nature portfolio

Peer Review File

Functionally overlapping intra- and extralysosomal pathways promote bis(monoacylglycero)phosphate synthesis in mammalian cells

Corresponding Author: Dr Robert Zimmermann

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

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In this manuscript by Bulfon et al, the authors attempt to identify BMP synthesizing enzymes using limited screens of various lipid enzymes. They successfully identified various isoforms of PLA2 group IV as intracellular mediators of Hemi-BMP/BMP synthesis in vitro/intact cells as well as an endothelial lipase as an extracellular mediator of BMP synthesis based on in vitro and in vivo data.

BMP is emerging as a critical disease-relevant phospholipid in a growing number of diseases, including lysosomal storage disorders and late life neurodegenerative diseases. Its synthesis is known to be highly complex and to involve multiple enzymes. In this manuscript, the authors made significant progress in identifying some of the key relevant enzymes and make a strong case that one of the relevant pathways involves conversion of PG to hemi-BMP, which in turn is converted to BMP. This pathway may be a major alternative to the PG-to-LPG-to-BMP pathway which is believed to be the predominant one, at least in some instances. Interestingly, the key PLA2G4 isoforms identified act primarily as transacylases rather than hydrolases, and mediate Hemi-BMP synthesis. Generally the paper does a great job making a case that multiple enzymes and enzyme families are involved, acting either intracellularly or extracellularly. While the paper potentially represents a significant milestone in the field, there are a few points that need to be addressed to generally increase the impact of the work for the broad readership of Nature Comms. These are as follows

Main issues:

1. Lysosomal PLA2 (PLA2G15) was identified as one of the BMP synthesizing enzymes (PMID 36823305) and should be both discussed upfront in the Introduction and tested experimentally, to determine how it compares to the other PLA2 isoforms presented in the manuscript under consideration, particularly for the intracellular PLA2 isoforms like the group IV isoforms.

2. It seems that there may be some PLA2 isoform redundancy for hemi-BMP and BMP synthesis as well as hints of cell type specificity in the relative importance of various PLA2 isoforms vis-a-vis the synthesis of these lipids. The authors should perform knockdown (or knockout) of some of the key group IV isoforms in cell lines/types other than HEK293.

3. In the PG and other phospholipid supplementation experiments in Figure 1I, the authors should match the fatty acyl composition of PG/PE/PS to make sure that differences in fatty acyl composition between do not account for differences in functional outcome (like endocytosis or ILV formation); also, as mentioned below, the authors should mention what specific fatty acyl species are used in the main text. This also applies to the PG species used for the PG supplementation experiments in other parts of the manuscript, including in vivo studies.

4. More importantly, the evidence provided in Figure 1I does not demonstrate that PG triggers endocytosis; it could simply be taken up via constitutive endocytosis. Similarly, the LysoSensor phenotype is not easily interpretable. Is the PG treatment causing more acidification of lysosomes or a proliferation of lysosomes via lysosomal biogenesis. In 18 hours, this is entirely possible. If the authors want to demonstrate PG treatment (and BMP generation) affects the lysosomal pH, they should measure the actual pH and provide absolute values. Additionally, when providing microscopy images like those shown in

Figure 1I, it is critical to perform proper quantifications and data replication.
5. The EM analysis of ILVs in Suppl. Figure 1 E should also be supported by morphometry. It's not trivial, because vesicular compartments without ILVs may not be endolysosomal. Proper identification of the endolysosomal compartment at the EM level requires the presence of endocytic tracers like HRP, which can generate electron-dense material visible at the EM level. Since this is going to be a lot of work, I suggest exclusion of this data, and perhaps a substitution of this data with data from anti-BMP antibody stains showing potentially appearance of BMP in the endolysosomal compartment upon PG supplementation (which of course would have to be generated).

6. In Figure 2A, the authors use a limited screen of about 200 candidate mouse lipid enzymes overexpressed in HEK293 cells to identify the hemi-BMP synthesizing activities and both PLA2G4D and E came up as top hits. This is a powerful approach, but what evidence is there that all 200 mouse lipid enzymes are actually expressed? Do they have mRNA assessments showing proof of overexpression or tagged versions of the said 200 lipid enzymes to monitor actual overexpression at the protein levels? It looks like His tags are used for purification, so theoretically anti-His western blots can be performed for all their overexpressors (or use some other techniques to quantify protein expression).

7. For all the PLA2 isoforms identified as top hits (PLA2G4D, PLA2G4E, and LIPG), the authors also need to overexpress the catalytically dead mutant versions of the enzymes to determine if the Hemi-BMP or BMP-synthesizing activities are lost.

8. The Ca2+ sensitivity for PLA2Gx isoforms' transacylase activity is interesting. The authors should test whether modulation of intracellular Ca2+ levels affects Hemi-BMP and BMP levels in intact WT cells or cells lacking the key PLA2 group IV isoforms identified as Ca2+-sensitive. They can potentially use Ca2+ ionophores, or inducers of Ca2+ release from intracellular stores (e.g., ER, mitochondria or lysosomes). Since human PLA2G4B is Ca2+ sensitive, the corresponding KO in HEK293 cells may show a Ca2+ induced phenotype in intact cells, whereas the PLA2G4C KO may be less sensitive to Ca2+.

9. For the Lipg KO mouse study in Figure 5, it seems that measuring BMP levels in plasma is important, if the enzyme is actually secreted/extracellular.

10. An important gap in this manuscript is some demonstration that reducing BMP synthesis by knocking out/down the top PLA2G4 hits identified causes defects consistent with BMP deficiency. For instance, is there any evidence of lysosomal unesterified cholesterol (filipin), gangliosides or glucocerebrosidase substrate accumulation in the knockouts of PLA2G4B and C? This would dramatically increase the impact of the manuscript. Similarly, any cholesterol/ganglioside/GCase substrate accumulation in the tissues of the Lipg KO showing reducing BMP levels?

Minor comments:

11. As the authors present the literature on BMP, it's always helpful to indicate whether the key findings they refer to are described for cell-free studies, cell culture studies and/or in vivo studies. For instance, in lines 50-52, it's unclear whether the data they refer to is solely based on cell free studies or other types of studies. This is important because it addresses physiological relevance.

12. In addition to GRN and NPC disease biology, there is an emerging link between BMP and other neurodegenerative diseases, which should probably be cited to increase the significance of the manuscript. For instance, PMID 36854767 and 25653221 for LRRK2/Parkinson, PMID: 22134919 and 35351864 for APOE4 and Alzheimer's.

13. In the Intro paragraph starting with line 53, it's also helpful to mention the cell free work of Sandhoff and colleagues on GCase and SapC (PMID: 28126847)

14. In the same paragraph, it's important to present the role of BMP in egress of cholesterol from the endolysosomal system giving also credit to the seminal work of Gruenberg and colleagues and citing some of the primary literature on this topic (for instance, PMID: 10559883 and 18644787)

15. In lines 63-66, regarding the Grn mutant work, it's important to mention the in vivo data show a global deficiency of all BMP species analyzed in various tissues (e.g. brain, liver) and biofluids (plasma, CSF). Cell culture experiments, at least in Logan et al, suggests upregulation of specific species of BMP, like 18:1/18:1, in KO BMDMs, while PUFA species are downregulated. Therefore, cell culture Grn loss of function models do not always a global deficiency of all BMP species.

16. In line 67-68, the statement "it is unclear whether PGRN directly interacts with BMP or if it regulates its metabolism" is slightly inaccurate, as both studies Boland et al and Logan et al show PGRN binds directly to BMP in vitro. Perhaps the authors meant the following: "It is unclear if PGRN regulates BMP metabolism directly by modulating its stability via a physical interaction or whether it regulates BMP levels indirectly via modulation of BMP-metabolizing enzymes".

17. In PG supplementation experiments in Figure 1 (but also throughout the paper), the authors should consistently mention the specific fatty acyl composition of PG (regular or 13C-labeled) in the main text, and figure legends.

18. Line 466, the following statement "Hemi-BMP synthesis was most efficient at neutral pH and absent at pH values ≤ 5.5 (Figure 1F), indicating that hemi-BMP synthesis rather occurs at membranes facing the cytosol than within acidic organelles." can be complemented with another scenario, whereby hemi-BMP synthesis may be also occurring in the ectoplasmic leaflet of the plasma membrane, since the extracellular environment is also neutral, at least theoretically.

19. In Figures 1G and 1H (hemi-BMP and BMP formation), it seems that the time course could benefit from inclusion of additional time points between 2h and 24h to increase time resolution when the bulk of synthesis occurs.

20. Line 479, please indicate the duration of the PG treatment; it can help the reader interpret the effects on the endocytic compartment

21. Line 511, the six isoforms of group IV should include PLA2G4F in addition to PLA2G4A-E

22. In the Discussion, it would be beneficial to discuss findings from the CRISPR screen identifying regulators of BMP levels using an anti-BMP antibody (PMID: 34936700) and check whether any of the hits overlap with those from the manuscript under consideration. If not, perhaps discuss how different the approaches are.

23. The Discussion seems a bit long and could be a bit more concise.

Reviewer #2

(Remarks to the Author)
In this manuscript the authors present a series of experiments aimed at understanding the biosynthetic pathway for the lipid bis(monoacylglycerol)-phosphate, BMP. This is an important and intriguing topic, and the manuscript describes results from extensive experimentation. They performed screens of libraries of known (and related) hydrolase and transacylase enzymes using a glycerol labeled PG substrate, and identified two specific enzymes, PLA2G4D and PLA2G4E, which increase Hemi-BMP and, to a much lesser extent, increase BMP itself. Overall they provide additional support for previously proposed steps and also a number of additional insights into potential synthetic pathways for BMP. Nevertheless the overall conclusions are based largely on in vitro data with semipurified enzymes in cell free system, with the cellular and animal data not robustly supporting the proposed intra- and extra-cellular synthetic routes. The proposal that PG is first converted to hemi-BMP prior to hydrolysis to BMP is weakly supported by limited time course studies. Importantly, moreover, a recent publication has robustly identified a novel BMP synthase as the product of the CLN5 gene (doi: 10.1126/science.adg9288). Overall, the paper while interesting leaves many uncertainties and represents a somewhat modest advance in understanding of BMP biosynthesis. The following are specific comments.

1. A recent Science paper by Medoh et al. identifies the product of the CLN5 gene as the enzyme responsible for BMP synthesis, seemingly occurring directly from 2 molecules of PG. This is obviously very different from the biosynthetic scheme proposed here. As the present study used a target gene approach, the CLN5 was not identified. Given this new finding plus the fact that knockout of the PLA2G4D, PLA2G4E, and LIPG had only modest if any effects on BMP synthesis, it is uncertain that the intracellular and extracellular enzymes identified play key physiological roles in endogenous BMP biosynthesis.

2. While there are some interesting observations about BMP synthesis that are shown in this paper (i.e strong evidence that the process does not require breakdown and resynthesis of the glycerol-phosphate-glycerol backbone/headgroup; that acyl CoA is not a precursor for fatty acylation (supporting transacylation reaction), the level of solidly supported new information is modest.

3. The authors purport that endothelial lipase and members of the pancreatic lipase family (PLRPs) acting in the circulation are involved in BMP synthesis (extracellular pathway), in addition to intracellular pathway. While they show that these enzymes are capable of transacylation and hydrolytic activities, and indeed some of the PLRP's had been previously shown to use BMP as substrate, there is no evidence that these activities have physiological relevance. The authors' own data show virtually no PG substrate in serum of unsupplemented animals. Additionally, despite identification of endothelial lipase (LIPG gene) in the in vitro screen, no reduction in conversion of PG to hemi-BMP or BMP in circulation was found in LIPG knockout mice, and the reductions in tissue BMP levels in this mouse were small in the few tissues where decreases were found.

4. Kinetics of BMP and hemi-BMP formation does not strongly support precursorproduct relationship. Figure 1G and 1H y axes are almost 10-fold different for Hemi-BMP and BMP, making the time course of BMP formation look flat when it might not be. Further, although in the Expi cells about 50-fold more hemi-BMP is

formed compared to BMP, in the HEK cells the absolute amount of BMP is much greater than Hemi-BMP. Can the investigators discuss this cell-dependent discrepancy, and what it might indicate for precursor-product relationship?

5. If Hemi-BMP is the intermediate/precursor for BMP, it seems surprising that at time zero, prior to adding PG, there is little to no hemi-BMP in both cell types. This would imply no regulation of the conversion of hemi-BMP to BMP and hence of BMP level, perhaps surprising given its apparently important functions. This should be commented on.

6. Time course of product formation from PG should be followed using the stable isotope labeled PG. It may well be that Hemi-BMP is the major intermediate/precursor but the present data are not strong enough, especially with the inconsistencies between different “WT” cell lines.

7. pH dependence of the Hemi-BMP formation is shown to be 7.0 and the authors interpret this as showing that the reaction occurs facing the cytosol. These data were generated using cell lysates. What is the pH dependence after PG addition in intact cells? Also is the pH dependence of BMP formation also peaking at 7? More information is necessary to reach the conclusions drawn from the cell-free system.

8. The substrate preparations need clarification. Were the final concentrations used for samples to be analyzed by LCMS 0.64mM? And for samples to be analyzed by TLC were the samples treated with 1mM lipid? These seem quite high, please discuss the need for the high levels and whether this may not be physiologically coherent.

