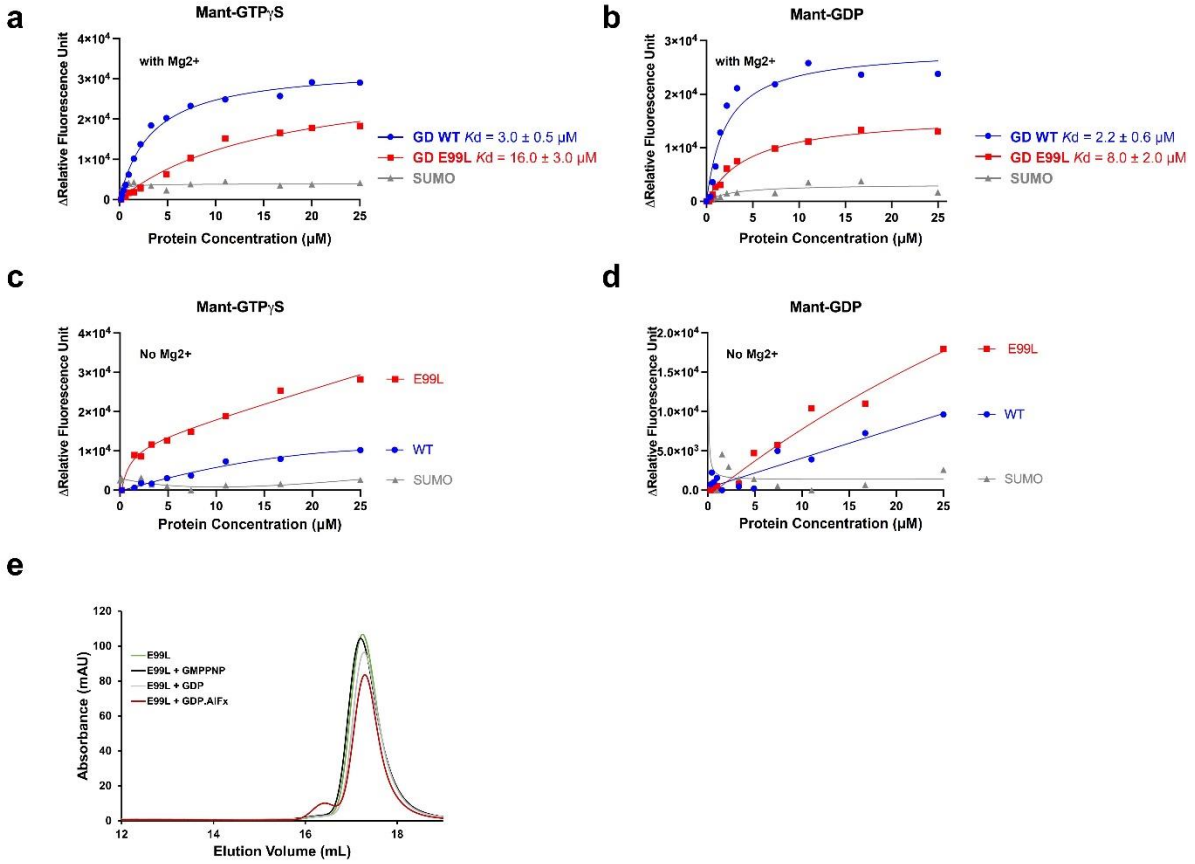


Figure 7 (Figure 3c-d and S5b-d) Characterization of GBP2GD E99L. (a-b) Comparison of GTP (a) and GDP (b) binding affinities of GBP2GD WT (blue) and E99L mutant (red). SUMO (gray) is included as a negative control. (c-d) Comparison of GTP (c) and GDP (d) binding affinities of GBP2GD WT (blue) and E99L mutant (red) in the absence of Mg2+. The graphs shown in (a-d) are representative of at least three independent experiments. (**e**) SEC profiles of GBP2GD E99L mutant in nucleotide-free form (green), GMPPNP-bound form (black), GDP·AlFx-bound form (red), and GDP-bound form (gray).

4. Figure 4: Full-length GBP2

Since the nucleotide-free GBP2 wt structure has already been published before and is very similar to the current nucleotide-free structure of the GBP2 mutant, these data currently do not contribute much to the story. Is there anything one can learn from this structure concerning the role of Glu99 in GBP2?

The nucleotide-free WT GBP2 structure (PDB ID: 7E58) misses electron densities for key G1 and G2 motif residues. The nucleotide-free GBP2^{K51A} structure reported in this manuscript, however, has continuous densities for all G motif residues, thus revealing a conformation not available from the WT GBP2 structure. We have expanded our analysis of the GBP2^{K51A} G1/G2/G3 conformation and included the comparison as Supplementary Figure 9b-d.

"Superposing the G domain structure in GBP2^{K51A} to the GBP2GD·GDP structure reveals conformational changes in the nucleotide binding pocket, in particular the G2/switch I region, the loop following the G3 motif, and the guanine cap (Supplementary Figure 9a). In the GBP2^{K51A} structure, the N-terminal portion of the G2 motif moves toward the guanine cap, pushing it outward. The G1 motifs in the two structures adopt very similar mainchain conformations, though the sidechains of Y47 and R48 point outward in the GBP2^{K51A} structure (Supplementary Figure 9b). The G2/switch I region, however, displays drastic rearrangement. In the nucleotide free GBP2^{K51A} structure, the G2 motif moves outward, no longer able to support GDP binding (Supplementary Figure 9c). Similarly, the G3 motif also slightly moves away from the nucleotide binding pocket (Supplementary Figure 9d). Collectively, the nucleotide binding pocket in the GBP2^{K51A} structure is more open than in the GBP2GD·GDP structure." (lines 308-319)

5. Figure 5: Model of GBP2 conformation changes.
The novelty of this story relates to the glutamate finger and its role in the GTPase cycle (if it turns out to be true). The model should therefore summarize details of the molecular changes in the catalytic center rather than repeat the overall GTPase cycle to which the current data contribute very little. For example, a movie showing the structural rearrangements of residues in the catalytic center during the entire GTPase cycle could be provided and further illustrated in the figure.

We thank the reviewer for their insightful suggestions. We have revised Figure 5 to include snapshots of GBP2 catalytic center conformational changes through the GTPase cycle: nucleotide-free, GMPPNP-bound, GDP·AlFx-bound, and GDP-bound (Figure 5a). In addition, we generated a movie of the Chimera conformational morph as Supplementary Video 1.

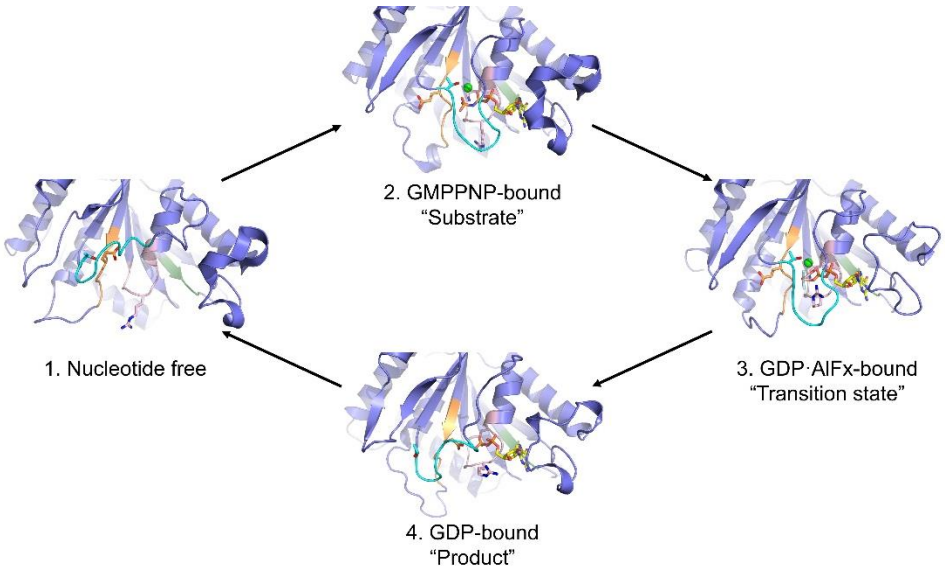


Figure 8 (Figure 5a) GBP2 Catalytic site conformation changes throughout the GTPase cycle. G1-4 motifs are colored as in Figure 2. Key residues R48, K51, T75, and E99 are shown as sticks. Guanine nucleotides and magnesium ions are represented as sticks and green spheres, respectively. The structures used are 7M1S (nucleotide free), 1F5N (GMPPNP-bound), 2B92 (GDP·AlFx-bound), and 6VKJ (GDP-bound).

6. General implications:

How conserved is the glutamate in dynamin superfamily proteins, e.g. in the closely related atlastins and in other dynamin superfamily proteins? Could this be a general mechanism? If it was not conserved elsewhere, which residues are at the corresponding positions in other dynamin superfamily proteins?

The glutamate residue E99 is conserved in the GBP family but not the dynamin superfamily (see the alignment below and Supplementary Figure 5a). In atlastin the corresponding position is occupied by glutamine (Q), similar to the glutamate in GBP family. In other members, the position is occupied by proline (P). We added the sentence to the revised manuscript.

"Moreover, E99 is conserved in the GBP family but not in other dynamin family GTPases (Supplementary Figures 1 and 5a)." (lines 229-230)

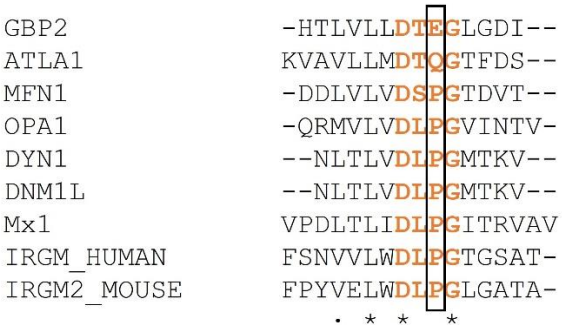


Figure 9 (Figure S5a) Alignment of the G3 motif residues from representative dynamin superfamily members. The G3 residues are colored orange. E99 in GBP2 and corresponding residues in other dynamin members are boxed in black.

Minor:

7. The GDP-bound structure should be carefully checked for the presence and absence of water molecules: Some placed water molecules have little density while some clearly defined electron densities in hydrogen bond distance to the protein are not filled with waters. There is a prominent, unfilled electron density at the carbonyl of the guanine base. Is it a phosphate? Some attempts should be performed to model this density.

We are working with the RCSB staff scientist to update the GDP-bound structure. The electron density near the guanine base could be a phosphate ion. As we tried to fit a phosphate into that density, the geometry is off, and next round of refinement produced negative density in the difference map. It could be a smaller ion, though the identity of the ion is difficult to determine.

