Response to Reviewers' Comments

Uncovering the Functional Roles of Plasma Membrane Proteins in Foraminiferal biocalcification

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We thank Dr. Marleen Stuhr for the careful evaluation of our manuscript and for the constructive and insightful comments. We greatly appreciate the time and expertise devoted to reviewing our work. Below, we provide a detailed point-by-point response to each comment and outline the revisions that we propose to incorporate into the revised manuscript in order to address the issues raised and improve the clarity and interpretation of our study.

Dr. Marleen Stuhr Comments	Response
1. Absence of biological replication renders proteomic conclusions unreliable: The most fundamental limitation of the study is the apparent absence of biological replication. Based on the Methods, specimens were collected from a single sampling event at a single intertidal site in February 2024. The protein extraction appears to have been performed on one pooled sample per fraction (total, cytoplasmic, membrane-associated), with no indication of independent biological replicates. No statistical analysis is presented for the proteomic data. From my perspective, for mass spectrometry-based proteomics, biological replication is not optional, but the minimum requirement for assessing whether detected proteins are consistently present rather than artefacts of extraction, contamination, or preparation. Without at least three independent biological replicates, it is impossible to determine whether the protein identifications are reproducible, to apply any form of statistical testing, or to make quantitative comparisons between fractions. The authors acknowledge that their analysis is qualitative, but this does not circumvent the need to demonstrate reproducibility of detection across independent samples. Hence, the authors are encouraged to: (i) provide clarity on the number of independent extractions performed, (ii) if replication was indeed absent, explicitly acknowledge this as a major limitation of the study, and (iii) refrain from drawing strong mechanistic conclusions from non-replicated proteomic data.	We agree that independent biological replication is essential for robust statistical and quantitative proteomic analyses. We would first like to clarify the sampling procedure and the rationale for selecting the study site. Surface sediment was collected randomly from multiple points across the intertidal site to obtain representative material and to ensure the recovery of the large number of foraminiferal specimens required for the analyses. The site was selected because previous ecological observations indicated that the target coastal foraminiferal taxa are consistently present and abundant in this area. In addition, independent surveys conducted at the same site in 2024 and 2025 documented the recurrent presence and high abundance of the same taxa (Costanzi, 2026, PhD thesis). The sampling and experimental approach followed the procedures described by Prada et al. (2024 https://doi.org/10.1073/pnas.2417845121). Although the use of specimens collected from a single site limits the broader ecological generalization of the results, the purpose of the present study was not to investigate spatial variability among populations or sites. Rather, the aim was to provide a preliminary qualitative characterization of the proteins identified in different cellular fractions and to establish an initial protein inventory for abundant coastal foraminiferal taxa, including the cosmopolitan and symbiont-free genus *Ammonia*. This preliminary inventory may provide a

	useful baseline and starting point for future replicated and comparative studies. Accordingly, the study was not designed as a quantitative comparative proteomic analysis. Our interpretation of the LC-MS dataset was based on protein identification and functional annotation, without statistical comparisons of protein abundance. Nevertheless, we acknowledge that the LC-MS proteomic analysis was performed without independent biological replicates. We agree that this limits the assessment of the reproducibility of protein detection and precludes statistical evaluation of the proteomic dataset. We will therefore explicitly acknowledge this limitation in the Discussion and revise the manuscript to avoid overinterpretation or strong mechanistic conclusions, presenting the proteomic findings instead as preliminary and exploratory observations. However, we would like to clarify that the absence of independent biological replicates applies specifically to the LC-MS proteomic analysis. The protein extraction procedures and subsequent Western blot analyses were independently repeated on different dates to verify the reproducibility of sample preparation and immunodetection. To document this procedural reproducibility, we have included the blot images obtained from the different extractions performed on the indicated dates using the two annexins, A13 and A6 (Fig. R1). Should the reviewer consider it appropriate, these figures can also be included in the Supplementary Material or in an appendix to the revised manuscript. Finally, we will revise the Materials and Methods section to distinguish more clearly among the sampling procedure, the LC-MS proteomic analysis, the repeated protein extractions, and the Western blot experiments, thereby avoiding any ambiguity regarding the extent and type of replication performed in the study.
2. Detection of a protein does not establish its functional role in calcification: Throughout the discussion, functional roles in biomineralization are inferred from the mere presence of proteins in the foraminiferal proteome (which btw is also always a holobiont proteome). This logical leap is is a stretch too far from the data. For example, the detection of Annexin A13 in the membrane-associated fraction is used to suggest a role in calcium-dependent vesicular trafficking	We agree that the detection of a protein alone does not demonstrate its direct functional involvement in biomineralization and that our original wording may, in some instances, have implied stronger conclusions than warranted by the data. The primary objective of this study was to provide a qualitative characterization of the protein repertoire associated with different cellular fractions and to discuss these findings in the context of the current literature on foraminiferal biomineralization. Our