9. Preparation of substrate was done with a total of 30 seconds of sonication, according to the methods section. Even with a probe sonicator, the liposomes formed after 30s will very likely be highly heterogeneous in size and will be multilamellar. They will vary depending on the lipid type, and for some of the lipids there could possibly be different phases (e.g. the PE will be in hexagonal rather than lamellar phase, the LPG in micellar phase). This makes the comparisons between the lipids as enzyme substrates difficult, and the results uncertain. Especially 30sec sonication of mM concentration of phospholipid would likely have mostly multilamellar structures such that little would be available to the enzymes (outer leaflet of outer lamella only), and the heterogeneity would vary from lipid to lipid, even for lamellar-forming phospholipids.

10. In vitro assay for Hemi-BMP and BMP formation indicates 50ul of PG substrate, does this mean 50ul of 50uM, for a final concentration of 25uM? It is suggested that throughout the manuscript and including in the figure legends, the final concentrations of all compounds be given. The current presentation is ambiguous. Another example is further on in the same section, where 20ul of substrate containing 1mM of PG or LPG is indicated, but the final volume is not given, or is 20ul the final reaction volume? Again, final concentrations rather than “volume of substrate” where the substrate concentration is not given close by, are needed.

11. Protocol for transfection of HEK cells as written would seem to have 4h of transfection done with 0.63ml total volume for a 100 mm culture dish. Such a small volume would not likely promote uniform incubations.

Reviewer #3

(Remarks to the Author)
Comments for authors:

In the manuscript entitled "The endo-lysosomal phospholipid bis(monoacylglycero)phosphate is synthesized via intra- and extracellular pathways" by Dominik Bulfon et al., the authors search for enzymes that catalyze bis(monoacylglycero)phosphate (BMP) production. They reported that several cytosolic phospholipase A2 (PLA2G4 family) enzymes are responsible for intracellular BMP production from phosphatidylglycerol (PG), via hemi-BMP or lyso-PG as intermediate metabolites. They also identified several lipases including endothelial lipase (LIPG), as extracellular BMP producing enzymes.
The reviewer agrees with the identifying the importance of BMP synthetic pathway, as BMP has a unique structure among mammalian phospholipids. The experiments are technically well done, but the overall research lacks cohesion. Many data are presented, but few are conclusive.

Therefore, the reviewer does not recommend that the manuscript to be published in the journal in its current form.

Point-by-point criticism:
In Figs 1-2, the authors characterize the cellular Hemi-BMP-producing activity as transacylase(s), but without identifying the acyl donor, it may be acyltransferase(s). Negative results by acyl-CoA addition in Figs 1D and 1E are not sufficient to exclude acyltransferase.
In Fig 2, the authors characterize PLA2G4 family enzymes as BMP-producing enzymes. The authors could have directly addressed BMP production from hemi-BMP by monitoring hemi-BMP (not FFA) as a hydrolysis product. Fig 2E and 2F are indirect to reach conclusion.
In Fig 3, the authors performed cellular KO experiments only for PLA2G4B and PLA2G4C. It is desirable to test other isoforms using cells that endogenously express the enzymes.
In Fig 4, it is not clear whether extracellular BMP synthesis is relevant for endolysosomal BMP. The authors show LIPG and other lipases as possible extracellular BMP synthetic enzymes, but their effect on intracellular BMP was not validated.
In Fig 5, the in vivo experiment is performed only for the extracellular BMP synthetic pathway. Although panels A-E are informative to demonstrate in vivo BMP synthesis from PG, but in panel F, analysis of LIPG-KO mice provides limited information, which is not enough to make the whole picture as shown in Fig 6.
In Fig 6, the reviewer feels that the scheme shown in Panel A is very tentative as a conclusion.

Minor points:
Fig 1C: The structure of BMP is incorrect and that of LPG is inappropriate. Acyl groups should be at sn-1/3, not sn-2. Similar problems are found in Fig 6.
Fig 1G-H: Addition of one or two time points between 2h-24h is desirable.
Fig 4: In the legend, "shown in (E)" should be corrected as “shown in (C)”.
Figure 5: Panel F t-tests need correction for multiple comparisons (since the authors did not have a prior hypothesis that LIPG-KO causes a difference in a particular tissue).

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have addressed all my questions and concerns

Reviewer #2

(Remarks to the Author)
In this substantially revised manuscript, the authors have presented essential new data showing that even in the absence of the endolysosomal enzhyme CLN5/BMPS, synthesis of BMP occurs in cells. They offer the interesting hypothesis that in this alternative pathway the potential precursors to BMP, phosphatidylglycerol (PG) and lysoPG (LPG), are first secreted from the cell, then acylated in the circulation to produce hemi-BMP or BMP, respectively, and these are then taken back up into the cell for completion of BMP synthesis from hemi-BMP via lipolytic activity.

There are several strengths of the paper. The authors have provided a great deal of information regarding the substrate specificities of numerous phospholipases/transacylases in cell free systems and in some cases cells. As noted before, they show conclusively that BMP synthesis does not require breakdown of the glycerol-phosphate- backbone/headgroup and that acyl CoA is not a required for acylation, supporting a transacylation reaction. By adding the data in CLN5 deficient cells, they now show that there are multiple pathways involved in the synthesis of BMP. While not surprising, since numerous phospholipids and neutral lipids are known to have multiple biosynthetic routes, these new experiments are an important addition.

What is not well supported, though, is the precursor secretion-acylation-uptake-final synthesis model. Although the authors have added necessary time course studies showing the disappearance of labeled PG and the appearance of hemi-PG, the data do not strongly support precursor-product as a major contributor to BMP synthesis. Also not addressed is the relative importance of the present pathway compared to the CLN5/BMP synthase pathway. Specific points:

1. Figure 1 raises several questions, the most puzzling is why (1E) the 13C-hemi BMP remains constant from about 6h through almost 20h, while 13C-BMP continues to rise. Expectation for precursor-product would be reciprocal curves. Quantitatively, there is much more 13C-BMP (eg Fig 1D at 10h, about 1nmol/mg) but 20-fold less 13C-hemi BMP (Fig 1E at 10h, about .05nmol/mg). Additional comments about Figure 1 a) Figures 1H and 1I show data for 2 lipids but the y axis labels indicate values for only one lipid. b) Additionally, please show the unlabeled hemi-BMP in 1E, as is done for BMP and LPG in 1D and 1F.
2. As noted before, the mouse data supporting their overall hypothesis remain quite weak, and in fact the authors have now ruled out a role for LIPG, robustly identified in their TLC screen of serum-based data, because of the in vivo data. This cannot help but raise concerns about the physiological importance of the findings overall.
3. The identification of PLA2G4 enzymes in the screen for hemi BMP synthesis is also very robust (2A), but the changes in

cellular Hemi-BMP and BMP upon deletion of the identified family members is modest at best, whether at basal levels or upon incubation with PG substrate, and deletion of the major PLA2G4 found in HEK cells, PLA2G4C (2I), doubles the levels of hemi BMP rather than reducing it (2J-L). This too raises questions about the physiological importance of the proposed pathway.
4. The PLA2G15 data are important, including consideration of its acting as both a biosynthetic enzyme and a degradative enzyme. Suggest including.
5. The authors interpretations would imply that there no PLA2G15 contribution to BMP synthesis in the CLN5 ko cells. This experiment should be performed.
6. Cell lysate experiments were used to examine the pH dependence of the conversion of PG to hemi-PG, and the results show a neutral to slightly basic pH optimum. Authors conclude that PG acylation occurs outside of acidic organelles. How does the PG, which is presumably taken up as vesicles or lipoproteins in situ via endocytosis, get outside of the endocytic vesicle system? Where are the transacylases localized within the cell, and how do the authors propose that PG coming in from the circulation by endocytosis is accessed? The lysate experiments support a cell-based conversion, but does this occur in intact cells for PG added externally (or coming in from the serum which is part of the authors' proposed scheme)?
7. While the CLN5 ko data add important support to the idea that there are multiple ways for BMP to be made, data in Figure 4 seem to suggest that the non-CLN5 path is not major. It is suggested that the data for panels B-E be rearranged to two panels, one showing BMP levels in WT and ko in the absence and presence of PG (all with the same y axis), and the other showing LPG levels similarly, and all with the same y axis). What becomes apparent is that in the WT cells, PG leads to 2.5 fold increase in BMP whereas in the CLN5 ko, the increase only about 35%. The relative importance/contributions of the different pathways should be explicitly considered.
8. Figure 5—Mouse data must be placed in a separate figure, not bundled along with in vitro data.
9. An overall issue that comes up with the manuscript, which contains a laudable, enormous amount of data, is that it is difficult to follow which evidence is obtained from cell-free experiments, which from lysates, which from intact cells, which from naïve cells and which from cells with overexpression of enzymes, and which from mouse. Each paragraph is well written, that is not the issue, but the overall flow does not adequately distinguish between the levels of evidence that support (or do not support) the scheme presented in the title and Figure 6.

Other comments:
Methods and figure legends—The authors have mostly clarified their procedures and the actual concentrations of various reagents used. Still, some of the assays are missing information. The transacylase assays of cell supernatants and plasma do not indicate time of incubation, nor temperature. The transacylase assay for cell lysates uses a single time point. Was linearity with time and linearity with protein concentration established? Same for the other two transacylase assays.
Hemi-BMP synthase activity, a single 4h time point was used; was linearity for time and protein established?
Hydrolase activity assay, a single 30 min time point was used; was linearity for time and protein established?
Hemi-BMP hydrolysis by partially purified PLA2G4, a single 30 min time point was used; was linearity for time and protein established?
Hemi-BMP hydrolysis by PNLIP enzymes, no incubation time is provided; was linearity for time and protein established?
Figure S1, COS cells incubated with phospholipids—for how long?
Figure 1H through K, what was the incubation time for these data?
Figure 2 most panels, times of incubation are not provided.

Reviewer #3

(Remarks to the Author)
In the revised manuscript, the authors have included the results of new experiments on some of the points raised by the reviewers. The reviewer believes that this has improved the paper to some extent. Although the authors provide evidence in this paper for the involvement of the PLA2G4 family and secreted enzymes in the production of BMPs, most of the enzymatic aspects remain unclear. Thus, the main contribution of this paper is limited to the demonstration of a lysosome/CLN5-independent pathway for BMP production. In this regard, the authors have added experiments using CLN5 KO cells, which add some certainty to the claim of the existence of a CLN5-independent BMP production pathway. In light of this, the reviewer considers this revised paper to be worthy of publication to the community.

Version 3:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
The authors have provided reasonable answers to most of the reviewer questions and comments, and have made additional minor modifications to the wording of their interpretations. Several statements are now somewhat less definitive, commensurate with remaining uncertainties. The data presentation, including clarification of in vitro versus in vivo results, is also improved now (the combination in new Figure 6 is fine). The omissions/clarifications in methods have been largely dealt with.
The following final suggestions are made:
1. The following title is more representative of the data and would be better at putting the present findings in context of current literature: The endolysosomal phospholipid BMP *can be* synthesized via *cytosolic* and extracellular pathways, *in addition to lysosomal generation*. (or lysosomal synthesis). The title closest to what was actually demonstrated in this

manuscript is the final phrase of the Abstract: Functionally overlapping pathways promote BMP synthesis in mammalian cells.
2. The PLA2G15 data provided in review should be incorporated into the manuscript; at the least, in Supplementary Information, and in that case it should be referred to and well-discussed in the main manuscript. Again, this would serve to underline the multiple pathways (and seemingly spatially separated pathways) of BMP synthesis, and provide better integration with the current literature.

We want to thank the reviewers for their valuable and constructive comments.

Based on these new findings and the reviewer's suggestions, we have corrected large parts of the manuscript. We now show that cytosolic and secreted transacylases are capable of promoting BMP synthesis independently of the recently identified lysosomal BMP synthase CLN5. Please find the point-to-point response below.

As described in the Method section, we determined lipid concentrations based on internal standards and on external calibration curves. During revision, we found an error in the calculation of hemi-BMP concentrations. Corrections led to changes in the absolute hemi-BMP concentrations in Figure 1J, 2E, 2G, 2L, 3E, 3I, and 3K, while relative abundances remain the same.

All authors agreed to add Martin Tischitz and Clara Zitta as co-authors, both of whom contributed significantly to the experiments performed during revision.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In this manuscript by Bulfon et al, the authors attempt to identify BMP synthesizing enzymes using limited screens of various lipid enzymes. They successfully identified various isoforms of PLA2 group IV as intracellular mediators of Hemi-BMP/BMP synthesis in vitro/intact cells as well as an endothelial lipase as an extracellular mediator of BMP synthesis based on in vitro and in vivo data.