Fig. S5B:

8. What is the motivation for these experiments? Has it been shown before that the isolated CHD can interact with the GTPase domain of GBP1? Without a reference or positive control, it is not conclusive to claim that the CHD does not regulate GTPase domain enzymatic activity - it may do so in the context of the full-length protein.

The reason for these experiments came from our observation that at the same concentration, the GTPase activity of GBP2FL is always lower than the GTPase activity of GBP2GD (represented in Figure S5e), raising the question whether the C-terminal helical domain (CHD) regulates the activity of GTPase domain. We appreciate the reviewer's comment and thus revised the text to read as

"GBP2GD activity remained the same in the presence of increasing concentration of GBP2CHD (Supplementary Figure 5g), suggesting it does not affect the GTPase activity *in trans*." (lines 251-252)

9. L55: ‘GBP1 can bind both GTP and GDP with equimolar affinity. However, the physiological role of this nucleotide preference is still unknown.’ PMID 32433976 could be added in this regard.

We have revised the sentence and added the citation PMID 32433976 as ref 13 for further clarification. Now it reads “GBP1 binds GTP, GDP, and GMP with similar affinities, yet it cannot hydrolyze external GDP to GMP [11,12]. However, the physiological importance of this nucleotide preference is unclear. Once GTP bound, GBP1 exhibits high intrinsic rates of GTPase and GDPase activity that occurs in a structurally conserved two-step reaction [9]. Interestingly, the GBP1-mediated GMP production is necessary for inflammasome activation, but dispensable for the restriction of *Chlamydia trachomatis* growth [13].” (lines 60-66)

10. L156: The here described structural rearrangements within the GTPase domain were also observed in GBP1. PMID 10970849, 16511497, 38267655 could be added.

We have added the citations PMID 10970849, 16511497, and 38267655 here as refs 30, 9, and 19. (line 169)

11. L249: The observed salt bridge network between the GTPase domain and helix a12/13 has also been described for GBP1. PMID 32352209 and 20450919 could be added.

We have added the citations PMID 32352209 and 20450919 here as refs 37 and 38. (line 274)

12. L338: ‘Mutants in full-length GBP2 and GBP2GD showed similar reduction in activity, suggesting the elongated C-terminal helical domain does not have any additional contribution to nucleotide binding or GTP hydrolysis.’

The presented biochemical data do not allow for this conclusion. Additional GTPase assays in a concentration-dependent setup and nucleotide binding assay comparing full-length GBP2 and GBP2GD would be necessary. Since both GBP1 and GBP2 show a salt bridge network between GD and CHD, one would envisage the CHD to have a regulative function in GTP hydrolysis.

We agree with the reviewer that additional GTPase assays and nucleotide binding assays are necessary to elucidate the role of CHD in regulating GTP hydrolysis by the GTPase domain. We have revised the sentence to “We also noticed GBP2GD appeared to be slightly more active than full-length GBP2, yet the C-terminal helical domain (CHD) did not affect GBP2GD activity when added *in trans* (Supplementary

Figure 5e-f). However, given the salt bridge network between GD and CHD in both GBP1 and GBP2 [37,38], it is likely that CHD has a regulatory role only in full-length GBP proteins. Carefully conducted GTPase activity assays and nucleotide binding assays are necessary to further elucidate the role of CHD." (lines 381-387)

13. L355: ‚Such elongated structures are necessary for coating bacterial membranes as has been shown for GBP1 (ref 15)'. PMID 38267655 and the preprint 10.1101/2023.03.28.534355 could be included. These references might as well be added in the introduction as well as PMID 32510692.

We have added the two citations here. The two citations are added in the Introduction together with PMID 32510692 as refs 17-19. (lines 76 and 403).

14. L358: 'We propose that in solution, α12 and α13 are capable of assuming any possible conformation in dimeric GBP2, from closely interacting with the GD of the other protomer to fully stretching out (Figure 5c).' In regard to this conclusion, PMID 37314846 could be discussed.

We have added a brief discussion regarding PMID 37314846 (ref 44). "Alternatively, the intrinsic flexibility of GBP proteins, in particular the α12-α13 region, may allow the dimer to adopt a more open dimer conformation mediated by the G domain and the α13 helix, as shown by a recent integrative structural analysis on GBP1 [44]". (lines 409-412)

Reviewer #3 (Remarks to the Author):

Roy et al attempted to elucidate the structural plasticity of human GTPase, GBP2 upon nucleotide binding through crystallographic analysis. They determined the structure of the free K51A mutant and the GBP2 G-domain in complex with GDP. They have also performed biochemical assays such as GTP hydrolysis and size-exclusion chromatography experiments.

The manuscript has several issues:

1. The main objective of the paper has not been clearly defined in the context of current literature available on biochemical function and structural elucidation (Febs J 2019, PNAS 2021, and Biochemistry 2023).
2. It is not clear how this study will provide knowledge on this protein as well as on its homologs.

3. The manuscript is not well written. Several information they have reported, are already available in the literature.

We thank the reviewer's comments. We have carried out additional experiments and revised the manuscript to improve clarity. Suggested literatures are cited to place this study on GBP2 in the context of known GBP functions and biochemistry.

4. Several experimental issues:

The authors described in the text that they used analytical size-exclusion chromatography to determine the oligomeric state of the protein, but the elution volume presented in Fig 1b and the column material shown in Fig 1c legend together indicate that it is not the analytical size-exclusion chromatography set up. More importantly, why there is a huge difference in the elution volume of the full-length protein observed between Fig 1b and 1C? Although the oligomeric state has been mentioned, nowhere does it mention the estimated molecular mass. Without this, commenting on the oligomeric state does not make sense. In Fig 1b, what is the oligomeric state of CHD? Another important point is to be noted in Fig 1c: The elution volume of the WT+GDP.AlFx is lower than that of the protein marker (158 kDa). How do they conclude that the WT protein exists as a dimer in the presence of GDP.AlFx.

We understood the concerns of the reviewer and revised the manuscript.

In Figure 1b, we used a HiLoad 16/600 Superdex 200 column for a preliminary characterization of GBP2 and the two individual domains. In Figure 1c and 1d, we used a Superdex 200 10/300 column to resolve the oligomeric states of GBP2 (FL or the GTPase domain) in the presence of different nucleotides. The Superdex 200 10/300 column is a preparative purification/analysis dual purpose column as described by the manufacturer Cytiva (previously known as GE Healthcare). It is very common to use the Superdex 200 10/300 column to characterize protein molecular weight/oligomeric status [1,2].

The HiLoad 16/600 column and the Superdex 200 10/300 column have bed volumes of 120 and 24 mL, respectively, therefore, the same protein is expected to elute at very different volumes on those two columns. The elution volumes of protein standards with known molecular weights demonstrate the nominal difference in the two columns. In Figure 1b, GBP2 eluted at 72.0 mL from a HiLoad 16/600 column, corresponding to the molecular weight of ~ 80 kDa based on protein standard calibration; in Figure 1c, GBP2 (GDP or GMPPNP bound) eluted from a Superdex 200 10/300 column at the positions of 13.7-13.8 mL, corresponding to the molecular weight of 70.9 – 74.9 kDa. The molecular weights deduced from the two columns agree well

with each other, and both are consistent with the monomeric form of GBP2 with a molecular weight of 67.2 kDa.

For Figure 1b, we carried out size-exclusion chromatography coupled multi-angle light scattering (SEC-MALS) to examine the oligomeric state of CHD. SEC-MALS revealed a molecular weight of 34.05 kDa, consistent with a monomeric CHD (calculated MW 32.83 kDa).

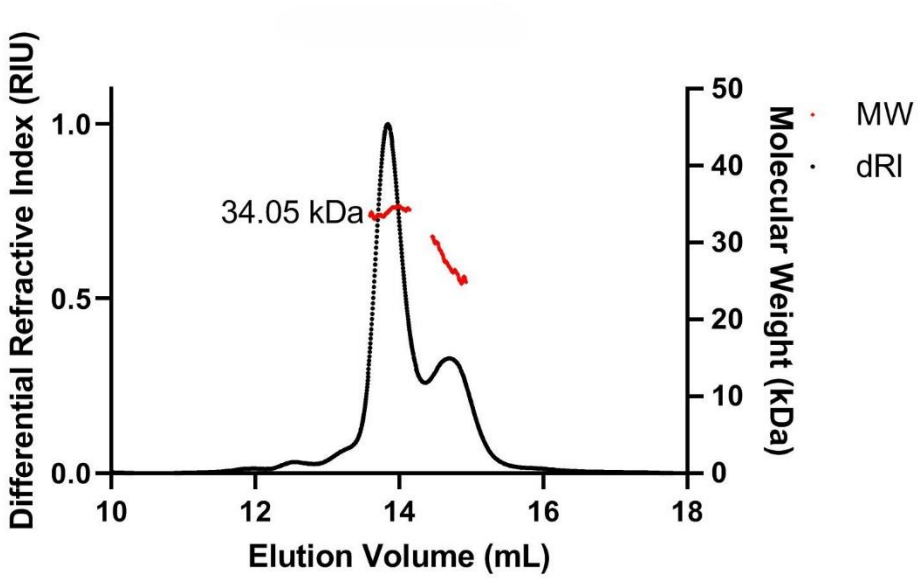
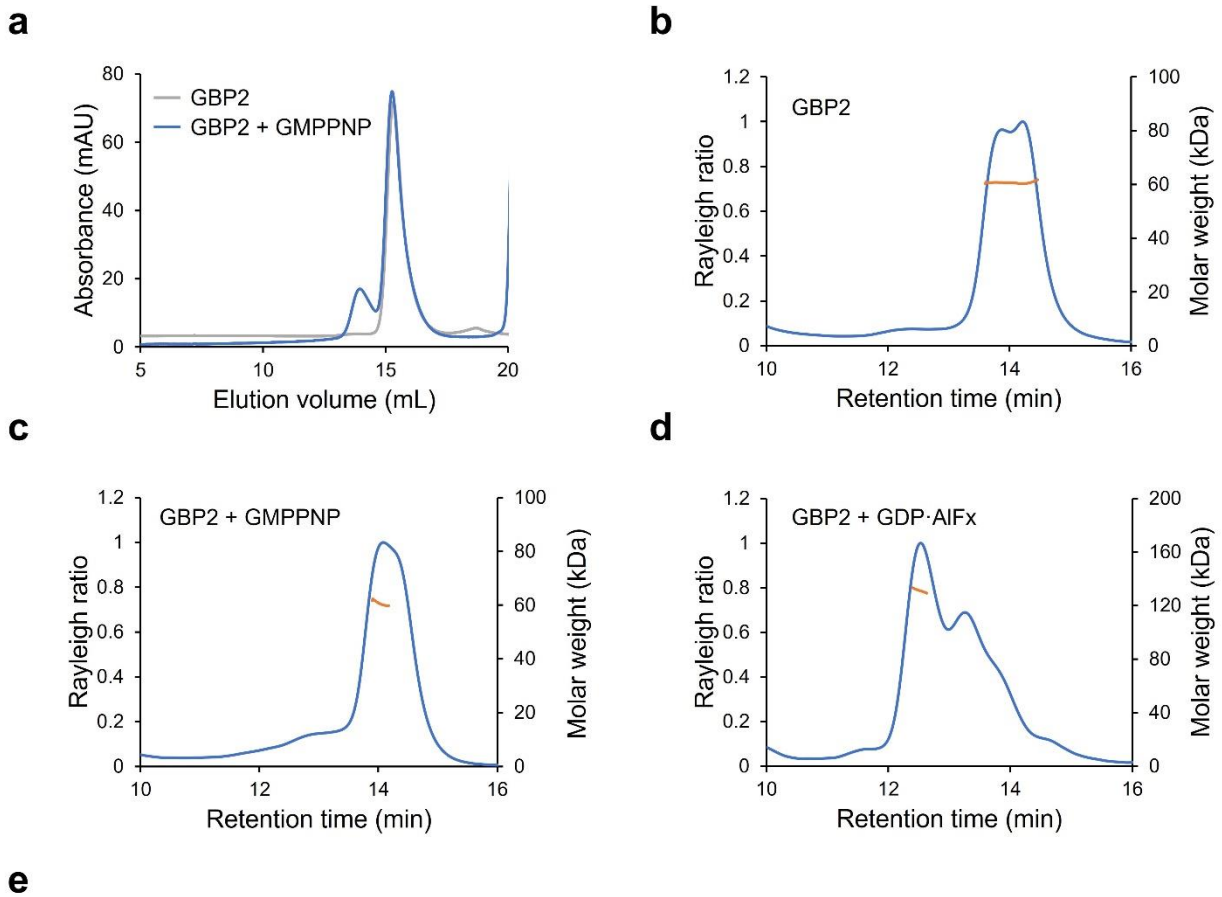


Figure 10 SEC-MALS profile of human GBP2 CHD domain. The refractive index is plotted as the black curve; the molecular weight across the main peak is plotted as the red curve. The minor peak is degradation product.

In Figure 1c the elution volume of the WT+GDP·AlFx (red trace) is indeed lower than the 158 kDa marker, suggesting the GBP2+GDP·AlFx molecular weight is higher than 158 kDa, which is in turn higher than the molecular weight of GBP2 dimer (134.4 kDa). However, it is known that the shape of a protein affects its elution position as well as the size. GBP proteins are known as elongated proteins, and they do elute earlier than globular proteins of the same molecular weight [2,3]. For GBP2, we experimentally confirmed the molecular mass of GBP2+GDP·AlFx with SEC-MALS, revealing a molecular mass of 1.31*10^5 g/mol, consistent with a dimer (calculated to be1.34*10^5 g/mol, or 134.4 kDa). The SEC-MALS results are shown below (panels b-e) and included in Supplementary Figure 2. We have revised the text to read "… however, binding to GDP·AlFx, the GTP hydrolysis transition state mimic, shifted full-length GBP2 completely to an earlier, presumably dimer elution position (Figure 1c, Suppl. Figure 2a). We further confirmed the oligomeric status of GBP2 in different nucleotide binding states via multi-angle light scattering (MALS). Consist with size-exclusion

chromatography results, MALS showed that GBP2 was dimeric only in the presence of GDP·AlFx, but not in GMPPNP-binding form or in nucleotide free form (Supplementary Figure 2b-e).” (lines 123-128)



e

Sample	Molecular mass (g/mol)
GBP2	6.063×10^4 (± 0.271%)
GBP2 + GMPPNP	6.062×10^4 (± 0.453%)
GBP2 + GDP·AlFx	1.312×10^5 (± 0.423%)

Figure 11 (Figure S2) Oligomeric status of full-length GBP2 in different nucleotide binding states. (a) SEC profiles of nucleotide-free full-length GBP2 in regular running buffer (gray) and GBP2+GMPPNP with 100 μM GMPPNP in the running buffer (blue) from a Superdex 200 10/300 column. (b-d) SEC-MALS profiles of full-length GBP2 in nucleotide-free state (b), in the presence of GMPPNP (c), and in the presence of GDP·AlFx (d). Rayleigh ratios and the molecular weights across major SEC peaks are shown as blue and orange traces, respectively. (e) Summary of molecular masses of the three GBP2 states shown in (b-d).

5. To elucidate the structural plasticity of GBP2, it is appropriate to compare the structure of this full-length protein in the free form and with GppNHp or GDP. However, they used the free K51A mutant instead of the WT whose structure is already available

(PNAS 2021). For the comparison, they used the known structure of the GppNHp-bound hGBP1, which is not appropriate.

We understand the concern of the reviewer. Comparison of the nucleotide free K51A mutant with the published GBP2 WT mutant is to show the mutant assumes highly similar conformation as the WT GBP2. The K51A mutant structure reveals additional structural information on the G1 and G2 motifs, which is not available in the nucleotide-free WT structure.

For comparison with other conformations, we agree that it is most appropriate to compare the nucleotide free structure with GppNHp or GDP-bound forms. However, our search of the PDB repository reveals no structure of full-length GBP2 in complex with GppNHp or GDP. GBP1 structures in different nucleotide-binding forms are available. GBP1 and GBP2 share high sequence similarity, and the individual domains of GBP1 and GBP2 show high overall structure features. Therefore, we postulate GBP1 and GBP2 share similar conformational changes and mechanisms in their GTPase cycles. Comparing the GBP2 structure with GBP1 structures likely will reveal the shared plasticity in all GBP family proteins.

6. For the activity assay, the malachite green phosphate detection method was used, which is not very sensitive. Additionally, the substrate hydrolysis was measured instead of the production estimation. Authors can use more sensitive methods, such as HPLC or others.

We agree that the malachite green assay is not very sensitive. We used the malachite green assay because it serves our purpose of qualitatively comparing the GTPase activities of a number of mutants.

The malachite green assay measures the level of inorganic phosphate (Pi), one of the products of GTP hydrolysis. The major hydrolysis products of GTP by GBP2 is GDP and inorganic phosphate (Pi), GTP → GDP + Pi, meaning the decrease of GTP is equimolar to the increase of Pi. We have confirmed that GMP production by GBP2 is negligible by HPLC (see the Figure below). Based on this information, we believe measuring Pi levels faithfully reflects the rate of substrate hydrolysis, enabling us to assess the GTPase activities of WT and mutant GBP2 proteins.

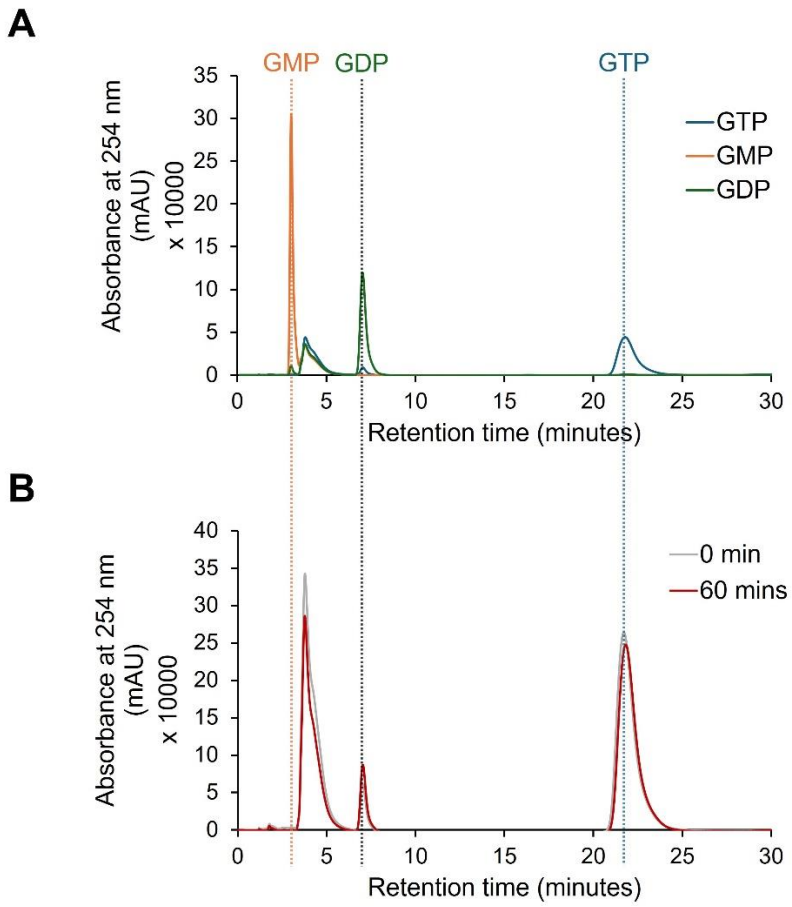


Figure 12 HPLC analysis of GBP2-mediate GTP hydrolysis. (A) Retention times of GTP (blue), GDP (green), and GMP (orange) standards. (B) Retention times of GBP2 mixed with GTP at reaction time 0 (gray) and 60 min (red). No GMP is detected at 60 min.

7. Why did they use the S73A mutant as a control? Not even discussed. I think the study on the S73A mutant of GBP2 has already been reported.

We included the S73A mutant because S73 is present in G2 motif but does not contact the phosphate in the GBP2GD·GDP structure (lines 225-227). Hence, we do not expect the mutation S73A to cause any deficiency in the GTPase activity of GBP2.

S73A mutation has been studied for hGBP1, mGBP2, and hGBP5 with contrasting results. While S73A mutation affects hGBP1 and mGBP2 efficiency [4,5], it shows little effect on hGBP5 GTPase activity [6], suggesting the effect of S73A mutation varies in different GBP members. Considering this, we believe experimentally characterizing hGBP2 S73A GTPase activity serves two purposes: validating our structural observation and expanding our knowledge on the variable roles of S73 in the GBP protein family.

8. In the text, inconsistency in the writing of GppNHp, the same is written somewhere as GMPPNP.

We have used GMPPNP, another name of GppNHp, throughout the manuscript.

9. Page 3, confusing sentence, GBP1 can bind both GTP and GDP with equimolar affinity to produce guanosine-5'-monophosphate (GMP). GBP1 can bind both GTP and GDP with equimolar affinity, but cannot hydrolyze external GDP to GMP. This should be mentioned.