BMP is emerging as a critical disease-relevant phospholipid in a growing number of diseases, including lysosomal storage disorders and late life neurodegenerative diseases. Its synthesis is known to be highly complex and to involve multiple enzymes. In this manuscript, the authors made significant progress in identifying some of the key relevant enzymes and make a strong case that one of the relevant pathways involves conversion of PG to hemi-BMP, which in turn is converted to BMP. This pathway may be a major alternative to the PG-to-LPG-to-BMP pathway which is believed to be the predominant one, at least in some instances. Interestingly, the key PLA2G4 isoforms identified act primarily as transacylases rather than hydrolases, and mediate Hemi-BMP synthesis. Generally the paper does a great job making a case that multiple enzymes and enzyme families are involved, acting either intracellularly or extracellularly. While the paper potentially represents a significant milestone in the field, there are a few points that need to be addressed to generally increase the impact of the work for the broad readership of Nature Comms. These are as follows

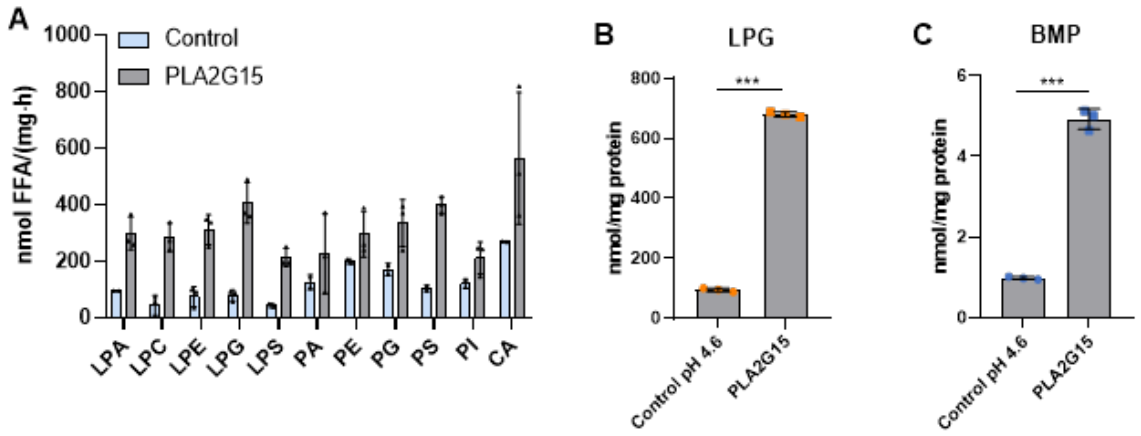
Main issues:

1. Lysosomal PLA2 (PLA2G15) was identified as one of the BMP synthesizing enzymes (PMID 36823305) and should be both discussed upfront in the Introduction and tested experimentally, to determine how it compares to the other PLA2 isoforms presented in the manuscript under consideration, particularly for the intracellular PLA2 isoforms like the group IV isoforms.

Following the reviewer's recommendation and taking recently published results into consideration, the following sentence has been added "Recent observations suggested that LPG is produced from PG in acidic organelles by lysosomal phospholipase A2 (PLA2G15)[50] and subsequently acylated by the lysosomal transacylase CLN5[22], resulting in BMP formation."

In addition, we detected PLA2G15 activity in screening experiments using phospholipids as substrates. The enzyme degrades lysophospholipids and phospholipids at lysosomal pH (4.6, Fig. R1A), but not at neutral pH.

To investigate PLA2G15 function experimentally, we monitored PG-induced BMP formation at pH 4.6 using lysates from cells overexpressing PLA2G15 and control cells. Overexpression of PLA2G15 increased LPG (Fig. R1B) and BMP formation (Fig. R1C) several-fold, confirming previously published data. These observations indicate that PLA2G15 delivers LPG for CLN5-mediated BMP synthesis. As PLA2G15 is active in lysosomes and the focus of our manuscript is on extra-lysosomal BMP synthesis pathways, we did not include these observations in the manuscript.



<u>Figure R1</u>: In vitro activity of PLA2G15: (A) Phospholipid hydrolysis of PLA2G15 at pH 4.6. FFA release was determined using colorimetric kits (NEFA-HR(2), Wako Chemicals). Data derive from screening experiments with different phospholipids. (B, C) Reaction products of PLA2G15 (at pH 4.6) using di-oleoyl PG as substrate. Lysates of Expi293F cells transfected with mouse PLA2G15 or empty vector (control) were used as source of enzymatic activity. Data are shown as mean ± SD. Statistical differences were determined by student's *t*-test (p***<0.001).

2. It seems that there may be some PLA2 isoform redundancy for hemi-BMP and BMP synthesis as well as hints of cell type specificity in the relative importance of various PLA2 isoforms vis-a-vis the synthesis of these lipids. The authors should perform knockdown (or knockout) of some of the key group IV isoforms in cell lines/types other than HEK293.

Taking the reviewers recommendations into consideration we searched for a suitable cell line. To this end, we compared PLA2G4 mRNA expression in the microglial cell line BV-2, the astrocyte cell line C8D1A, and the neuronal cell line CAD (Cath.-a) (Fig. R2). BV-2 cells barely express PLA2G4 mRNA, while C8-D1A cells show a similar expression profile as HEK293 cells. Cath.a cells express all PLA2G4 members except PLA2G4C. Considering that all of the expressed enzymes possess transacylation activity, we cannot perform loss-of-function experiments for all enzymes within a reasonable time frame. In addition, combined deletion could result in cell death, as observed in HEK293 cells lacking PLA2G4C&B. Furthermore, lack of a single PLA2G4 isoform does not necessarily affect BMP levels, since PG/LPG can be acylated by secreted transacylases and by lysosomal CLN5.

To discriminate between lysosomal and non-lysosomal pathways, we instead deleted CLN5 in HEK293 cells and monitored the effect on cellular BMP levels. Our observations demonstrate that

members of the PNLIP and PLA2G4 family promote BMP accumulation in CLN5-deficient cells. Therefore, our data suggest that BMP synthesis is promoted by secreted, cytosolic, and lysosomal pathways resulting in functional redundancy. The new results derived from CLN5-KO cells have been added to the manuscript under the section 'PLA2G4 and PNLIP family members increase BMP formation in CLN5-deficient cells' and are presented in Fig. 4.

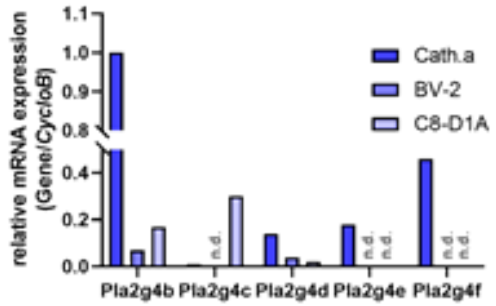


Figure R2. Relative mRNA expression of PLA2G4 family members in neuronal cell lines. Cyclophilin B (CycloB) was used as housekeeping gene. Expression was normalized to *Pla2g4b* in Cath. a cells (n.d. = not detected)

3. In the PG and other phospholipid supplementation experiments in Figure 1I, the authors should match the fatty acyl composition of PG/PE/PS to make sure that differences in fatty acyl composition between do not account for differences in functional outcome (like endocytosis or ILV formation); also, as mentioned below, the authors should mention what specific fatty acyl species are used in the main text. This also applies to the PG species used for the PG supplementation experiments in other parts of the manuscript, including in vivo studies.

All phospholipid substrates used in this study were esterified with oleic acid, which is now mentioned in figure legends, main text, and method section.

4. More importantly, the evidence provided in Figure 1I does not demonstrate that PG triggers endocytosis; it could simply be taken up via constitutive endocytosis. Similarly, the LysoSensor phenotype is not easily interpretable. Is the PG treatment causing more acidification of lysosomes or a proliferation of lysosomes via lysosomal biogenesis. In 18 hours, this is entirely possible. If the authors want to demonstrate PG treatment (and BMP generation) affects the lysosomal pH, they should measure the actual pH and provide absolute values. Additionally, when providing microscopy images like those shown in Figure 1I, it is critical to perform proper quantifications and data replication.

We agree with the reviewer's consideration that the results presented in Fig 1I are insufficient to dissect and discuss the mode of PG uptake. Our intention was to confirm the suitability of our cell line system to study BMP synthesis. We did not investigate uptake mechanisms or organelle acidification. Nevertheless, we believe that quantification using LysoSensor to demonstrate that exogenously provided PG promotes the formation of acidic organelles remains an important confirmation of cell system suitability and confirms previous observations. As this observation is not novel, we moved the microscopy study to Fig. S1 and added a more detailed procedure in the

method section under "Fluorescence microscopy". We performed quantifications using LysoSensor revealing that PG supplementation promotes the formation of acidic organelles.

5. The EM analysis of ILVs in Suppl. Figure 1 E should also be supported by morphometry. It's not trivial, because vesicular compartments without ILVs may not be endolysosomal. Proper identification of the endolysosomal compartment at the EM level requires the presence of endocytic tracers like HRP, which can generate electron-dense material visible at the EM level. Since this is going to be a lot of work, I suggest exclusion of this data, and perhaps a substitution of this data with data from anti-BMP antibody stains showing potentially appearance of BMP in the endolysosomal compartment upon PG supplementation (which of course would have to be generated).

According to the reviewer's suggestion, we excluded EM from the manuscript, because we cannot clearly demonstrate that observed vesicular structures are LE/lysosomal compartments.

6. In Figure 2A, the authors use a limited screen of about 200 candidate mouse lipid enzymes overexpressed in HEK293 cells to identify the hemi-BMP synthesizing activities and both PLA2G4D and E came up as top hits. This is a powerful approach, but what evidence is there that all 200 mouse lipid enzymes are actually expressed? Do they have mRNA assessments showing proof of overexpression or tagged versions of the said 200 lipid enzymes to monitor actual overexpression at the protein levels? It looks like His tags are used for purification, so theoretically anti-His western blots can be performed for all their overexpressors (or use some other techniques to quantify protein expression).

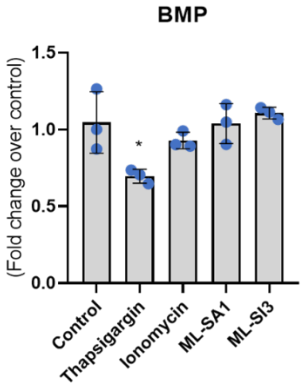
As mentioned in the manuscript, we selected protein families for the screening experiments, where at least one family member has been reported to exhibit lipid hydrolase or transacylase activity. Protein expression was confirmed by Western blotting analysis of overexpressed candidates (now added to the Supplemental Table S2, and the online data repository DOI: 10.17632/hcs7crxk3z.1) revealing that 86% of the proteins were expressed. Some enzymes were not detected, possibly due to cleavage of a peptide containing His- or Strep-tags. However, we additionally performed activity-based screening assays with multiple substrates and detected enzymatic activity for some enzymes, which we could not detect by Western blotting. Such screens were performed using 23 different lipid species. Together, we could confirm the expression of 93% of genes either by Western blotting analysis or using activity-based assays.

7. For all the PLA2 isoforms identified as top hits (PLA2G4D, PLA2G4E, and LIPG), the authors also need to overexpress the catalytically dead mutant versions of the enzymes to determine if the Hemi-BMP or BMP-synthesizing activities are lost.

As suggested by the reviewer, we cloned loss-of-function mutants for the identified top hits. As shown in Fig. 2D and Fig. 3B (Insert), the exchange of the active serine with an alanine resulted in loss of transacylation activity.

8. The Ca2+ sensitivity for PLA2Gx isoforms' transacylase activity is interesting. The authors should test whether modulation of intracellular Ca2+ levels affects Hemi-BMP and BMP levels in intact WT cells or cells lacking the key PLA2 group IV isoforms identified as Ca2+-sensitive. They can potentially use Ca2+ ionophores, or inducers of Ca2+ release from intracellular stores (e.g., ER, mitochondria or lysosomes). Since human PLA2G4B is Ca2+ sensitive, the corresponding KO in HEK293 cells may show a Ca2+ induced phenotype in intact cells, whereas the PLA2G4C KO may be less sensitive to Ca2+.

Taking the reviewers suggestion into considersation, we tested whether modulation of Ca^{2+} affects BMP levels in HEK293 cells. As shown in Fig. R3, Ionomycin, or the lysosomal TRPML1 channel agonist (ML-SA1) and antagonist (ML-SI3) had no effect, while Thapsigargin moderately reduced BMP (prolonged exposure to Thapsigargin reduced cell viability). These observations indicate that modulation of Ca^{2+} signaling has no strong impact on BMP levels in HEK293 cells. We did not include these experiments in the manuscript, as the investigation of the role of Ca^{2+} in the regulation of BMP synthesis requires more detailed studies in different cell types.



<u>Figure R3</u>. Effect of Ca^{2+} modulation on cellular BMP levels. HEK293 cells were supplemented with 5 µM Thapsigargin, 5 µM Ionomycin, 10 µM of the lysosomal TRPML1 channel agonist ML-SA1, 10 µM of TRPML1 channel antagonist (ML-SI3). After an overnight incubation, lipids were extracted and BMP levels were determined by LC-MS. Data are shown as mean ± SD (n=3). Statistically significant differences were determined by one-way ANOVA corrected for multiple comparisons against control by Dunnett's post hoc test (levels of statistically significant differences are: *$p < 0.05$).

9. For the Lipg KO mouse study in Figure 5, it seems that measuring BMP levels in plasma is important, if the enzyme is actually secreted/extracellular.