We have revised the paragraph to clearly state GBP1 cannot hydrolyze external GDP to GMP. This part now reads "GBPs possess distinctive features, for example, GBP1 binds GTP, GDP, and GMP with similar affinities, yet it cannot hydrolyze external GDP to GMP [11,12]. However, the physiological importance of this nucleotide preference is unclear. Once GTP bound, GBP1 exhibits high intrinsic rates of GTPase and GDPase activity that occurs in a structurally conserved two-step reaction [9]. Interestingly, the GBP1-mediated GMP production is necessary for inflammasome activation, but dispensable for the restriction of Chlamydia trachomatis growth [13]." (lines 60-66)

10. Helical domain does not contribute to GTP hydrolysis. This information has already been reported (2019).

We have cited the reference PMID 31199074 as ref. 35. (line 252)

References

1 Kuhm, T. *et al.* Structural basis of membrane targeting and coatomer assembly by human GBP1. *bioRxiv*, 2023.2003.2028.534355 (2023). https://doi.org/10.1101/2023.03.28.534355

2 Kutsch, M., Ince, S. & Herrmann, C. Homo and hetero dimerisation of the human guanylate-binding proteins hGBP-1 and hGBP-5 characterised by affinities and kinetics. *The FEBS journal* **285**, 2019-2036 (2018). https://doi.org/10.1111/febs.14459

3 Syguda, A. *et al.* Tetramerization of human guanylate-binding protein 1 is mediated by coiled-coil formation of the C-terminal alpha-helices. *The FEBS journal* **279**, 2544-2554 (2012). https://doi.org/10.1111/j.1742-4658.2012.08637.x

4 Ghosh, A., Praefcke, G. J., Renault, L., Wittinghofer, A. & Herrmann, C. How guanylate-binding proteins achieve assembly-stimulated processive cleavage of GTP to GMP. *Nature* **440**, 101-104 (2006). https://doi.org/10.1038/nature04510

5 Kravets, E. *et al.* The GTPase activity of murine guanylate-binding protein 2 (mGBP2) controls the intracellular localization and recruitment to the parasitophorous vacuole of Toxoplasma gondii. *J Biol Chem* **287**, 27452-27466 (2012). https://doi.org/10.1074/jbc.M112.379636

6 Cui, W. *et al.* Structural basis for GTP-induced dimerization and antiviral function of guanylate-binding proteins. *Proc Natl Acad Sci U S A* **118** (2021). https://doi.org/10.1073/pnas.2022269118

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

The authors have addressed the concerns raised in the initial review. The manuscript is now clearer in the methodology and results sections.
We thank the reviewer's positive evaluation of our revised manuscript.

Reviewer #2 (Remarks to the Author):

The new data, in particular the nucleotide-binding data, are very informative and contribute neatly to the story. However, they also point to an obvious problem of the structure: Despite a clear Mg2+-dependency in GDP binding and the presence of 5 mM Mg2+ in the crystallization solution, there is no Mg2+ visible in the catalytic center (one should see it at 2 Angstrom resolution). There is a clear deviation of the biochemical and the structural data!

As stated before, this could be consequence of the acidic pH (as it may be for GMP-bound GBP1 structure). In an appropriate microenvironment (e.g. when forming a salt bridge to Lys51), the local pKa of Glu99 can be quite different from the expected value of a glutamate in solution (see for example PMID 19324049). As also stated before, it does not make much sense that a negatively charged glutamate bonds with the beta-phosphate, e.g. in all likelihood, Glu99 is protonated in the crystal structure. Since the conformation of Glu99 is at the heart of the current study, the authors need to address this issue in more detail.
First, they should repeat the nucleotide-binding experiments at pH 6 (as previously requested) and include these data and the pH measurements of the crystallization solution, reported in the rebuttal letter, in the manuscript. Based on the outcome of these experiments, they should discuss why Mg2+-dependent GDP binding was observed only in the biochemical experiments, but no Mg2+ was seen in the structure (line 345 – 'The presence of GDP in the active site likely induces a low-energy conformation that precludes binding of magnesium ions' does not make much sense since the biochemical data show just the opposite). Finally, they should also discuss the differences of the wt protein and E99L in GDP-binding in the absence of Mg2+ – after all, the idea that E99 pushes GDP out of the catalytic pocket by charge repulsion when Mg2+ has left the catalytic pocket is supported by these data.

We appreciate the reviewer's evaluation of our new data in the previous round of revision and understand the concern regarding the absence of Mg2+ in the crystal structure.

Following the reviewer's suggestion, we carried out nucleotide binding at pH 6.0 in the presence and absence of Mg2+ for both GBP2GD WT and E99L proteins (Figure 3e, 3g, Suppl. Figure 5f-i; for convenience, they are organized as Figure 1 below). In the presence of Mg2+, GBP2GD WT bound GTPγS with comparable affinities at pH 8.0 and pH 6.0; GBP2GD WT binding affinity to GDP also remained very similar at pH 8.0 and pH 6.0. In the presence of Mg2+, the GBP2GD E99L mutant bound GTPγS and GDP with elevated affinities at pH 6.0 than at pH 8.0. Interestingly, at pH 6.0, both GBP2GD WT and the E99L mutant bound to GTPγS and GDP with detectable affinities in the absence of Mg2+, with E99L mutant binding tighter to GTPγS and GDP (Figure 3e, 3g, Suppl. Figure 5f-I, and Figure 1 below). At pH 6.0, the presence of Mg2+ enhanced WT protein binding to both GTPγS and GDP by 2-4 folds, but slightly reduced E99L binding to GTPγS and GDP. The new set of nucleotide binding results is consistent with the crystallographic structure of GBP2GD·GDP, in which GDP binds in the absence of Mg2+ at pH ~ 6.0.

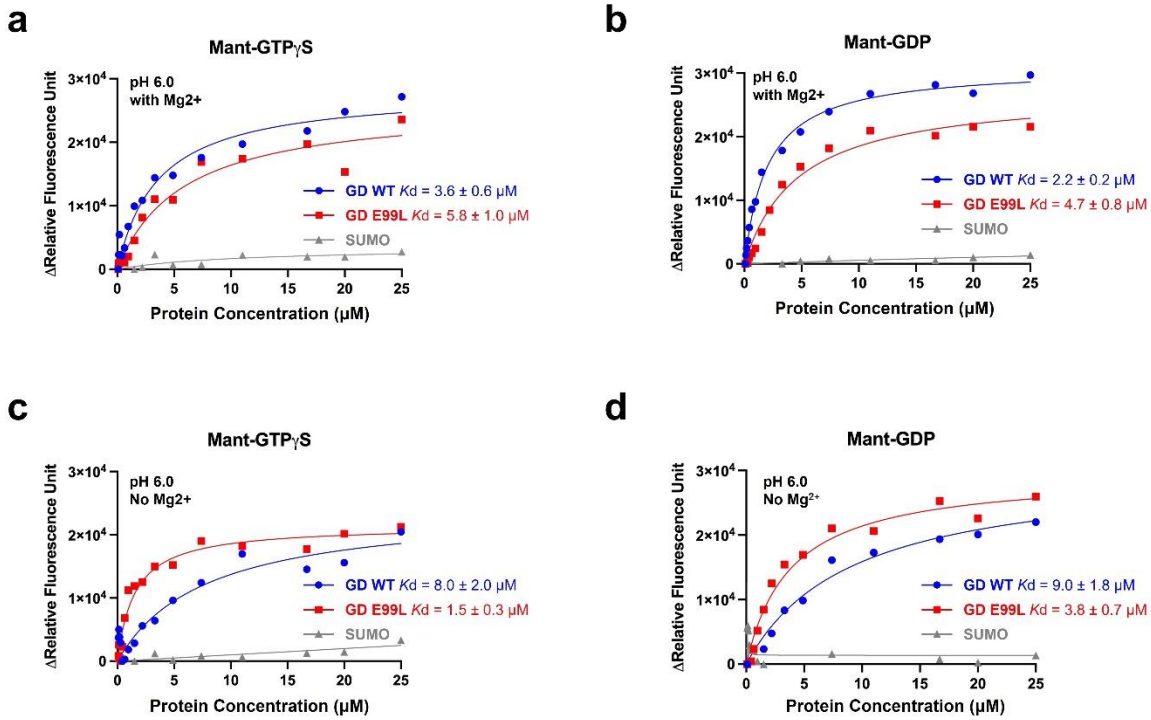
At both pH 8.0 and 6.0, WT protein binding to GDP is weaker in the absence of Mg2+ than in the presence of Mg2+, indicating removal of Mg2+ weakens the interaction between WT and GDP. In contrast, the E99L mutant bound to GDP with comparable affinities with or without Mg2+ at pH 6.0. The differential behaviors of WT and E99L proteins support the hypothesis that after Mg2+ leaving the catalytic site, the charge repulsion between E99 sidechain and β-phosphate favors the release of GDP.

We have included the new results and revised the manuscript in Results (lines 251-270) and Discussion (lines 395-399).