In general, LIPG and other PNLIP members are constitutively secreted enzymes. LipG as well as other serum enzymes of the PNLIP family are active at the surface of endothelial cells and/or can be detected in the circulation (PMID: 35458682). Our study clearly demonstrates that plasma contains hemi-BMP/BMP synthase activity

10. An important gap in this manuscript is some demonstration that reducing BMP synthesis by knocking out/down the top PLA2G4 hits identified causes defects consistent with BMP deficiency. For instance, is there any evidence of lysosomal unesterified cholesterol (filipin), gangliosides or glucocerebrosidase substrate accumulation in the knockouts of PLA2G4B and C? This would dramatically increase the impact of the manuscript. Similarly, any cholesterol/ganglioside/GCase substrate accumulation in the tissues of the Lipg KO showing reducing BMP levels?

We agree with the reviewer that lack of BMP can be associated with lysosomal lipid accumulation. However, our data suggest that lack of a single PLA2G4 or PNLIP enzyme does not necessarily affect BMP levels due to functional redundancy within and between extra- and intracellular tranacylases. Even cells lacking CLN5 have normal BMP content when cultured under standard conditions (Figure 4).

We hope that the reviewer understands that investigating the changes in lipid metabolism in cells with defective BMP metabolism will be a major topic in our laboratory in the future. In the current study, we aim to understand BMP metabolism.

Minor comments:

11. As the authors present the literature on BMP, it's always helpful to indicate whether the key findings they refer to are described for cell-free studies, cell culture studies and/or in vivo studies. For instance, in lines 50-52, it's unclear whether the data they refer to is solely based on cell free studies or other types of studies. This is important because it addresses physiological relevance.
corrected, all studies were performed in vitro

12. In addition to GRN and NPC disease biology, there is an emerging link between BMP and other neurodegenerative diseases, which should probably be cited to increase the significance of the manuscript. For instance, PMID 36854767 and 25653221 for LRRK2/Parkinson, PMID: 22134919 and 35351864 for APOE4 and Alzheimer's.

We now mention this link in the introduction: Altered BMP levels are lipidomic signatures of common neurodegenerative diseases such as Parkinson's [13] and Alzheimer's disease[14,15].

13. In the Intro paragraph starting with line 53, it's also helpful to mention the cell free work of Sandhoff and colleagues on GCase and SapC (PMID: 28126847)

We now mention this work in the introduction: BMP also interacts with lipid-binding and -transport proteins. It supports the presentation of glycolipids to catabolic enzymes by sphingolipid activator proteins[7,8].

14. In the same paragraph, it's important to present the role of BMP in egress of cholesterol from the endolysosomal system giving also credit to the seminal work of Gruenberg and colleagues and citing some of the primary literature on this topic (for instance, PMID: 10559883 and 18644787)

We now mention this work in the introduction: Furthermore, BMP facilitates cholesterol export from lysosomes[9,10], which depends on Niemann-Pick type C1 and C2 proteins (NPC1 and NPC2).

15. In lines 63-66, regarding the Grn mutant work, it's important to mention the in vivo data show a global deficiency of all BMP species analyzed in various tissues (e.g. brain, liver) and biofluids (plasma, CSF). Cell culture experiments, at least in Logan et al, suggests upregulation of specific species of BMP, like 18:1/18:1, in KO BMDMs, while PUFA species are downregulated. Therefore, cell culture Grn loss of function models do not always a global deficiency of all BMP species.

Answered together with comment 16.
16. In line 67-68, the statement "it is unclear whether PGRN directly interacts with BMP or if it regulates its metabolism" is slightly inaccurate, as both studies Boland et al and Logan et al show PGRN binds directly to BMP in vitro. Perhaps the authors meant the following: "It is unclear if PGRN regulates BMP metabolism directly by modulating its stability via a physical interaction or whether it regulates BMP levels indirectly via modulation of BMP-metabolizing enzymes".

Taking the reviewers comments 15/16 into consideration, we have added the following sentences to the Introduction: PGRN interacts directly with BMP, and mice lacking PGRN show global BMP deficiency. It is currently unclear whether the BMP/PGRN interaction modulates BMP stability or the activity of BMP-metabolizing enzymes[20,21].

17. In PG supplementation experiments in Figure 1 (but also throughout the paper), the authors should consistently mention the specific fatty acyl composition of PG (regular or 13C-labeled) in the main text, and figure legends.

All phospholipid substrates used in this study were esterified with oleic acid, which is now mentioned in figure legends, main text, and method section.

18. Line 466, the following statement "Hemi-BMP synthesis was most efficient at neutral pH and absent at pH values $\leq$ 5.5 (Figure 1F), indicating that hemi-BMP synthesis rather occurs at membranes facing the cytosol than within acidic organelles." can be complemented with another scenario, whereby hemi-BMP synthesis may be also occurring in the ectoplasmic leaflet of the plasma membrane, since the extracellular environment is also neutral, at least theoretically.

Taking the reviewers comments into consideration, we repeated the experiment with stably labeled PG and modified the statement (Figure 1 H&I) as follows: "Hemi-BMP synthase activity was strongly reduced at pH values ≤ 6.0, indicating that PG acylation occurs outside of acidic organelles."

19. In Figures 1G and 1H (hemi-BMP and BMP formation), it seems that the time course could benefit from inclusion of additional time points between 2h and 24h to increase time resolution when the bulk of synthesis occurs.

In accordance with the reviewer's suggestion, we repeated time-course experiments using stably labeled PG, increased time resolution, and included LPG in our measurements (Figure 1 D-Q). We now show that both hemi-BMP and LPG are rapidly formed in response to PG supplementation, while BMP increases at later time points. These observations suggest that the conversion of PG to BMP requires transacylation and hydrolase reactions, with either hemi-BMP or LPG as intermediate.

20. Line 479, please indicate the duration of the PG treatment; it can help the reader interpret the effects on the endocytic compartment

Following the reviewer's recommendation, the incubation period has been indicated in the Figure legend. Microscopy data are now shown in Fig. S1.

21. Line 511, the six isoforms of group IV should include PLA2G4F in addition to PLA2G4A-E
Corrected

22. In the Discussion, it would be beneficial to discuss findings from the CRISPR screen identifying regulators of BMP levels using an anti-BMP antibody (PMID: 34936700) and check whether any of the hits overlap with those from the manuscript under consideration. If not, perhaps discuss how different the approaches are.

There is no overlap between the genome-wide CRISPRs screen described by Lu *et al.* and our observations. The most important reason is probably that serum contains substantial transacylase

activity, which affects cellular BMP levels. As shown in our study, CLN5-ko cell have normal BMP levels under standard conditions and severe BMP deficiency when cultured in the presence of heat-inactivated serum (Figure 4). Lu *et al.* did not use heat-inactivated serum, which is likely the main reason why CLN5 and potentially other important genes in this pathway were missed.

23. The Discussion seems a bit long and could be a bit more concise.

The discussion was modified taking our new data and reviewer suggestions into consideration.

Reviewer #2 (Remarks to the Author):

In this manuscript the authors present a series of experiments aimed at understanding the biosynthetic pathway for the lipid bis(monoacylglycerol)-phosphate, BMP. This is an important and intriguing topic, and the manuscript describes results from extensive experimentation. They performed screens of libraries of known (and related) hydrolase and transacylase enzymes using a glycerol labeled PG substrate, and identified two specific enzymes, PLA2G4D and PLA2G4E, which increase Hemi-BMP and, to a much lesser extent, increase BMP itself. Overall they provide additional support for previously proposed steps and also a number of additional insights into potential synthetic pathways for BMP. Nevertheless the overall conclusions are based largely on in vitro data with semipurified enzymes in cell free system, with the cellular and animal data not robustly supporting the proposed intra- and extra-cellular synthetic routes. The proposal that PG is first converted to hemi-BMP prior to hydrolysis to BMP is weakly supported by limited time course studies. Importantly, moreover, a recent publication has robustly identified a novel BMP synthase as the product of the CLN5 gene (doi: 10.1126/science.adg9288). Overall, the paper while interesting leaves many uncertainties and represents a somewhat modest advance in understanding of BMP biosynthesis. The following are specific comments.

1. A recent Science paper by Medoh et al. identifies the product of the CLN5 gene as the enzyme responsible for BMP synthesis, seemingly occurring directly from 2 molecules of PG. This is obviously very different from the biosynthetic scheme proposed here. As the present study used a target gene approach, the CLN5 was not identified. Given this new finding plus the fact that knockout of the PLA2G4D, PLA2G4E, and LIPG had only modest if any effects on BMP synthesis, it is uncertain that the intracellular and extracellular enzymes identified play key physiological roles in endogenous BMP biosynthesis.

We agree with the reviewers that the recently reported findings of Medoh *et al.*, which were published after we have submitted our manuscript, are helpful if not essential for the interpretation of our observations. The presence of cytosolic, extracellular, and lysosomal pathways significantly complicates the investigation of the importance of individual pathways. To circumvent this problem, we now show the effect of the newly identified transacylases on BMP synthesis in cells lacking *CLN5* (Figure 4). Members of the PNLIP and PLA2G4 family are capable of promoting BMP synthesis independently of CLN5. Furthermore, we highlight that cell culture conditions, specifically the effect of serum and heat-inactivated serum, strongly affect BMP levels in *CLN5*-deficient cells.

2. While there are some interesting observations about BMP synthesis that are shown in this paper (i.e strong evidence that the process does not require breakdown and resynthesis of the glycerol-phosphate-glycerol backbone/headgroup; that acyl CoA is not a precursor for fatty acylation (supporting transacylation reaction), the level of solidly supported new information is modest.

Here, we have to disagree with the reviewer. We demonstrate for the first time that PNLIP and PLA2G4 enzymes catalyze hemi-BMP/BMP synthesis. In the revised manuscript, we now demonstrate that extra-lysosomal pathways strongly promote BMP synthesis in CLN5-deficient cells. These results highlight the considerable redundancy of BMP biosynthetic pathways.

3. The authors purport that endothelial lipase and members of the pancreatic lipase family (PLRPs) acting in the circulation are involved in BMP synthesis (extracellular pathway), in addition to intracellular pathway. While they show that these enzymes are capable of transacylation and hydrolytic activities, and indeed some of the PLRP's had been previously shown to use BMP as substrate, there is no evidence that these activities have physiological relevance.

To our best knowledge, the only enzyme of the PNLIP (PLRP) family, which has previously been shown to hydrolyze BMP and hemi-BMP, is PNLIPRP2. We are not aware of any previous reports describing transacylation reactions as reported in this manuscript. The physiological relevance of serum transacylases is indicated by the following observations:

- **(i) Serum contains substantial hemi-BMP/BMP synthase activity (Figure 3A and Figure 5A-C). These reactions are catalyzed by several members of the PNLIP family (Figure 3C-F).**
- **(ii) PG treatment leads to the formation of hemi-BMP and BMP in vivo (Figure 5E&F).**
- **(iii) The identified transacylases promote hemi-BMP/BMP formation in *CLN5*-deficient cells (Figure 4).**
- **(iv) *CLN5*-deficient cells cultured under standard conditions (10% FBS) exhibit unchanged BMP levels compared to wild-type cells and develop severe BMP deficiency after prolonged exposure to heat-inactivated FBS lacking transacylase activity (Figure 4).**

The authors'own data show virtually no PG substrate in serum of unsupplemented animals.

Indeed, PG is present in low, but detectable concentrations in serum. However, as also mentioned in the discussion, it is a highly abundant lipid in plant and bacterial membranes. Accordingly, it can be assumed that PG is present in substantial amounts in the human diet. Our study indicates that circulating PG is rapidly degraded or acylated, which might be causal for its low abundance. BMP is present in serum of mice and humans and, as demonstrated in this study, can derive from circulating PG. Most importantly, however, we demonstrate with stable isotope labeled PG that the backbone of circulating PG is efficiently incorporated into tissue BMP stores implicating that dietary PG can act as precursor lipid of BMP and thereby promote BMP synthesis. As mentioned in the discussion section, this observation could be relevant for the treatment of lysosomal storage disorders.

Additionally, despite identification of endothelial lipase (LIPG gene) in the in vitro screen, no reduction in conversion of PG to hemi-BMP or BMP in circulation was found in LIPG knockout mice, and the reductions in tissue BMP levels in this

mouse were small in the few tissues where decreases were found.

Our in vivo studies suggest that LipG can promote BMP synthesis in specific tissues, but is clearly not rate-limiting for extracellular hemi-BMP/BMP synthesis. This is consistent with the observation that other enzymes of the PNLIP family can mediate PG acylation (Figure 3C-F). In the revised manuscript, we now demonstrate that LIPG is responsible for ~ 30% of hemi-BMP synthase activity in heparinized mouse plasma (Figure 5C). Thus, other serum enzymes, such as PLA1A, LIPC, and LIPH, substantially contribute to transacylation reactions (Figure 3C-F).

4. Kinetics of BMP and hemi-BMP formation does not strongly support precursor product relationship. Figure 1G and 1H y axes are almost 10-fold different for Hemi-BMP and BMP, making the time course of BMP formation look flat when it might not be. Further, although in the Expi cells about 50-fold more hemi-BMP is formed compared to BMP, in the HEK cells the absolute amount of BMP is much greater than Hemi-BMP. Can the investigators discuss this cell-dependent discrepancy, and what it might indicate for precursor-product relationship?

In general, it is difficult to compare hemi-BMP/BMP formation in cell culture and cell-free studies, as for example the generated products can be rapidly metabolized in cellular environments. Independent of the synthesis pathway, BMP formation requires the acylation of the headgroup of PG or LPG and this reaction can be demonstrated in cell culture and cell-free experiments at neutral and, for LPG, at acidic pH. Based on transacylation activities detected in cell lysates and on the accumulation of possible intermediates upon PG-supplementation (Figure 1), we hypothesized that both PG and LPG can be acylated. We repeated time-course experiments using stably labeled PG and higher time resolution, and included LPG in our measurements. We now show that both hemi-BMP and LPG are rapidly formed in response to PG supplementation, while BMP increases at later time points (Figure 1D-G). These observations suggest that the conversion of PG to BMP requires transacylation and hydrolase reactions, with either hemi-BMP or LPG as intermediate. In addition, we would like to also refer to our response to comment 6.

5. If Hemi-BMP is the intermediate/precursor for BMP, it seems surprising that at time zero, prior to adding PG, there is little to no hemi-BMP in both cell types. This would imply no regulation of the conversion of hemi-BMP to BMP and hence of BMP level, perhaps surprising given its apparently important functions. This should be commented on.

Considering that BMP is the end product of this biosynthetic pathway, its presence in higher amounts compared to the intermediates seems not unusual, as biosynethetic intermediates tend to be rapidly metabolized and are therefore barely detectable under basal conditions. On the contrary, under specific stress conditions, such as addition of exogenous PG, the biosynethtic intermediates can accumulate. In addition, we extracted larger amounts of cells and were able to detect hemi-BMP under basal levels, though in the low pM range (data not shown).

6. Time course of product formation from PG should be followed using the stable isotope labeled PG. It may well be that Hemi-BMP is the major intermediate/precursor but the present data are not strong enough, especially

with the inconsistencies between different “WT” cell lines.

Following the reviewer’s recommendation, we repeated time-course experiments using stably labeled PG, a higher time resolution, and included LPG in our measurements. BMP increases in a linear manner, while hemi-BMP and LPG accumulate already 30 min after PG supplementation (Figure 1D-G).

7. pH dependence of the Hemi-BMP formation is shown to be 7.0 and the authors interpret this as showing that the reaction occurs facing the cytosol. These data were generated using cell lysates. What is the pH dependence after PG addition in intact cells? Also is the pH dependence of BMP formation also peaking at 7? More information is necessary to reach the conclusions drawn from the cell-free system.

Take the reviewers concerns into consideration, we have performed additional experiments comparing the pH dependence of transacylation reactions mediating the acylation of LPG and PG. LPG is most efficiently acylated in the acidic pH range with an optimum of 6.5, and the reaction does not generate hemi-BMP (Figure 1H). The observed pH dependance of BMP formation is consistent with the recently identified lysosomal BMP synthase CLN5. Highest hemi-BMP synthase activity was observed in the neutral and slightly alkalic pH range (Figure 1I). Taken together, these observations suggest that LPG and PG are acylated by different enzymes. The pH dependence of hemi-BMP formation suggested that this metabolite is formed outside of acidic organelles and this initial hypothesis was later confirmed by the identification of cytosolic and secreted transacylases. Furthermore, additional experiments using *CLN5*-KO HEK293 cells now demonstrate that extra-lysosomal transacylation reactions promote BMP synthesis (Figure 4).

8. The substrate preparations need clarification. Were the final concentrations used for samples to be analyzed by LCMS 0.64mM? And for samples to be analyzed by TLC were the samples treated with 1mM lipid? These seem quite high, please discuss the need for the high levels and whether this may not be physiologically coherent.

We would like to refer to our response to comment 9 below, addressing both comments together.

9. Preparation of substrate was done with a total of 30 seconds of sonication, according to the methods section. Even with a probe sonicator, the liposomes formed after 30s will very likely be highly heterogeneous in size and will be multilamellar. They will vary depending on the lipid type, and for some of the lipids there could possibly be different phases (e.g. the PE will be in hexagonal rather than lamellar phase, the LPG in micellar phase). This makes the comparisons between the lipids as enzyme substrates difficult, and the results uncertain. Especially 30sec sonication of mM concentration of phospholipid would likely have mostly multilamellar structures such that little would be available to the enzymes (outer leaflet of outer lamella only), and the heterogeneity would vary from lipid to lipid, even for lamellar-forming phospholipids.

We are in agreement with the reviewer that the form of substrate presentation varies depending on the lipid type. PG, especially the exclusively employed di-oleoyl PG, predominantly forms monolamellar vesicles under the applied conditions, which differ in size (PMID: 16150420, and

personal communication with G. Pabst, Biophysics, Institute of Molecular Bioscience, University of Graz, Graz, Austria). However, the presented substrate is only partially accessible for enzymes, which can lead to high apparent K_M values. This is a general problem using lipid substrates. Therefore, concentrations of 1 mM are usual lipid concentrations in (phospho)lipase assays, but clearly do not reflect physiological conditions.

To gain a better understanding of the potential bias associated with the employed lipid substrate preparation method, we studied the enzymatic conversion of reaction of di-oleoyl PG as substrate in the presence of semi-purified PLA2G4D. We observed the formation of hemi-BMP, LPG, BMP, and BDP (Fig. R4A) using sonicated lipid preparations. Saturation kinetics revealed apparent K_M values of ~ 2 mM for hemi-BMP and LPG (Fig. R3B&C). These products directly derive from PG and can be detected even at the lowest PG concentration used (0.1 mM).

Altering the lipid preparation method by using a lipid extruder (100 nm filter), detergents, or a mixture of different lipids moderately affected the activity of PLA2G4D at a final substrate concentration of 0.5 mM under otherwise identical assay conditions. Extrusion of PG or a PC/PG mixture slightly increased hemi-BMP but not LPG formation. Addition of NP-40 reduced hemi-BMP but not LPG formation, while addition of CHAPS had no effect. Taken together these additional experiments revealed that independent of lipid concentration and preparation methods, comparable results were produced and that the enzymatic reaction always produced hemi-BMP, LPG, BMP, and BDP. Similar results were obtained with other enzymes (data not shown). As in general *in vitro* conditions will hardly ever be able to exactly mimic the *in vivo* environment, we opted for the simplest and most time efficient method of substrate preparation.

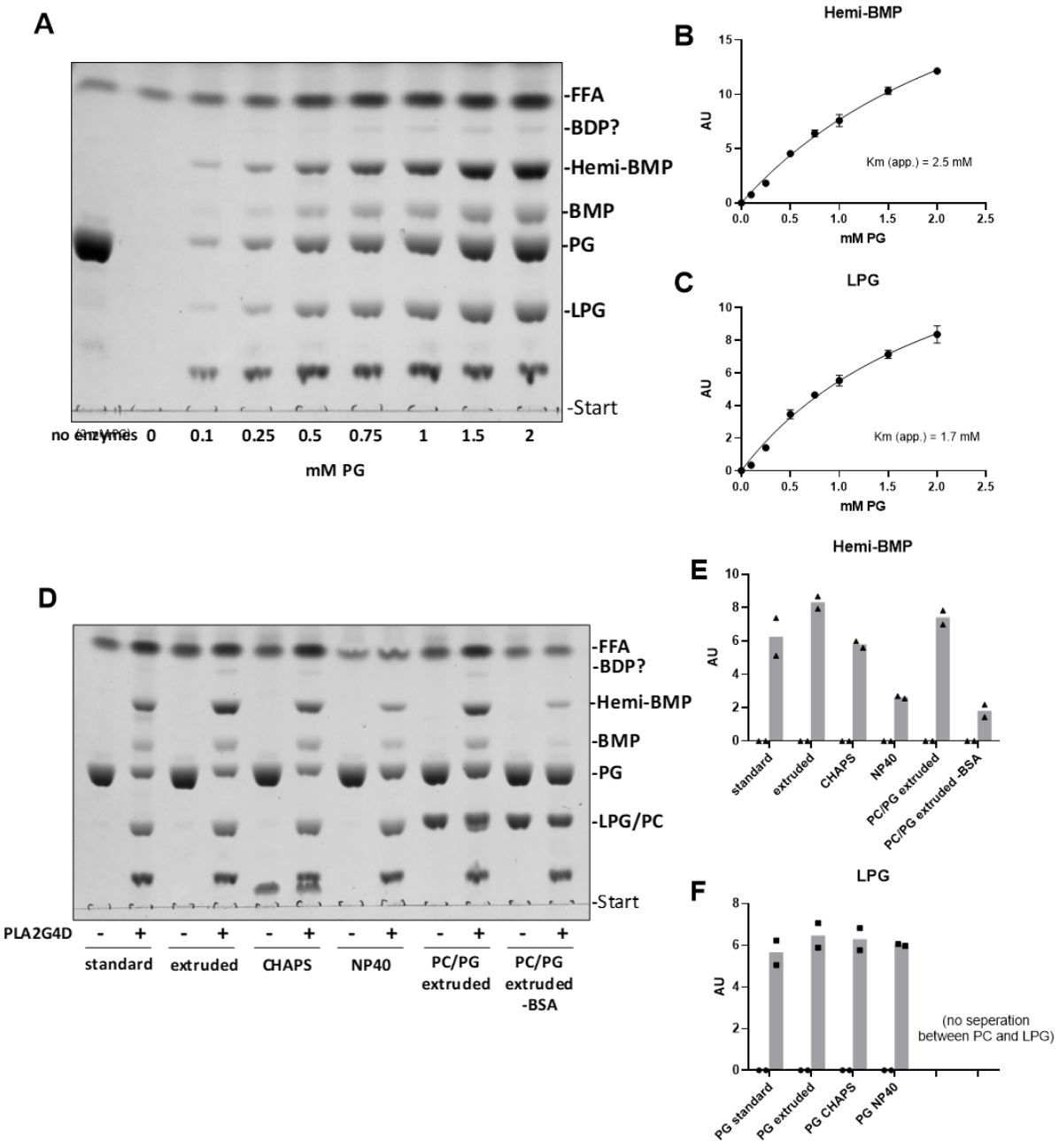


Figure R4. (A) Representative TLC showing the reaction products of semi-purified PLA2G4D (1µg/assay) incubated with the indicated concentrations of PG for 1 hour at 37°C in a volume of 20 µl. The substrate was prepared by sonication with a SONOPULS ultrasonic homogenizer (3 x 10s in PBS, 15% amplitude). (B, C) Band intensities of hemi-BMP and LPG were quantified with ImageJ. Apparent Km values were calculated using GraphPad Prism 8.0.1. Data are shown as mean ± SD (n=3).(D) PG transacylase/hydrolase activity assay using different substrate preparations procedures. All assays in a final volume of 20 µl contained PG at a concentration 1 mM and 1 µg enzyme protein. The substrate was prepared by sonication (standard), using a lipid extruder with a 100 nm filter (extruded), mixed with equimolar concentrations of PC and extruded (PG/PC extruded), prepared in the presence of 2.5 mM CHAPS (CHAPS), or prepared in the presence of 0.1% NP-40 (NP40). (E, F) Band intensities of hemi-BMP and LPG were quantified with ImageJ. Data are shown as single values of duplicate determinations.

10. In vitro assay for Hemi-BMP and BMP formation indicates 50ul of PG substrate, does this mean 50ul of 50uM, for a final concentration of 25uM? It is suggested that throughout the manuscript and including in the figure legends, the final concentrations of all compounds be given. The current presentation is ambiguous. Another example is further on in the same section, where 20ul of substrate containing 1mM of PG or LPG is indicated, but the final volume is not given, or is 20ul the final reaction volume? Again, final concentrations rather than "volume of substrate" where the substrate concentration is not given close by, are needed.

We apologize for the confusion. Following the reviewer's recommendation, we now provide a more detailed description of enzyme assays in the Method section and always describe the final concentrations and assay volume.

11. Protocol for transfection of HEK cells as written would seem to have 4h of transfection done with 0.63ml total volume for a 100 mm culture dish. Such a small volume would not likely promote uniform incubations.

The method section in question has been clarified and corrected in accordance with the reviewers recommendation.

Reviewer #3 (Remarks to the Author):

Comments for authors:

In the manuscript entitled "The endo-lysosomal phospholipid bis(monoacylglycero)phosphate is synthesized via intra- and extracellular pathways" by Dominik Bulfon et al., the authors search for enzymes that catalyze bis(monoacylglycero)phosphate (BMP) production. They reported that several cytosolic phospholipase A2 (PLA2G4 family) enzymes are responsible for intracellular BMP production from phosphatidylglycerol (PG), via hemi-BMP or lyso-PG as intermediate metabolites. They also identified several lipases including endothelial lipase (LIPG), as extracellular BMP producing enzymes.
The reviewer agrees with the identifying the importance of BMP synthetic pathway, as BMP has a unique structure among mammalian phospholipids. The experiments are technically well done, but the overall research lacks cohesion. Many data are presented, but few are conclusive.
Therefore, the reviewer does not recommend that the manuscript to be published in the journal in its current form.

Point-by-point criticism:
In Figs 1-2, the authors characterize the cellular Hemi-BMP-producing activity as transacylase(s), but without identifying the acyl donor, it may be acyltransferase(s). Negative results by acyl-CoA addition in Figs 1D and 1E are not sufficient to exclude acyltransferase.