"It is perplexing that Mg2+ is critical for GTP or GDP binding (Supplementary Figure 5c and 5d), yet we do not observe any Mg2+ in the GBP2GD·GDP crystal structure (Figure 2b and 2d). GBP2GD·GDP crystallized at pH ~ 6.0 (Supplementary Figure 5e), prompting us to examine the behaviors of GBP2GD under acidic conditions. In the presence of Mg2+, GBP2GD WT binding affinities to GTP and GDP at pH 6.0 are comparable to those at pH 8.0 (Figure 3c, 3f, Supplementary Figure 5f, 5g, and 5i). The E99L mutant, however, bound GTP and GDP with elevated affinities at pH 6.0 than at pH 8.0 (Figure 3c, 3f, Supplementary Figure 5f, 5g, and 5i). While Mg2+ is essential for stable GTP and GDP binding at pH 8.0, at pH 6.0, both GBP2GD WT and E99L bound guanine nucleotides with detectable affinities even in the absence of Mg2+ (Figure 3e, 3g, Supplementary Figure 5h). The new set of nucleotide binding results clearly demonstrated that in solution, GBP2GD can bind to GDP in the absence of Mg2+ under acidic conditions, consistent with our observation in the crystal structure. At pH 8.0 and

6.0, GBP2GD WT binding to GDP is weaker in the absence of Mg2+ than in the presence of Mg2+ (Figure 3e-3f, Supplementary Figure 5b, 5d, 5g, and 5i), indicating removal of Mg2+ weakens the interaction between GBP2GD and GDP. In contrast, the E99L mutant binds to GDP with comparable affinities with or without Mg2+ at pH 6.0 (Figure 3e, 3g, Supplementary Figure 5g, 5i). The differences between WT and E99L proteins in GDP binding in the absence of Mg2+ suggest that E99 promotes GBP2 activity by facilitating GDP release after Mg2+ leaving the catalytic site. “

“The differential behaviors of WT and E99L proteins in binding GDP in the presence and absence of magnesium ions further corroborate the hypothesis that after the magnesium ion leaves the catalytic site, the charge repulsion between E99 sidechain and the β-phosphate favors the release of GDP (Figure 3e-g, Supplementary Figure 5b, 5d, 5g, and 5i).”



e

Kd (μM)	pH 8.0, + Mg2+	
	GTPγS	GDP
GBP2GD WT	3.0 ± 0.5	2.2 ± 0.6
GBP2GD E99L	16.0 ± 3.0	8.0 ± 2.0

f

Kd (μM)	pH 8.0, - Mg2+	
	GTPγS	GDP
GBP2GD WT	N.D.	N.D.
GBP2GD E99L	N.D.	N.D.

g

Kd (μM)	pH 6.0, + Mg2+	
	GTPγS	GDP
GBP2GD WT	3.6 ± 0.6	2.2 ± 0.2
GBP2GD E99L	5.8 ± 1.0	4.7 ± 0.8

h

Kd (μM)	pH 6.0, - Mg2+	
	GTPγS	GDP
GBP2GD WT	8.0 ± 2.0	9.0 ± 1.8
GBP2GD E99L	1.5 ± 0.3	3.8 ± 0.7

Figure 1 Characterization of nucleotide binding capacities of GBP2GD and E99L mutation at pH 6.0 and 8.0. (**a**-**b**) Comparison of GTPγS (**a**) and GDP (**b**) binding affinities of GBP2GD WT (blue) and E99L mutant (red) at pH 6.0 in the presence of Mg2+. (**c**-**d**) Comparison of GTPγS (**c**) and GDP (**d**) binding affinities of GBP2GD WT (blue) and E99L mutant (red) at pH 6.0 in the absence of Mg2+. The graph shown in (**a**-**d**) is representative of at least two independent experiments. SUMO (gray) is included as a negative control. (**e**-**h**) Summarization of GTPγS and GDP binding affinities of GBP2GD WT and GBP2GD E99L at pH 8.0 or pH 6.0 in the presence or absence of Mg2+.

Some other minor issues:

1. Abstract

Binding of GDP induces a distinct closed conformation in the G motifs and changes in

the distal regions, which are further transmitted to the C-terminal helical domain, leading to large-scale conformational rearrangements.
It has not been shown in this manuscript that an observed structural changes in the active site are further transmitted to the C-terminal helical domain, leading to large-scale conformational rearrangements. This statement is confusing for the current paper, please reformulate.

We have revised the sentence to “Comparison between the nucleotide-free full-length GBP2 structure with homologous structures reveal notable movement in the C-terminal helical region, along with conformational changes in the G domain.” (lines 27-30)

2. Figure 5: The new figure needs higher magnification into the catalytic site for each nucleotide-loading state, including some labeling of important elements and highlighting the molecular changes (for example, ‘Glu99 in’ vs ‘Glu99 out’ etc).

We thank the reviewer’s suggestion. Accordingly, we revised Figure 5a (below), increasing the magnification at the catalytic site. We also labeled the E99 orientation as “in” or “out”. (lines 830, 834-835)

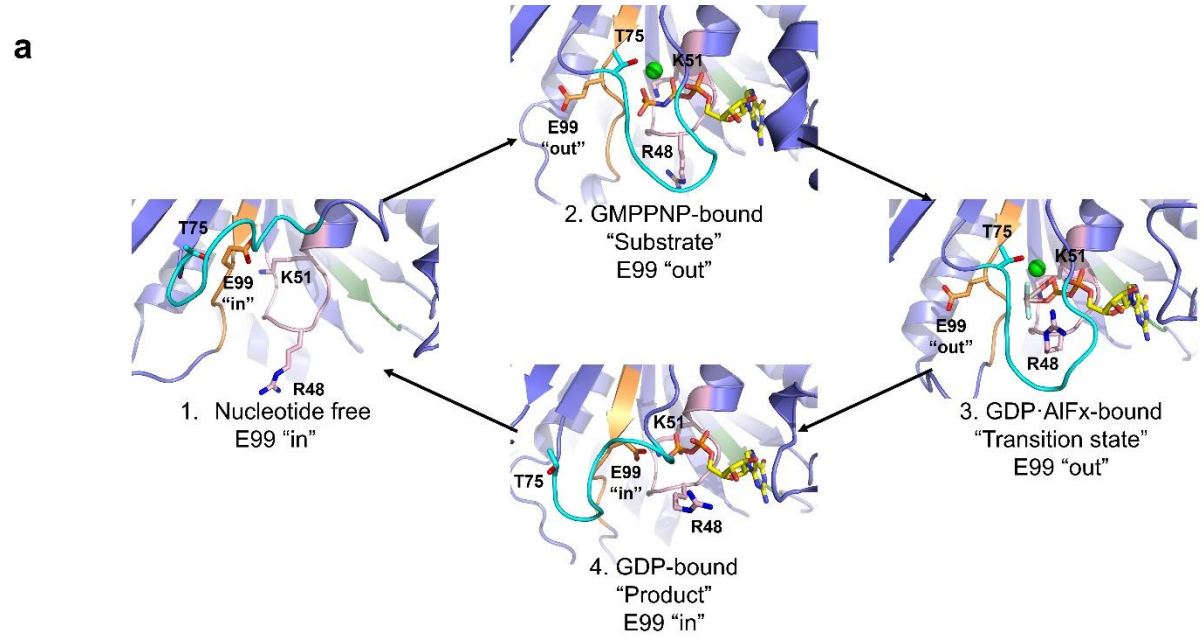


Figure 2 (Figure 5a in manuscript) Revised conformational changes at GBP2 catalytic site, highlighting the movements of key residues.

3. Figure S1 needs higher resolution.

We have updated Figure S1 to a higher resolution.

4. Figure S2B-D: Please report the elution volume, not the retention time, for all three graphs to allow a direct comparison with Figure S2A.

In Figure S2B-D, the size-exclusion chromatography coupled multi-angle light scattering (SEC-MALS) experiments were carried out at SIBYLS beamline at Advanced Light Source in Berkeley, California. At SIBYLS, separation of analytes is typically accomplished using an Agilent 1260 series HPLC with Shodex analytical column (30 cm * 8 mm, particle size 5 μm) at a flow rate of 0.65 ml/min. In Figure S2A, we analyzed the elution positions at home, using the AKTA pure system with a Superdex 200 10/300 column (30 cm * 10 mm, particle size 8.6 μm). As the size-exclusion columns used in Figure S2A and Figure S2B-D are of different brands and parameters, we cannot directly compare the elution volumes of the protein samples. We believe the differences in retention times, hence the elution volumes, of the three GBP2 samples (Figure S2B-D) and the molecular masses summarized in Figure S2E unambiguously state that full-length GBP2 dimerizes in the presence of GDP·AlFx but not GMPPNP.

5. Supplementary Video: In principle, this video could be neat and helpful if the transitions between the two states were more slowly, with short breaks and clear labeling of the individual nucleotide-loading states.

We thank the reviewer for their suggestions. In the updated video we paused the video at each state and slowed down the transition between states. We also labeled the four key residues: R48, K51, T75, and E99.

6. Line 251 GBP2GD activity remained the same in the presence of increasing concentration of GBP2CHD (Supplementary Figure 5f), suggesting it does not affect the GTPase activity in trans.
Maybe better: suggesting that the isolated GBP2CHD does not interact with the GBP2GD to affect the GTPase activity in trans.

We thank the reviewer's suggestion. We have changed the sentence as suggested. (lines 275-277)

Reviewer #3 (Remarks to the Author):

For clarity and brevity, in the responses below we refer the manuscript text after first round review and revision as "R1" and the current text after second round review and revision as "R2".

1. Line 47. Grammatical error

We have revised lines 45-47 to “Among the thousands of genes induced by interferons is a prominent family of GTPases, which accounts for twenty percent of the total population of interferon-stimulated genes.” (lines 45-47)

2. Line 34-35, may facilitate the development of targeted therapeutic strategies against intracellular pathogens. I think it is an overstatement.

We have rephrased it to “open avenues for exploring how the unique functions of GBPs could be leveraged to combat pathogen invasion.” (lines 34-35)

3. Line 59, GBPs can homodimerize via their G domains. It is not mentioned that the intermediate region in the G domain of GBPs plays an important role in dimerization because, in the absence of the intermediate region, the catalytic domain cannot dimerize. It is already known in GBP1 and GBP2 and references should be provided.

We have revised the sentence to “Like dynamins, GBPs can homodimerize via their G domains -- including both the small GTPase like region and the intermediate region -- in a parallel fashion [9-11].” (lines 59-60). We add the citation Ref #11 PMID 20923658 in R2 text (Abdullah N, Balakumari M, and Sau AK. "Dimerization and its role in GMP formation by human guanylate binding proteins." *Biophys J*. 2010; 99(7):2235-2244.)

4. Line 66, GBP1-mediated GMP production is also essential for anti-HCV activity. This is not stated and no reference is given.

We added the description “GBP1-mediated GMP production is also essential for its anti-HCV activity [15].” (lines 67-68) We add the citation cited Ref #15 PMID 27071416 in R2 text (Pandita E, et al. Tetrameric assembly of hGBP1 is crucial for both stimulated GMP formation and antiviral activity. *Biochem J.* 2016; 473(12):1745-1757.)

5. Line 97, GBP2 is one of the two most highly induced GBPs 33 yet it is not well studied as GBP1 or GBP5. This statement is not entirely true. There are four papers published on GBP2 including Febs Letter, 1996. However, two papers were published on GBP5.

Most of the publications on GBP proteins focused on their biological roles. In this sense, GBP5 is better studied than GBP2, especially among early publications.