The reviewer raises important issues. We did not search for acyl donors due to the catalytic mechanism of serine hydrolases (all identified enzymes are serine hydrolases). The catalytic

reaction starts with the binding of the lipid substrate and the attack of nucleophilic serine on the carbonyl carbon atom of the ester bond. This leads to the cleavage of the substrate, the release of the alcohol product, and to the formation of an acyl-enzyme intermediate, where the substrate-derived FA is covalently bound to the active serine. The next step is the deacylation of the acyl-enzyme intermediate, in which a water molecule hydrolyzes the covalent bond, leading to the release of the free FA and the regeneration of the enzyme. Alternatively, the acyl-enzyme intermediate reacts with an alcohol instead of water leading to the formation of an ester bond and enzyme regeneration. In cases where the acyl acceptor is a lipid, this mechanism enables transacylase reactions that catalyze the transfer of FAs between different lipid molecules. Based on this mechanism, we assume that any hydrolyzed substrate can also act as acyl donor. Most of the identified phospholipases have broad substrate specificity, indicating that many lipids can act as donor. In our study, we did not compare different donor lipids. However, our in vivo data show that a large part of di-oleoyl PG is acylated with saturated fatty acids (Fig. S5E-F). Furthermore, PG-derived BMP undergoes tissue-specific FA remodeling (Fig. 5I). These observations strongly indicate that other lipids than PG can act as acyl donor.

Our assays revealed that cell lysates are capable of acylating PG independent of the addition of acyl-CoA. Based on the detected activity, we identified the enzymes as transacylases and demonstrate that these enzymes can promote hemi-BMP/BMP synthesis. To our knowledge, the detection of acyl-CoA-dependent acyltransferase activity requires the addition of acyl-CoA to the reaction. We agree with the reviewer that we cannot completely exclude that acyl-CoA-dependent mechanisms could also mediate head group acylation of PG or LPG further providing biosynthetic redundancy, as many acyltransferases are not expressed in HEK293 cells and were not tested in our targeted screening approach. However, *CLN5*-deficient cells develop severe BMP deficiency, only when cultured in heat-inactivated serum lacking transacylase activity (Figure 4). This observation indicates that BMP synthesis in HEK293 requires transacylation reactions catalyzed by extra- or intra-lysosomal transacylases.

In Fig 2, the authors characterize PLA2G4 family enzymes as BMP-producing enzymes. The authors could have directly addressed BMP production from hemi-BMP by monitoring hemi-BMP (not FFA) as a hydrolysis product. Fig 2E and 2F are indirect to reach conclusion.

According to the reviewer's suggestion, we digested hemi-BMP with semi-purified enzymes. Dependent on the activity of different enzymes, digestion of hemi-BMP resulted in the formation of BDP, BMP, PG, and LPG (Fig. S2M).

In Fig 3, the authors performed cellular KO experiments only for PLA2G4B and PLA2G4C. It is desirable to test other isoforms using cells that endogenously express the enzymes.

Taking the reviewers comment into consideration, we tested PLA2G4 expression in several mouse cell lines, described in more detail in response to Reviewer 1 comment 2. However, based on the observations that hemi-BMP/BMP can be synthesized by multiple enzymes in different compartments, we came to the conclusion that deletion of a single enzyme does not necessarily result in reduced BMP levels as even CLN5-KO cells presented BMP levels similar to WT cells in presence of 10% FBS (Figure 4). In addition, combined deletion of multiple enzymes has an elevated risk of causing cell death, as observed upon double deletion of PLA2G4B&C.

To discriminate between lysosomal and non-lysosomal pathways, we instead deleted CLN5 in HEK293 cells and monitored the effect of extra-lysosomal hemi-BMP/BMP synthases on cellular BMP levels. Our observations demonstrate that members of the PNLIP and PLA2G4 family members increase BMP accumulation in CLN5-deficient cells, confirming the notion of redundant pathways (Figure 4).

In Fig 4, it is not clear whether extracellular BMP synthesis is relevant for endolysosomal BMP. The authors show LIPG and other lipases as possible extracellular BMP synthetic enzymes, but their effect on intracellular BMP was not validated.

Taking the reviewers comment into consideration, the revised manuscript now shows that cell-associated hemi-BMP levels are significantly increased in LIPG-, LPL-, PLA1A- overexpressing cells after a 4 h incubation period (Figure 3K). LIPH- and PLA1A-overexpressing cells exhibited elevated cellular BMP levels (Figure 3L). Furthermore, we show that overexpression of PLA2G4D and all tested PNLIP members (LipG, LipH, LipC, PLA1A) increase cellular BMP levels in *CLN5*-KO cells (Figure 4F&G)

In Fig 5, the in vivo experiment is performed only for the extracellular BMP synthetic pathway. Although panels A-E are informative to demonstrate in vivo BMP synthesis from PG, but in panel F, analysis of LIPG-KO mice provides limited information, which is not enough to make the whole picture as shown in Fig 6.

We performed experiments with LIPG-KO mice, as LIPG was the most potent hemi-BMP synthase in our enzyme screens. To further expand on the role of LIPG, we now demonstrate that LIPG is only responsible for ~30% of the hemi-BMP synthase activity detected in heparinized mouse plasma (Figure 5C), suggesting that other serum transacylases, such as PLA1A and LIPC, substantially contribute to this reaction. This observation is supported by in vitro assays demonstrating that several PNLIP members promote hemi-BMP/BMP formation (Figure 3C-F).

In Fig 6, the reviewer feels that the scheme shown in Panel A is very tentative as a conclusion.
According to the reviewer's suggestion, we removed Fig.6A. In the new Figure 6A, we propose that secreted, cytosolic, and lysosomal PG/LPG transacylases promote BMP synthesis. This scheme is based on the observation that members of the PLA2G4 and PNLIP family promote BMP synthesis in CLN5-deficient and wild-type cells (Figure 3 and 4).

Minor points:
Fig 1C: The structure of BMP is incorrect and that of LPG is inappropriate. Acyl groups should be at sn-1/3, not sn-2. Similar problems are found in Fig 6.

We are in agreement with the reviewers concerns that intermediates of BMP synthesis could have acyl chains in sn-1/3 position, while mature BMP has been reported as acylated primarily in sn-2/2' position. Fig. 1C is intended as a simplified overview of BMP synthesis, which does not consider the position of acyl groups and the specific stereochemistry. These aspects are described in more detail in the Discussion section. In the updated Fig.1C, we opted to depicted the acyl chains on the 2-positions representing mature BMP to prevent unnecessary confusion of the inclined reader due to potential acyl migration requirements and adapted the legend to better highlight this intent: "(C) Schematic depiction of BMP synthesis starting from its precursor PG. Acyl group positions and the stereoconfiguration of these metabolites are described in the Discussion section."

Fig 1G-H: Addition of one or two time points between 2h-24h is desirable.

Following the reviewer's recommendation, we repeated time-course experiments with stably labeled PG, increased time resolution, and included LPG in our measurements (Figure 1D-G).

Fig 4: In the legend, "shown in (E)" should be corrected as “shown in (C)”.

Corrected in accordance with the reviewer’s suggestion.

Figure 5: Panel F t-tests need correction for multiple comparisons (since the authors did not have a prior hypothesis that LIPG-KO causes a difference in a particular tissue).

In Figure 5, we individually compare the BMP content of specific tissues deriving from two groups, LIPG-KO and wild-type littermates, since BMP levels and composition are highly tissue specific (PMID: 32350080). To the best of our knowledge, a t-test should be sufficient in this case.

POINT-TO-POINT RESPONSE

We again want to thank the reviewers for their valuable and constructive comments. Please find the point-to-point response below.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have addressed all my questions and concerns

Reviewer 1 did not ask any further questions.

Reviewer #2 (Remarks to the Author):

In this substantially revised manuscript, the authors have presented essential new data showing that even in the absence of the endolysosomal enzhyme CLN5/BMPS, synthesis of BMP occurs in cells. They offer the interesting hypothesis that in this alternative pathway the potential precursors to BMP, phosphatidylglycerol (PG) and lysoPG (LPG), are first secreted from the cell, then acylated in the circulation to produce hemi-BMP or BMP, respectively, and these are then taken back up into the cell for completion of BMP synthesis from hemi-BMP via lipolytic activity.

There are several strengths of the paper. The authors have provided a great deal of information regarding the substrate specificities of numerous phospholipases/transacylases in cell free systems and in some cases cells. As noted before, they show conclusively that BMP synthesis does not require breakdown of the glycerol-phosphate- backbone/headgroup and that acyl CoA is not a required for acylation, supporting a transacylation reaction. By adding the data in CLN5 deficient cells, they now show that there are multiple pathways involved in the synthesis of BMP. While not surprising, since numerous phospholipids and neutral lipids are known to have multiple biosynthetic routes, these new experiments are an important addition.

What is not well supported, though, is the precursor secretion-acylation-uptake-final synthesis model. Although the authors have added necessary time course studies showing the disappearance of labeled PG and the appearance of hemi-PG, the data do not strongly support precursor-product as a major contributor to BMP synthesis. Also not addressed is the relative importance of the present pathway compared to the CLN5/BMP synthase pathway.

Specific points:

1. Figure 1 raises several questions, the most puzzling is why (1E) the 13C-hemi BMP remains constant from about 6h through almost 20h, while 13C-BMP continues to rise. Expectation for precursor-product would be reciprocal curves. Quantitatively, there is much more 13C-BMP (eg Fig 1D at 10h, about 1nmol/mg) but 20-fold less 13C-hemi BMP (Fig 1E at 10h, about .05nmol/mg).

Additional comments about Figure 1 a) Figures 1H and 1I show data for 2 lipids but the y axis labels indicate values for only one lipid. b) Additionally, please show the unlabeled hemi-BMP in 1E, as is done for BMP and LPG in 1D and 1F.

As outlined by the reviewer, the expected precursor to product relationship in an in vitro experiment tends to be reciprocal for enzymatic reactions that catalyze the direct, single step conversion of the precursor (substrate) to its corresponding product. To-date no single step

conversion of PG to BMP has been reported. In case of the recently reported CLN5-dependent conversion of PG to BMP, a two-step mechanism involving the intermediate LPG was proposed. In this manuscript, we report the CLN5-independent conversion of PG to BMP via the intermediate hemi-BMP, as indicated in Fig. 1C. In a two or multi-step conversion it is often observed that intermediates exhibit a quasi steady state, as their formation and conversion/degradation attain temporary equilibrium. As observed by the reviewer, the intermediate ^{13}C-hemi-BMP in Fig. 1E displays a dynamics steady state concentration after already ~2h, analogous to the dynamics steady state observed for intermediate ^{13}C-LPG in Fig. 1F. The reciprocal precursor to product relationship can be observed between cellular ^{13}C-PG (36:2+36:1) and ^{13}C-BMP levels in Fig. 1D and 1G. Nevertheless, it needs to be taken into consideration that the observed relationship between cellular ^{13}C-PG and ^{13}C-BMP levels is partially obscured due to continued exogenously supply of ^{13}C-PG precursor from the cell medium. Thus, we are of the strong believe that the observed dynamic steady-state of intermediates hemi-BMP and LPG in the described cell based in-vitro assay in Fig. 1D-G is well in line with the proposed two-step mechanism indicated in Fig. 1C. We cannot deduce from this experiment that either hemi-BMP or LPG is a direct precursor of BMP. To be clearer, we modified our statement in the results section (Fig. 1D-G): "Thus, hemi-BMP and LPG accumulation precedes BMP synthesis, suggesting that both metabolites may act as intermediates in BMP synthesis."

Concerning the reviewer's comment, a) about the axis labelling in Fig. 1H and 1I, we agree with the reviewer and corrected the axis labelling accordingly.

Concerning the reviewers comment b) about Fig. 1E, we were not able to detect unlabeled hemi-BMP under the applied experimental conditions. To prevent potential misunderstandings by the inclined reader, we added a statement in the figure legend highlighting that unlabeled hemi-BMP was not detected.

2. As noted before, the mouse data supporting their overall hypothesis remain quite weak, and in fact the authors have now ruled out a role for LIPG, robustly identified in their TLC screen of serum-based data, because of the in vivo data. This cannot help but raise concerns about the physiological importance of the findings overall.

Our observations are not limited to LIPG and strongly suggest that several enzymes of the PNLIP family mediate extracellular hemi-BMP/BMP formation (Fig. 3). LIPG has moderate effects on BMP metabolism, which does not exclude the physiological relevance of the extracellular pathway catalyzed by several serum transacylases. Essentially, we found that other serum transacylases contribute substantially to PG/LPG transacylation reactions.

Cell-based experiments demonstrate that HEK293 cells require either CLN5 or serum transacylases to maintain normal BMP levels. These findings strongly argue for a physiological role of secreted transacylases in BMP metabolism.

We also show that PG is converted into hemi-BMP and BMP by serum transacylases/hydrolase *in vivo*. LIPG contributes to this reaction *in vitro*, but is clearly not rate-limiting in this process, again arguing for a major contribution of other serum transacylases. The contribution of other serum transacylases is now clearly mentioned in the paper: "Thus, serum transacylases can mediate head group acylation of both LPG and PG in vitro. The reaction is catalyzed by LIPG and, to a greater extent, by other transacylases."

We hope that the reviewer agrees that, according to our current knowledge, we cannot predict the physiological importance of different pathways in BMP metabolism as long as *Cln5*-ko mice have not been studied in detail (see also point 7). We currently do not have access to *Cln5*-ko mice.

However, based on personal discussions with other research groups, we know that there is sufficient space for CLN5-independent BMP synthesis pathways.

3. The identification of PLA2G4 enzymes in the screen for hemi BMP synthesis is also very robust (2A), but the changes in cellular Hemi-BMP and BMP upon deletion of the identified family members is modest at best, whether at basal levels or upon incubation with PG substrate, and deletion of the major PLA2G4 found in HEK cells, PLA2G4C (2I), doubles the levels of hemi BMP rather than reducing it (2J-L). This too raises questions about the physiological importance of the proposed pathway.

Our data suggest that HEK293 cells require either CLN5 or secreted serum transacylases to maintain normal BMP levels, implicating a minor role of PLA2G4 enzymes in BMP synthesis in this cell type. This is now mentioned in the discussion: "These observations confirm that CLN5 is the major intracellular BMP synthase in HEK293 cells, which also implies that endogenously expressed enzymes of the PLA2G4 family play a minor role in this cell type."

Nevertheless, gain-of-function experiments in CLN5-deficient cells (precultured in the presence of FBS) suggest that specifically PLA2G4D enhances BMP synthesis. This enzyme is not expressed in HEK293 cells (Fig. 2I). Based on mRNA expression levels, we assume that PLA2G4B and -C are the major cytosolic phospholipases in HEK293 cells (Fig. 2I). These enzymes can catalyze both the synthesis (Fig.2 E&F) and degradation hemi-BMP (Fig. S2K), which can contribute to the changes observed in *PLA2G4B*- and -*C* knockout cells. However, they cannot promote BMP synthesis in CLN5-KO cells (Fig. 4F&G).

4. The PLA2G15 data are important, including consideration of its acting as both a biosynthetic enzyme and a degradative enzyme. Suggest including.

5. The authors interpretations would imply that there no PLA2G15 contribution to BMP synthesis in the CLN5 ko cells. This experiment should be performed.

With regards to reviewer's comments 4 and 5, we agree with the reviewer that PLA2G15 could be important in modulating BMP levels by providing access to or degradation of BMP precursors, catalyzing transacylation reactions, or even degrading BMP. Nevertheless, we found that *CLN5*-KO HEK293 cells cultured in heat-inactivated serum exhibit severe BMP deficiency (Fig. 4I), suggesting that endogenously expressed PLA2G15 is insufficient to maintain normal BMP levels. We performed a gain-of function experiment in *CLN5*-ko cells. Overexpression of PLA2G15 reduced BMP levels in the absence and presence of exogenously added PG (Fig. R1). Furthermore, recent studies (peer-reviewed PMID: 38857781 and preprints PMID: 38895439, PMID: 37986778) suggest that PLA2G15 is involved in BMP degradation and that PLA2G15-deficient cells and tissues accumulate BMP, implicating that it is not essential for BMP synthesis.

PLA2G15 is a lysosomal phospholipase and the central aim of this study is to investigate the relevance of extra-lysosomal BMP synthetic pathways. The investigation of PLA2G15 function in BMP biosynthesis and degradation requires comprehensive studies that extend beyond the scope and objective of this study.

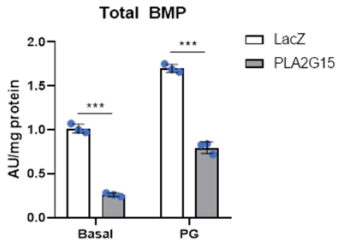


Fig. R1. Overexpression of PLA2G15 in CLN5-KO cells reduces BMP levels. *CLN5*-KO HEK293 cells overexpressing PLA2G15 or LacZ as control were incubated for 8 h in the absence or presence of 18:1 PG (50 µM). BMP content of cells was analyzed by LC-MS. Data are presented as mean ± SD (n=3). Statistically significant differences were determined by two-way ANOVA followed by Bonferroni post hoc analysis (level of statistically significant difference is ***p < 0.001).

6. Cell lysate experiments were used to examine the pH dependence of the conversion of PG to hemi-PG, and the results show a neutral to slightly basic pH optimum. Authors conclude that PG acylation occurs outside of acidic organelles. How does the PG, which is presumably taken up as vesicles or lipoproteins in situ via endocytosis, get outside of the endocytic vesicle system?

Where are the transacylases localized within the cell, and how do the authors propose that PG coming in from the circulation by endocytosis is accessed? The lysate experiments support a cell-based conversion, but does this occur in intact cells for PG added externally (or coming in from the serum which is part of the authors' proposed scheme)?

We agree with the reviewer that cellular PG uptake has been studied insufficiently to-date. We are also in agreement with the reviewer that one of the potential uptake mechanisms of extracellular PG might presumably proceed via endocytosis of PG vesicles. Though considering the current state of the literature, it seems too early to exclude potential alternative pathways or mechanisms.

It has been reported previously, that BMP is associated with a sub-population of extracellular vesicles produced by HEK293 cells (PMID: 32659447). It thus seems reasonable to consider that LPG (PG was not detected) can be secreted from late endosomal/lysosomal compartments via a similar mechanism.

As outlined in the manuscript, the studied PLA2G4 members, except PLA2G4C, are cytosolic enzymes that bind to membranes of organelles in a Ca^{++}-dependent manner. PLA2G4C, which does not contain C2 domains, is present on ER membranes and lipid droplets. PLA2G4-dependent PG/LPG acylation requires the presentation of the PG substrate on membranes accessible for cytosolic enzymes. Published data strongly suggest that PG can escape lysosomal degradation, since PG supplementation also increases cardiolipin synthesis (PMID: 29559686, PMID: 35504533). Unfortunately, the mechanism of lysosomal phospholipid export is poorly investigated. SPNS1 is a proton gradient-dependent transporter of Lyso-PC (PMID:36161949, PMID: 37075117) and a similar mechanism could exist for other phospholipids. Another possible export mechanism is the back-fusion or retrofusion of lumenal membranes (PMID: 34520711). In this process, phospholipids of intralumenal vesicles become part of the limiting membranes of endosomes, which is accessible for cytosolic phospholipases/transacylases and lipid transfer proteins.

The above discussed aspects have been added to the discussion, but final conclusion of PG/LPG uptake and secretion pathways will require extensive investigation in future studies and considerably exceeds the scope and objective of this study.

7. While the CLN5 ko data add important support to the idea that there are multiple ways for BMP to be made, data in Figure 4 seem to suggest that the non-CLN5 path is not major. It is suggested that the data for panels B-E be rearranged to two panels, one showing BMP levels in WT and ko in the absence and presence of PG (all with the same y axis), and the other showing LPG levels similarly,

and all with the same y axis). What becomes apparent is that in the WT cells, PG leads to 2.5 fold increase in BMP whereas in the CLN5 ko, the increase only about 35%. The relative importance/contributions of the different pathways should be explicitly considered.

Our data confirm that CLN5 is the major intracellular BMP synthase in HEK293 cells, as CLN5-ko cells cultured in hiFBS lack BMP. However, in the presence of FBS and ectopically expressed extra-lysosomal transacylases, CLN5-KO cells maintain normal BMP levels, which strongly indicates the presence of alternative BMP synthesis pathways. We have updated the discussion part of the manuscript to clearly mention this fact:

"Our experiments suggest that extra-lysosomal transacylation reactions can deliver BMP or precursors thereof in addition to lysosomal CLN5[22], indicating functionally overlapping pathways. To distinguish between acidic and neutral pathways, we investigated the role of secreted and cytosolic transacylases in BMP synthesis using CLN5-KO cells. These cells exhibit normal BMP content under standard conditions and severe BMP-deficiency when cultured in hiFBS lacking transacylase activity. Our observations confirm that CLN5 is the major intracellular BMP synthase in HEK293 cells, which also implies that endogenously expressed enzymes of the PLA2G4 family play a minor role in BMP synthesis in this cell type. Furthermore, our findings suggest that BMP synthesis in CLN5-KO cells is strongly dependent on secreted transacylases. We observed that overexpression of PNLIP enzymes increases the capacity of CLN5-KO cells to convert PG into BMP back to wild-type levels. Regarding the PLA2G4 family, only the overexpression of PLA2G4D led to increased BMP levels in CLN5-KO cells, which is not expressed in HEK293 cells. Overall, our observations indicate that secreted transacylases and cytosolic PLA2G4D can promote cellular BMP synthesis independently of CLN5 (summarized in Figure 7A)."

We also agree with the reviewer that CLN5-KO cells exhibit a strongly reduced capacity to convert PG into BMP. To emphasize this difference, we additionally calculated the difference between basal and PG-induced BMP content (Fig. 4E). We now write: "The PG-induced increase in BMP levels of CLN5-KO cells was reduced by 74 % compared to wild-type controls (Fig. 4E), confirming that CLN5-KO cells have a strongly reduced capacity to produce BMP"

In regard to the reviewer's comment "The relative importance/contributions of the different pathways should be explicitly considered": As mentioned above, we cannot predict the physiological importance of different pathways in BMP metabolism as long as *Cln5*-ko mice have not been studied in detail. Currently, it is unknown whether *Cln5*-ko mice lack BMP in specific tissues or have unchanged BMP levels. Published data on *Cln5*-ko mice suggest that the severity of the lysosomal storage disease in different tissues ranges from not to severely affected. Our data suggest that extra-lysosomal pathways can protect from BMP-deficiency in *Cln5*-ko tissues, likely in a tissue specific manner.

8. Figure 5—Mouse data must be placed in a separate figure, not bundled along with in vitro data.

Following the reviewer's suggestion, we have now separated Fig. 5 into two Figures, partially separating in vitro and in vivo results. The new Fig. 6 is entitled "Circulating PG is incorporated into tissue BMP stores".

Based on interrelated questions, we did not separate Fig. A-C from Fig. D-G. In Fig. A-G, we investigated the following interrelated questions: (A-C) Can mouse serum transacylases convert PG/LPG into hemi-BMP/BMP *in vitro* and is LIPG involved in this process? (D-F) Can mouse serum transacylases convert PG/LPG into hemi-BMP/BMP *in vivo* and is LipG involved in this process? (G) Does LipG-deficiency affect tissue BMP levels?

We now clearly mention that experiments shown in Fig. A-C are in vitro experiments. "Thus, serum transacylases can mediate head group acylation of both LPG and PG *in vitro*. The reaction is catalyzed by LIPG and, to a greater extent, by other transacylases".

9. An overall issue that comes up with the manuscript, which contains a laudable, enormous amount of data, is that it is difficult to follow which evidence is obtained from cell-free experiments, which from lysates, which from intact cells, which from naïve cells and which from cells with overexpression of enzymes, and which from mouse. Each paragraph is well written, that is not the issue, but the overall flow does not adequately distinguish between the levels of evidence that support (or do not support) the scheme presented in the title and Figure 6.

In accordance with the reviewer's suggestion, we made several corrections which indicate the level of evidence. Furthermore, we modified the legend of Figure 6 (now Fig. 7).

Other comments:
Methods and figure legends—The authors have mostly clarified their procedures and the actual concentrations of various reagents used. Still, some of the assays are missing information. The transacylase assays of cell supernatants and plasma do not indicate time of incubation, nor temperature. The transacylase assay for cell lysates uses a single time point. Was linearity with time and linearity with protein concentration established? Same for the other two transacylase assays. Hemi-BMP synthase activity, a single 4h time point was used; was linearity for time and protein established? Hydrolase activity assay, a single 30 min time point was used; was linearity for time and protein established? Hemi-BMP hydrolysis by partially purified PLA2G4, a single 30 min time point was used; was linearity for time and protein established? Hemi-BMP hydrolysis by PNLIP enzymes, no incubation time is provided; was linearity for time and protein established?

Figure S1, COS cells incubated with phospholipids—for how long?

Figure 1H through K, what was the incubation time for these data?

Figure 2 most panels, times of incubation are not provided.

In accordance with the reviewer's suggestion, we now added the reaction/incubation conditions for each experiment to figure legends.

Regarding linearity, reaction conditions are based on dose- and time-dependent experiments using lysates, semi-purified enzymes, or conditioned media as source of enzymatic activity. Some examples are shown below (Fig. R2 to R4). We did not consider linearity in some assays. E.g., assays shown in Fig. 2B were performed to monitor the products of PLA2G4D and -E catalyzed reactions. Transacylation activity in DMEM/10% FCS was determined to monitor hemi-BMP/BMP formation under settings mimicking cell culture conditions (Fig. 3A).

Dose-dependent experiments revealed a linear increase of BMP, BDP, and LPG in the presence of purified PLA2G4D up to 4 µg enzyme/reaction (Fig. R2A&B). It must be considered that PG transacylases also act as hydrolases and generate reaction products that can be further metabolized. E.g., the major product hemi-BMP can be acylated to BDP or hydrolyzed to BMP (or PG). Therefore, the reaction is only linear up to 1.5 µg enzyme/reaction for hemi-BMP (Fig. R1A&B). Time-dependent experiments revealed that BMP and BDP formation increases linearly up

to 120 min using 1 µg PLA2G4D/reaction, while hemi-BMP formation increased linearly up to 60 min (Fig. R2C&D).