Below is an excerpt of publications on GBP5 and GBP2 biological roles:

GBP5

1. Fellenberg F, et al. "GBP-5 splicing variants: New guanylate-binding proteins with tumor-associated expression and antigenicity." *J Invest Dermatol*. 2004; 122(6):1510-7.
2. Rupper AC and Cardelli JA. "Induction of guanylate binding protein 5 by gamma interferon increases susceptibility to Salmonella enterica serovar Typhimurium-induced pyroptosis in RAW 264.7 cells." *Infect Immun*. 2008; 76(6):2304-15.
3. Shenoy AR, et al. "GBP5 promotes NLRP3 inflammasome assembly and immunity in mammals." *Science*. 2012; 336(6080):481-5.
4. Krapp C, et al. "Guanylate binding protein (GBP) 5 is an interferon-inducible inhibitor of HIV-1 infectivity." *Cell Host Microbe*. 2016;19(4):504-14.
5. Feng J, et al. "Inducible GBP5 mediates the antiviral response via interferon-related pathways during influenza A virus infection." *J Innate Immun*. 2017; 9(4):419-435.
6. Li Z, et al. "GBP5 is an interferon-induced inhibitor of respiratory syncytial virus." *J Virol*. 2020; 94(21):e01407-20.
7. Haque M, et al. "Regulation of synovial inflammation and tissue destruction by guanylate binding protein 5 in synovial fibroblasts from patients with rheumatoid arthritis and rats with adjuvant‐induced arthritis." *Arthritis Rheumatol*. 2021; 73(6):943-954.
8. Yu X, et al. "GBP5 drives malignancy of glioblastoma via the Src/ERK1/2/MMP3 pathway." *Cell Death Dis*. 2021; 12(2): 203.
9. Yao X, et al. "Whole blood GBP5 protein levels in patients with and without active tuberculosis." *BMC Infect Dis*. 2022; 22(1): 328.
10. Ding K, et al. "GBP5 promotes liver injury and inflammation by inducing hepatocyte apoptosis." *FASEB J*. 2022; 36(1): e22119.

GBP2

1. Zhang J, et al. "Guanylate-binding protein 2 regulates Drp1-mediated mitochondrial fission to suppress breast cancer cell invasion." *Cell Death Dis*. 2017; 8(10): e3151.
2. Yu S, et al. "GBP2 enhances glioblastoma invasion through Stat3/fibronectin pathway." *Oncogene*. 2020; 39(27):5042-5055.
3. Ji G, et al. "GBP2 is a favorable prognostic marker of skin cutaneous melanoma and affects its progression via the wnt/β-catenin pathway." *Ann Clin Lab Sci*. 2021; 51(6):772-782.
4. Wang H, et al. "Subtyping of microsatellite stability colorectal cancer reveals guanylate binding protein 2 (GBP2) as a potential immunotherapeutic target." *J Immunother Cancer*. 2022. 10(4):e004302.

5. Ye S, et al. "GBP2 promotes clear cell renal cell carcinoma progression through immune infiltration and regulation of PD-L1 expression via STAT1 signaling." *Oncol Rep*. 2023; 49(3):1-14.

6. Line 100, While GBP2 is able to produce GMP as the end product, the efficiency is much less. I think a possible molecular basis of lower GMP formation in this GTPase has been reported. This is not described and no reference is given.

We added the sentence “This low efficiency has been attributed to sequence differences in GBP2 GTP-binding region.” (lines 104-105) The reference was already cited in R1 text as Ref #35 PMID 31199074 Rajan S, et al, *FEBS J*. 2019; 286(20): 4103-4121. In the current R2 version, the refence is #37.

7. Line 191, We then compared GBP2GD·GDP and GBP1GD·GMP structures to examine whether the product-bound structures of… This should have been done with the full-length protein with nucleotide. Otherwise, it may not give a complete picture.

We agree with the reviewer that it is more informative to compare structures of full-length protein bound with different nucleotides. However, there is no full-length GBP structure in complex with GDP or GMP, so we resort to the available GBP2GD·GDP and GBP1GD·GMP structures. We have added one sentence in Discussion “Structures of full-length GBP2 bound to various guanine nucleotides or functional mutants are needed to validate our observations of the GBP2 G domain and to fully elucidate the conformational changes occurring through the GTP hydrolysis cycle.” (lines 417-419)

8. Line 214, Together, conformational rearrangements in switch I and switch II regions dictate that once GTP is hydrolyzed to GDP, GBP2 can no longer form dimer. If so, why some amount of GMP is formed, because GMP formation is absolutely dependent on dimerization.

We have rephrased the sentence to “Together, conformational rearrangements in switch I and switch II regions dictate that once GTP is hydrolyzed to GDP, the majority of GBP2 can no longer form dimer.” (lines 218-219)

9. Line 227, We are particularly interested in the G3 motif residue E99 since its negative charge is positioned right next to the β-phosphate in the crystal structure (Figure 2d). This is based on the truncated protein structure with GDP. Thus, it may not give a clear picture in the absence of the full-length protein structure.

We agree with the reviewer that a structure of full-length protein in complex with GDP or other guanine nucleotides will reveal more information regarding GBP conformations. We added the following sentence in Discussion “Structures of full-length GBP2 bound to various guanine nucleotides or functional mutants are needed to validate our observations of the GBP2 G domain and to fully elucidate the conformational changes occurring through the GTP hydrolysis cycle.” (lines 417-419)

10. Line 330, On the opposite side, comparison of the two GBP2 structures reveals conformational changes in the α3-α3’ linker and α4’ region (Supplementary Figure 9e), which likely contribute to the movement of the C-terminal helical domain. This may be a correlation. Unless the study is done with a mutant which is defective in this process, one cannot make this conclusion.

We believe the reviewer refers to line 320 in R1 text. And we agree with the reviewer that the current observation may be a correlation. We added the following sentence in Discussion “Structures of full-length GBP2 bound to various guanine nucleotides or functional mutants are needed to validate our observations of the GBP2 G domain and to fully elucidate the conformational changes occurring through the GTP hydrolysis cycle.” (lines 417-419)

11. Line 336, we reason that the C-terminal helical domain does not allosterically regulate GD activity. This has already been reported in GBP2, however, it is not discussed and no reference is provided.

We believe the reviewer referred to the reference PMID31199074 Rajan S, et al, *FEBS J*. 2019; 286(20): 4103-4121. If so, this reference was cited in R1 version as Ref #35. In the current R2 version it is Ref #37. As studying the role of CHD is not the focus of this manuscript, we believe that additional discussion on CHD could shift the focus and potentially undermine the emphasis on our main findings.

12. Line 343, we propose that the captured crystal structure represents the second to last step in GTP hydrolysis – magnesium ion already released, but GDP remains binding (Figures 5a). This statement would be okay if the structure of full-length GBP2 with GDP had been done.

We agree with the reviewer that a structure of full-length protein in complex with GDP or other guanine nucleotides will reveal more information regarding GBP conformations. We added the following sentence in Discussion “Structures of full-length GBP2 bound to various guanine nucleotides or functional mutants are needed to validate our

observations of the GBP2 G domain and to fully elucidate the conformational changes occurring through the GTP hydrolysis cycle." (lines 417-419)

13. Line 385, it is likely that CHD has a regulatory role only in full-length GBP proteins. The CHD of GBP1 has a regulatory role in the GTP hydrolysis resulting in increased GMP formation, reported much earlier (I think JMB, 2009), yet not mentioned.

We believe that the reviewer referred to the reference PMID 19150356: Abdullah N, et al. *J Mol Biol*. 2009; 386(3): 690-703. We have added the reference as Ref #47. (line 415).

14. Line 466, Why the mobile phase does not have the nucleotide? In its absence, the complex is likely to be dissociated.

If the mobile phase refers to the running buffer of FPLC, it is true that the running buffer does not include any nucleotides. However, Figure 1c, 1d and Supplementary Figure 2a clearly demonstrated that under the same conditions (overnight incubation of GBP2 protein with specified nucleotide, no nucleotide in FPLC running buffer), GBP2GD forms dimer with GMPPNP but GBP2FL does not; similarly, overnight incubation with GDP·AlFx induces full dimerization of GBP2FL but less than half of GBP2GD. Therefore, our conclusion is that GBP2FL has a higher propensity to dimerize in the presence of GDP·AlFx than GBP2GD, while GBP2GD has a higher propensity to dimerize in the presence of GMPPNP than GBP2FL.

In the previous R1 round of revision, we addressed the issue per Reviewer #2's request (Supplementary Figure 2a). Even with 100 μM GMPPNP in the running buffer, the majority of GBP2FL remains monomeric, further reinforcing our conclusion that GBP2FL remains monomeric in the presence of GMPPNP.

15. Was the activity assay performed with the SUMO tag protein? Should be clarified in the results section.

All activity and binding assays were performed with tag-free proteins. We have described the protein purification steps in Materials and Methods: *Protein expression and purification*. We added this information in Results: *Mutation of G motif residues abrogated GTPase activity of GBP2* "All proteins used for activity assays are tag-free." (line 226).

16. Can the authors discuss which residues are important for the first and second steps

of GTP hydrolysis in the context of the present data since the protein has both GTPase and GDPase activity?

In this manuscript, we reported our findings on the structures of GDP-bound GBP2GD and nucleotide-free GBP2. Based on structure-guide mutagenesis and enzymatic assays, we identified K51, R48, and E99 as key residues for GTP hydrolysis and further investigated the role of E99 in detail. Malachite green assay does not differentiate between GDP and GMP productions. Under our assay conditions, HPLC analysis did not detect discernible levels of GMP (See response to Question 6 in R1 response and Question 20 in this R2 response). At this stage, we do not have sufficient data to hypothesize on the residues responsible for the individual steps in GTP hydrolysis. We believe that additional discussion without supporting data could shift the focus and potentially undermine the emphasis on our main findings.

17. In the GTPase assay, shown in Fig 3 a-b, what are the concentrations of enzyme and GTP used?

In Figure 3a-b, the GBP2 concentration is 500 nM for GBP2 FL and 300 nM for GBP2GD. GTP concentration is 200 μM. The information has been reported in Materials and Methods: *GTPase activity assay*. (lines 533-534)

18. GD WT and GBP2 FL WT, there is an 8-fold difference of Kd for the substrate binding affinity. Will it have any impact on the activity?

We did not carry out any substrate binding assay using GBP2FL WT. The substrate binding assay is carried out with GBP2GD WT and E99L mutant (Figures 3c, Suppl Figure 3c, 3f, and 3h).

19. In SEC data Fig d, is it possible GBP2 GD can be completely converted into a dimer if the GDP.ALFx is used with a higher concentration? Also, the mobile phase would have the same concentration of GDP.ALFx.

It is possible that increasing GDP·AlFx concentration can convert GBP2GD completely into a dimer. However, in our assay, GBP2GD was incubated overnight with 10-fold excess of nucleotide (20 μM protein vs 200 μM GDP·AlFx). We observed excessive GDP·AlFx eluted later in the SEC profile (Figure 3 below, the peaks at ~ 21 mL), arguing against the possibility that the GDP·AlFx concentration is insufficient to induce full dimerization of GBP2GD. Second, when incubated with the same concentration of GMPPNP (200 μM), almost all GBP2GD forms dimer, indicating at that concentration, GBP2GD can be fully converted to dimer with the proper nucleotide. Third, under that same condition – overnight incubation with 200 μM GDP·AlFx and no additional

GDP·AlFx in the FPLC running buffer, more than 90% of GBP2FL forms dimer (Figure 1c), while less than half of GBP2GD forms dimer. With the three lines of evidence, our conclusion is that GBP2FL has a higher propensity to dimerize in the presence of GDP·AlFx than GBP2GD.

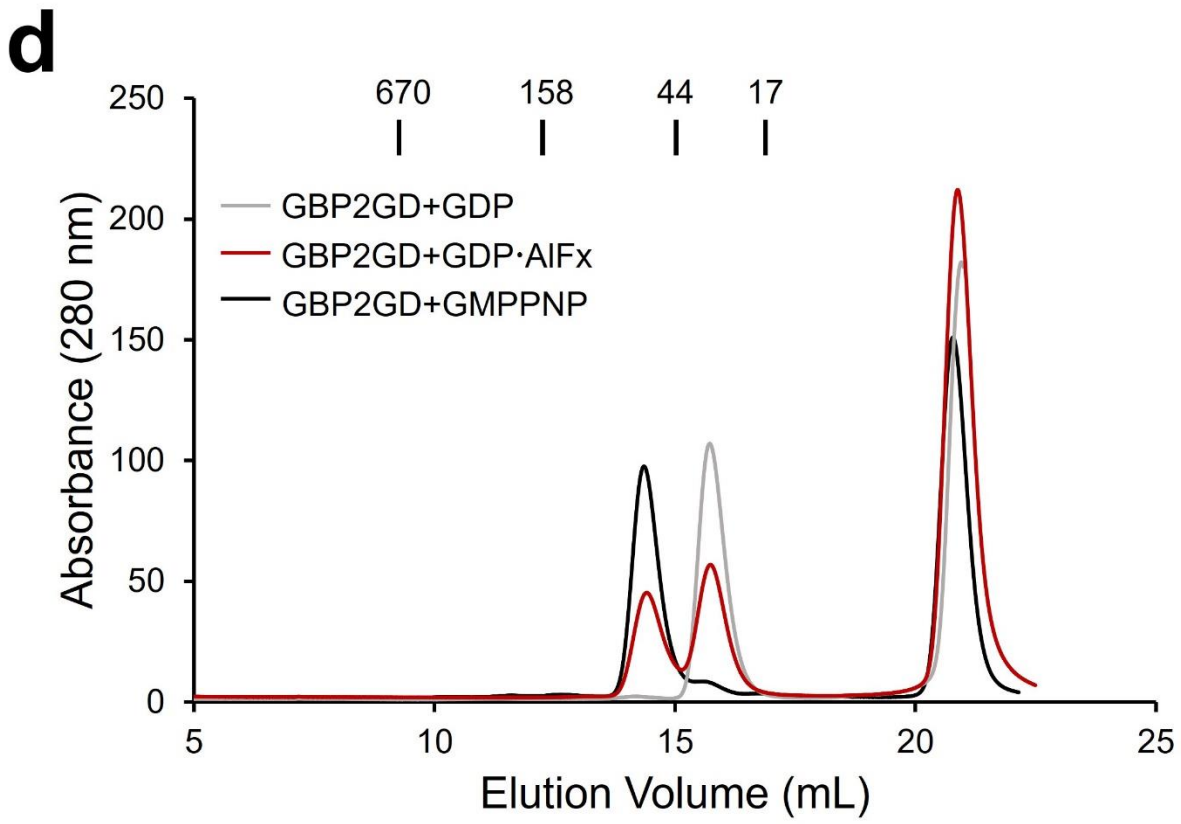


Figure 3 SEC profiles of GBP2GD incubated with GDP (gray), GDP·AlFx (red), and GMPPNP (black) from a Superdex 200 10/300 column. The profiles are extended to include the excessive guanine nucleotide peak at ~ 21 mL.

20. In the response, there is something wrong in Fig 12b. The GDP elution matches with the standard, but GMP does not. Moreover, how GMP became significant in GBP2-mediated GTP hydrolysis.

In the R1 response Fig 12B GBP2-mediated GTP hydrolysis products (Figure 4 below), we did not observe any GMP peak. The GMP standard peak is at ~ 3 min, while the peak we believe the reviewer referred to (now marked with a star *) eluted at ~ 3.8 min. The star-marked peak comes from the reaction buffer. It is also present in all three GTP/GDP/GMP standard traces (Figure 12A/4A below).

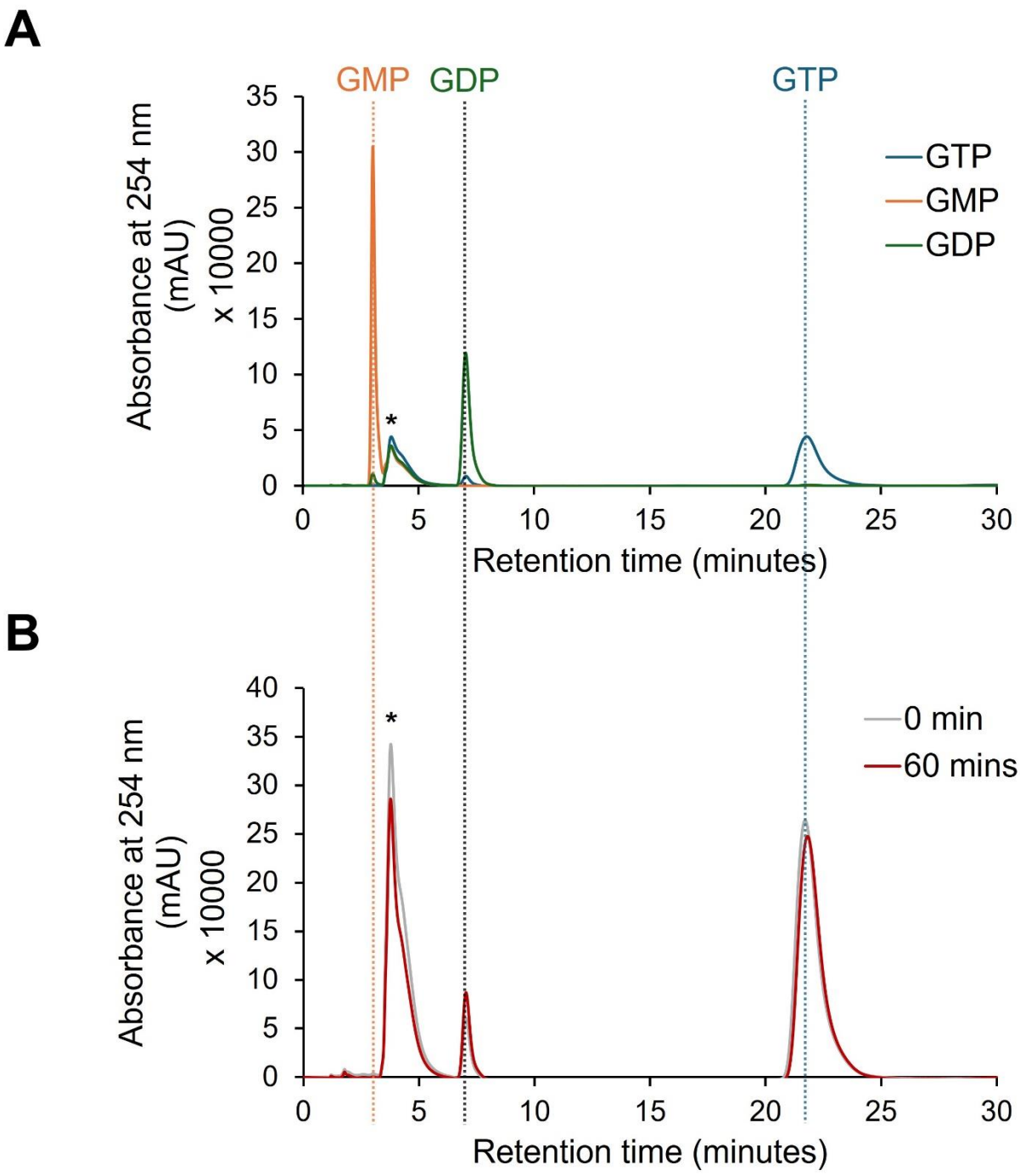


Figure 4 (Figure 12 in R1 response) HPLC analysis of GBP2-mediate GTP hydrolysis. (A) Retention times of GTP (blue), GDP (green), and GMP (orange) standards. (B) Retention times of GBP2 mixed with GTP at reaction time 0 (gray) and 60 min (red). No GMP is detected at 60 min. * marks the impurity peak from solutions.

21. In response to Q7, I checked literatures, S73A mutation has been studied in GBP1,

GBP2 and GBP5. This mutation in these three proteins has no or little effect on GTP hydrolysis. Since the study has already been reported, appropriate references should be given and discussed accordingly.

In R1 manuscript, we did cite literature reporting S73A function in hGBP1 and hGBP5 (Refs 9-10). In the current R2 version, we further added PMID 37042791: Mittal M, et al. *Biochemistry*. 2023; 62(9):1509-1526 as Ref #39.

As the S73A mutation does not affect hGBP2 activity, we focused on mutations that do affect hGBP2 activity, such as R48A, K51A, and in particular E99D/L/Q. We believe that additional discussion on the S73A mutation could shift the focus and potentially undermine the emphasis on our main findings.

22. Ideally, the structure of the K51 mutant of GBP2 should have been compared with the structure of GppNHp-bound GBP2 or GDP-bound GBP2. This would have been more informative in the context of the sequence of events during GTP hydrolysis.

We agree with the reviewer that the ideal situation would be comparing structures of full-length GMPPNP-bound and GDP-bound GBP2. As such structures have not been reported in any structure depository at the moment, we can only base our comparison on available structures: nucleotide-free full-length GBP2 (PDB ID 7E58), GMPPNP-bound full-length GBP1 (PDB ID 1F5N), and nucleotide-free farnesylated full-length GBP1 (PDB ID 6K1Z) (Figure S8a-c, Refs #10, 29, and 31). In Discussion, we added the sentence “Structures of full-length GBP2 bound to various guanine nucleotides or functional mutants are needed to validate our observations of the GBP2 G domain and to fully elucidate the conformational changes occurring through the GTP hydrolysis cycle.” (lines 417-419)

23. The authors should discuss how the K51-E99 contact in the GDP-bound GBP2 structure affects the switch I loop containing the catalytic residue/machinery.