Digestion of hemi-BMP with conditioned (serum-free) cell supernatants containing recombinant LIPH resulted in a dose-dependent, linear increase in FA release up to 40 µl medium (Fig. R3A&B). The generation of BMP and PG was linear up to 20 µl, because accumulating intermediary reaction products were further degraded (note that LPG becomes visible at 40µl). Using 20 µl conditioned medium/reaction, FA, BMP, and PG formation increased in a linear manner up to 120 min (Fig. R3C&D).

PLA2G4C exhibits moderate hemi-BMP synthase activity using PG as substrate in comparison to other enzymes of the PLA2G4 family (Fig. S2E). We observed that the enzyme is able to hydrolyze PG in dose- and time-dependent manner (Fig. R4).

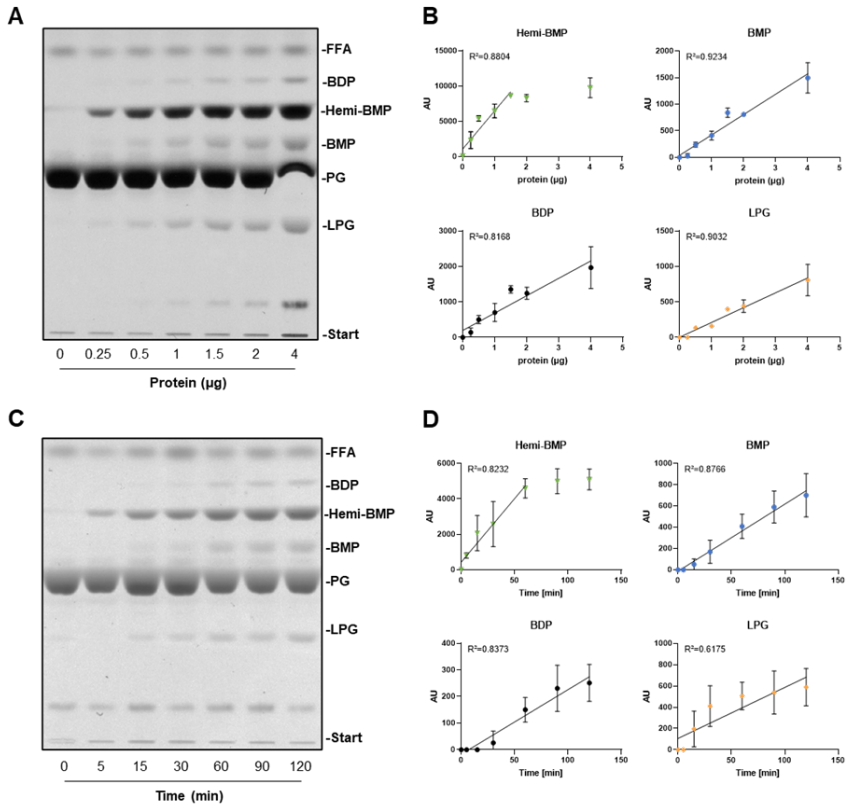


Figure R2. Dose- and time-dependency of PLA2G4E-catalyzed reactions. (**A&B**) Dose-dependent product formation in the presence of the indicated amounts of partially purified murine PLA2G4E/reaction. The final reaction conditions were 1 mM 18:1 PG, 1% FA^{-}-BSA, in a total volume of 40 µl (pH 7.4). Samples were incubated for 1 h at 37°C. The reaction was stopped with MTBE/MeOH and lipids were analyzed by TLC. (A) Representative TLC image. (B) Densitometric analysis of reaction products shown in (A) (n=3). (**C&D**) Time-dependent product formation using 1 µg of partially purified PLA2G4E/reaction. Samples were incubated with 18:1 PG under the same reaction conditions as described in (A) for the indicated time periods. (C) Representative TLC image. (D) Densitometric analysis of reaction products in (C) (n=3). Linear regression analysis was performed in GraphPad Prism 8.0.1.

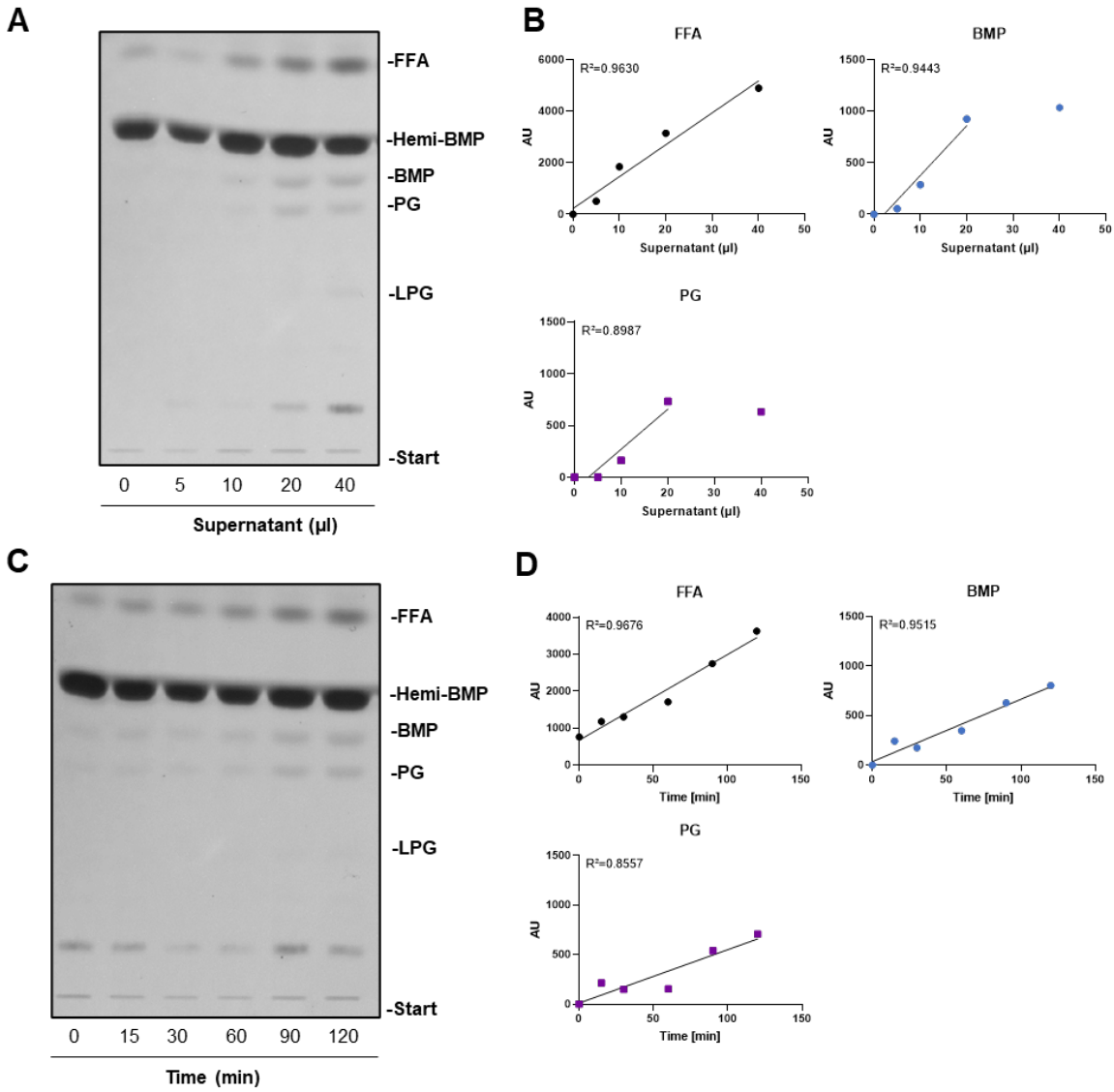


Fig. R3. Dose- and time-dependency of LIPH-catalyzed reactions. (**A&B**) Indicated amounts of conditioned medium containing recombinant murine LIPH were incubated with tri-oleoyl hemi-BMP for 1 h at 37°C. The final reaction conditions were 0.25 mM hemi-PG, 1% FA-BSA, and indicated amounts of medium. The reaction was stopped with MTBE/MeOH and lipids were analyzed by TLC. (**A**) Representative TLC image. (**B**) Densitometric analysis of reaction products in (**A**). (**C&D**) 20 µl of conditioned medium was incubated with hemi-BMP (final concentration and reaction conditions same as in **A**) for the indicated time periods. (**C**) Representative TLC image. (**D**) Densitometric analysis of reaction products in (**C**). Linear regression analysis was performed in GraphPad Prism 8.0.1.

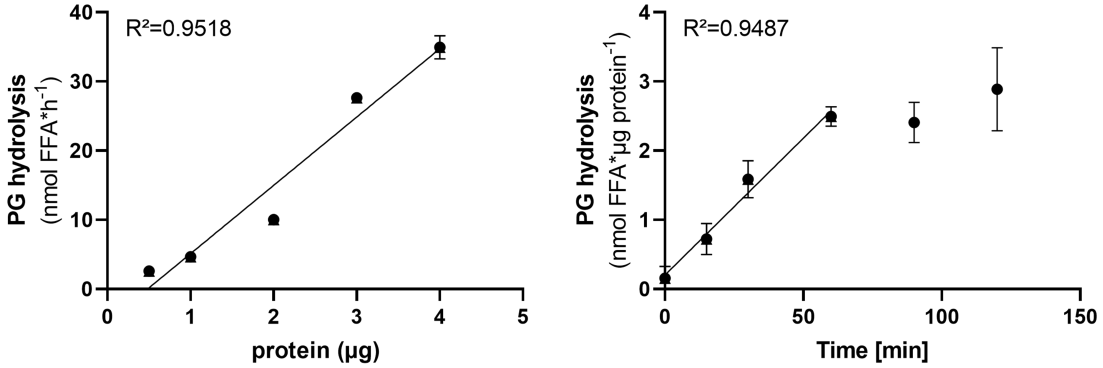


Fig. R4. Dose- and time dependent PG hydrolysis by PLA2G4C. (A) Dose-dependent FA release of 18:1 PG (1 mM) upon incubation with partially purified PLA2G4C for 1 h at 37°C. (B) Time-dependent FA release of PLA2G4C (2 µg) incubated at 37°C for the indicated time periods. FA release was determined using an enzymatic kit (NEFA-HR(2) Assay, Wako Chemicals). Data are presented as mean ± SD (n=3). Linear regression analysis was performed in GraphPad Prism 8.0.1.

Reviewer #3 (Remarks to the Author):

In the revised manuscript, the authors have included the results of new experiments on some of the points raised by the reviewers. The reviewer believes that this has improved the paper to some extent. Although the authors provide evidence in this paper for the involvement of the PLA2G4 family and secreted enzymes in the production of BMPs, most of the enzymatic aspects remain unclear. Thus, the main contribution of this paper is limited to the demonstration of a lysosome/CLN5-independent pathway for BMP production. In this regard, the authors have added experiments using CLN5 KO cells, which add some certainty to the claim of the existence of a CLN5-independent BMP production pathway. In light of this, the reviewer considers this revised paper to be worthy of publication to the community.

Reviewer 3 did not ask any further questions.

Point by point response

The authors have provided reasonable answers to most of the reviewer questions and comments, and have made additional minor modifications to the wording of their interpretations. Several statements are now somewhat less definitive, commensurate with remaining uncertainties. The data presentation, including clarification of in vitro versus in vivo results, is also improved now (the combination in new Figure 6 is fine). The omissions/clarifications in methods have been largely dealt with.

The following final suggestions are made:

1. The following title is more representative of the data and would be better at putting the present findings in context of current literature: The endolysosomal phospholipid BMP *can be* synthesized via *cytosolic* and extracellular pathways, *in addition to lysosomal generation*. (or lysosomal synthesis). The title closest to what was actually demonstrated in this manuscript is the final phrase of the Abstract: Functionally overlapping pathways promote BMP synthesis in mammalian cells.

*Following the suggestion of reviewer 2, we changed the title of the manuscript to "**Functionally overlapping intra- and extralysosomal pathways promote bis(monoacylglycero)phosphate synthesis in mammalian cells**".*

2. The PLA2G15 data provided in review should be incorporated into the manuscript; at the least, in Supplementary Information, and in that case it should be referred to and well-discussed in the main manuscript. Again, this would serve to underline the multiple pathways (and seemingly spatially separated pathways) of BMP synthesis, and provide better integration with the current literature.

We would like to exclude PLA2G15 experiments from the manuscript because they are beyond the scope of the study and lack novelty. The objective of this study is to investigate extra-lysosomal BMP synthesis pathways, whereas PLA2G15 acts as lysosomal phospholipase. The observation that PLA2G15 can generate LPG in lysosomes acting as precursor for BMP is not new and has been extensively discussed in previous studies (PMID: 36823305, PMID: 38447580, PMID: 37708259). Furthermore, recent comprehensive studies on PLA2G15 function suggest that the enzyme is primarily involved in BMP degradation and not BMP synthesis (PMID: 38857781, PMID: 38895439).