We have added the following sentence in Discussion “The K51-E99 interaction is closely linked to the repositioning of the switch I loop. When K51 and E99 interact, the switch I loop shifts outward, displacing T75 in the G2 motif away from the catalytic site. Disruption of this interaction allows the switch I loop to close in, positioning T75 to coordinate the magnesium ion essential for GTP binding and hydrolysis.” (lines 424-429)

Editor's request:
(1) Add sentence concerning absence of Mg2+ as suggested by R2
(2) Mention available structure of nucleotide-free GBP2 FL published in PNAS in 2021 in the introduction when describing current structural knowledge of GBP members.
(3) Authors should make clear that the GBP2 FL structure solved in the present manuscript is the K51A point mutant. Please avoid describing it as simply the GBP2 FL, instead you can use GBP2-K51A FL for instance, including in the heading of the results section.

(1) We have added a sentence in the Discussion section further emphasizing the absence of Mg2+ as suggested by R2. "Magnesium ions only moderately increased GTP or GDP binding affinity to GBP2GD at acidic pH (Figure 3e, 3g, Supplementary Figure 5f-i); the crystal lattice may have further stabilized the magnesium-free environment, explaining the absence of magnesium ions in the active site in the crystal structure." (lines 370-374).

(2) We have updated the Introduction section to include the structure of full-length, nucleotide-free GBP2 published in 2021. "The only reported GBP2 structure is the full-length, nucleotide-free GBP2 structure published in 2021 [10]." (lines 99-100)

(3) We have clarified the name of the structure as suggested in Abstract and the section title. Now the structure is described as "nucleotide-free full-length GBP2 with K51A mutation" (lines 25, 27, 281).

REVIEWERS' COMMENTS:

Reviewer #2 (Remarks to the Author):

The authors have adequately addressed my raised concerns and add new mechanistic insights which neatly contribute to the story. In my view, the manuscript is ready for publication.

We sincerely thank the reviewer for their thoughtful evaluation of our work. We are pleased that the revisions have addressed the reviewer's concerns, and that the manuscript is now deemed ready for publication. Thank you for your time, effort, and valuable feedback, which helped improve the clarity and impact of our study.

R2
Very minor:
One sentence could be added to the discussion why no Mg2+ was found in the crystal structure, e.g. something like 'Mg2+ only moderately increased affinity at acidic pH; the crystal lattice may have stabilized the Mg2+-free conformation, explaining the absence of Mg2+ in the active site in the crystal structure.'

The following sentence has been added to the discussion section:

"Magnesium ions only moderately increased GTP or GDP binding affinity to GBP2GD at acidic pH (Figure 3e, 3g, Supplementary Figure 5f-i); the crystal lattice may have further stabilized the magnesium-free environment, explaining the absence of magnesium ions in the active site in the crystal structure." (lines 370-374).

Reviewer #3:
Remarks to the Author:
1. Abstract (Line 426): The abstract incorrectly states that the authors determined the crystal structure of nucleotide-free full-length GBP2. Instead, they determined the structure of the nucleotide-free GBP2 K51A mutant. However, they have considered it a nucleotide-free full-length GBP2 structure, which is misleading and should be corrected.

If the reviewer is referring to the Abstract: line 25, we have clarified in Abstract that the structure we reported is "nucleotide-free full-length GBP2 with K51A mutation ($GBP2^{K51A}$)".

2. Literature Context (Line 104): The statement made in the manuscript is not accurate in the context of recent literature. This should be corrected and appropriately cited.

If the reviewer is referring to the basis of lower GMP production by GBP2, we have already cited Ref #37 that the sequence differences in the GTPase domain may contribute to the disparity of GMP production between GBP1 and GBP2.

3. Beta Phosphate Coordination (Line 157): The manuscript states that the beta phosphate is coordinated by the side chain of E99. Given that both are negatively charged, this interaction is unlikely and needs clarification. The authors should provide a detailed explanation.

We understand the concern of the reviewer regarding the apparent charge incompatibility between the β phosphate and the E99 side chain. The same concern has been raised by Reviewer 2 and addressed by us in the previous rounds of revisions. First, the negative charges on the β phosphate and the E99 side chain can be partially offset by the surrounding hydrogen bonding network, including K51, to mitigate the repulsion. Second, the repulsion is further reduced under the crystallization conditions (~ pH 6.0, Supplementary Figure S5e), where the side chain of E99 may be partially protonated, reducing the negative charge on E99 side chain. As suggested by Reviewer 2, we carried out comprehensive binding studies at pH 6.0. The binding results, summarized in Figure 3e, 3g, and Supplementary Figure 5f-i, clearly demonstrated that at acidic pH, GBP2GD binds to GTP or GDP with measurable affinities even in the absence of Mg2+, corroborating the observed interaction in the GBP2GD·GDP structure. Lastly, the close proximity between the β phosphate and the E99 side chain is consistent with the orientation of the α phosphate and the E99 side chain in the GBP1GD·GMP crystal structure (PDB ID: 2D4H, PMID: 16511497), suggesting a common mechanism between GBP1 and GBP2.

4. Heading Misrepresentation (Line 279): The heading inaccurately implies that the authors determined the crystal structure of the nucleotide-free full-length GBP2. This should be revised to reflect the actual structure studied (nucleotide-free GBP2 K51A mutant). In Fig 1b, can they clarify why the elution volume of CHD is lower than that of the GD because they have similar molecular masses?

We have changed the section title to “Crystal structure of nucleotide-free full-length GBP2 with K51A mutation reveals structural plasticity of CHD” (line 281).

The reviewer is correct that GBP2GD and CHD have similar molecular masses (34.8 kD vs. 32.9 kDa). While GD is globular, CHD assumes an elongate conformation, leading to larger gyration radius and its earlier elution position.

5. K51-E99 Contact: Based on the truncated GBP2 protein structure (devoid of the helical domain), the authors propose the presence of the K51-E99 contact. However, they did not measure the distance between their side chains in the full-length protein, which is critical to validating this claim. This is not described in the manuscript.

We understand the reviewer’s concern regarding the interaction between K51 and E99. Given the well-known large scale conformational changes in and around the active site of GTPases, it is natural that certain interactions are only observed in one nucleotide-binding state but not in others. Per our analysis of available GBP2 (and GBP1) structures through the GTP binding and hydrolysis cycle, K51-E99 contact formation is unique in the GDP-bound form, displacing switch I region and the T75 residue from the active site. The active site of GTPases is known to be flexible in the nucleotide-free state. Measuring the distance between K51 and E99 in the full-length, nucleotide-free GBP2 structure does not validate or invalidate the K51-E99 interaction observed in GBP2GD in the GDP-binding state. Future structures of full-length GBP2 bound to GDP will validate our observations in the GBP2GD·GDP structure.

6. Choice of Comparison Structure: The authors used the GBP2 K51A mutant protein structure for comparison, citing low yields of the wild-type protein. However, the full-length WT GBP2 protein structure has already been reported by another group in PNAS 2021. This structure was neither acknowledged nor discussed. Since it is available, the distance between K51 and E99 should be estimated and described.

We have added the nucleotide-free full-length GBP2 structure in Introduction “The only reported GBP2 structure is the full-length, nucleotide-free GBP2 structure published in 2021 [10].” (lines 99-100).

We already included the nucleotide-free full-length GBP2 structure (PDB ID: 7E58) in comparison and discussion (lines 314-321, Supplementary Figures 8a and 9a). This structure lacks continuous electron densities and misses some key residues in the G2 and G3 motifs (also known as the switch I and switch II regions), making it unreliable as a reference for more detailed structural comparison. We have discussed the relevance of K51-E99 distance in Response to Q5 above.

7. Figure 5a Legend: The legend is unclear and misleading. The nucleotide-free GBP2 structure should be explicitly used for comparison, with the appropriate PDB ID provided. Additionally, the subsequent use of GMPPNP-bound GBP1 should be clearly stated. The diagram shown in Figure 5a depicts the protein returning to the nucleotide-free state after step 4. If so, the authors need to explain how this reconciles with the lower amount of GMP observed in GBP2.

We understand the reviewer's concern. However, the nucleotide-free full-length GBP2 structure (PDB ID: 7E58) misses considerable switch I and II regions (G60-T75, D103-D108), preventing reliable comparison of key conformations; while our nucleotide-free full-length GBP2 structure with K51A mutation (PDB ID: 7M1S) contains all switch I and II regions. We provided the PDB ID (1F5N) for the GMPPNP-bound structure and stated several times in text this is the GBP1·GMPPNP structure.

It has been reported by different groups that the major product of GBP2-mediated GTP hydrolysis is GDP, not GMP. Under our experiment conditions and HPLC detection, we detected abundant GDP production but not GMP (Revision R2 response). Based on our own observations, we propose that after magnesium ion is released, "The presence of GDP in the active site likely induces a low-energy conformation that precludes rebinding of magnesium ions. Such binding modes may explain the inability of GBP2 to hydrolyze GDP …" Eventually, GDP has to be released for the next cycle of GTP binding and hydrolysis.

8. Homolog Comparison: GBP2 and GBP1 are close homologs. The authors used GMPPNP-bound GBP1 to explain their data on GBP2 and provided a model. However, using this model, can they explain why GBP1, a close homolog of GBP2, yields a higher amount of GMP?

Although GBP1 and GBP2 share significant structural similarity, subtle differences in their active site residues and surrounding regions may account for the observed variations in GMP production. We have already included the sentence "This low efficiency has been attributed to sequence differences in GBP2 GTP-binding region [37]." (Lines 105-106).
Comparing GMP production by GBP1 or GBP2 is not the main focus of this manuscript, we believe further discussion on this disparity would shift focus and potentially undermine the emphasis on our main findings.

9. Role of E99: The manuscript does not explain how E99 plays an important role in GTP hydrolysis. Is E99 a catalytic residue? If so, its role should be explicitly detailed.

We dedicated a considerable portion of the section "Mutation of G motif residues abrogated GTPase activity of GBP2" to the importance of E99 (lines 233-272). Figure 3a and 3b demonstrated the key role of E99 in GTP hydrolysis; Figure 3c-g and Supplementary Figure 5b-d, 5f-i further demonstrate the role of E99 in guanine nucleotide binding and GBP2 dimerization.

10. There is an 8-fold difference in Kd for substrate binding affinity between GD WT and GBP2 FL WT. The authors should address whether this difference impacts enzymatic activity. This concern was raised in a previous review but has not been adequately addressed in the revised manuscript.

As addressed in Q18 by Reviewer 3 in the previous round of review, we never conducted any substrate binding assay using GBP2 FLWT. The substrate binding assay is carried out with GBP2GD WT and E99L mutant (Figure 3c, 3e-g, Suppl Figure 5b-d, 3f-i).