during calcification, yet no experiment is presented that links annexin abundance, localization, or activity to the calcification process itself. The same reasoning is applied systematically throughout the discussion, where the presence of Hsp70, Hsp90, Rab GTPases, Na^+/K^+-ATPase, clathrin, and numerous metabolic enzymes is taken as evidence for their involvement in biocalcification. Many of these proteins are ubiquitous housekeeping proteins present in virtually all eukaryotic cells regardless of calcification status and may fulfil a variety of functions. Establishing a functional link to calcification would require, at minimum, one of the following: (a) differential abundance between calcifying and non-calcifying conditions; (b) spatial co-localization with active calcification sites (e.g., by immunofluorescence or immuno-EM); or (c) functional perturbation (e.g., inhibition of a target protein with demonstrated impact on test formation). The authors briefly acknowledge this in their statement that "functional studies are required to directly test this still speculative hypothesis", but this caveat is not consistently applied throughout the text, where the language frequently implies functional roles as if they were established findings. Therefore, I strongly suggest to substantially revise the manuscript to consistently and explicitly distinguish between what was detected and what is hypothesized to function. Phrasing such as "consistent with a role in" or "may contribute to" should replace language that implies established function.	intention was therefore to propose biologically plausible hypotheses rather than to establish causal functional relationships. In this context, the proteomic analyses presented by Prada et al. (2024) appeared particularly relevant to our biological model and provided a valuable methodological and interpretative framework for the present investigation. We therefore chose to follow a comparable approach to facilitate comparison between the two studies and to assess whether potentially common patterns could emerge from the respective datasets. Following the reviewer's suggestion, we will carefully revise the Discussion throughout the manuscript to clearly distinguish between protein detection and inferred biological function. Statements implying established roles will be replaced with more cautious wording, such as "may contribute to," "is consistent with a possible role in," "could be involved in," or "represents a candidate protein potentially associated with." We will also reinforce the statement that functional studies, including protein localization, differential expression analyses, and functional perturbation experiments, will be necessary to validate these hypotheses and establish the involvement of these proteins in biomineralization. We believe that these revisions will provide a more balanced interpretation of our findings while preserving the exploratory nature of the study.
3. Prior large-scale foraminiferal proteomics studies are inadequately cited: The literature review and citation practice in this manuscript are selective in ways that appear to disadvantage prior work. Although I am not feeling comfortable as a reviewer telling authors to include our studies, it cannot go unnoticed that our two large-scale quantitative proteomics studies on large benthic foraminifera (Stuhr et al. 2018, Scientific Reports; Stuhr et al. 2021, Oceans) are either not cited or are cited only in passing, despite the fact that the majority of proteins discussed in this manuscript as potentially novel foram proteins were already identified in those studies, and in some cases even discussed in the context of calcification and vesicular transport. A non-exhaustive comparison with the protein inventory from Stuhr et al. (2018) and Stuhr et al. (2021) reveals (check the supplementaries; all data is also deposited in	We agree that our discussion did not sufficiently acknowledge previous large-scale proteomic studies on larger benthic foraminifera, particularly those by Stuhr et al. (2018) and Stuhr et al. (2021), which represent important contributions to the characterization of the foraminiferal proteome. Following the reviewer's suggestion, we will thoroughly revise the manuscript to better integrate these studies throughout the Introduction, Discussion, and Table 1. In particular, we will add the appropriate citations where proteins or functional pathways identified in our study had already been reported or discussed in previous proteomic analyses of foraminifera. We will also carefully re-evaluate statements regarding novelty and modify the text where appropriate to avoid implying that proteins or pathways previously identified in foraminifera are

proteomeXchange) that the following proteins from the current manuscript's Table 1 were detected in those prior studies: **Tubulins (α and β):** Identified and regulated in both Stuhr et al. (2018) and Stuhr et al. (2021); discussed in the context of cytoskeletal dynamics and intracellular transport; **Actin isoforms:** Identified and regulated in both prior studies; **Heat Shock Proteins 70 and 90:** Central regulated proteins in both studies; their roles in protein quality control under stress are discussed extensively; **Polyubiquitin:** Detected in Stuhr et al. (2018) as a regulated host protein; **Clathrin heavy chain:** Detected in Stuhr et al. (2018) and (2021); **Na^+/K^+-ATPase and alpha-2 subunit:** Detected in Stuhr et al. (2018) in the host (Reticulomyxa filosa-assigned cluster), and further examined in Stuhr et al. (2021); **Rab2/RabB-family GTPase:** Detected in Stuhr et al. (2018); **Rab7:** Detected in Stuhr et al. (2018) in the host compartment... this is in fact the protein already cited as "Stuhr et al. 2021" in Table 1 of the current manuscript, yet the 2018 study, where it was first reported in this organism, is not cited; **14-3-3-like protein:** Detected in Stuhr et al. (2018); **Isocitrate dehydrogenase, aconitase, succinate-CoA ligase, dihydrolipoamide dehydrogenase:** All detected in Stuhr et al. (2018); **Calponin homology domain-containing protein:** Detected and regulated in Stuhr et al. (2018); **Kinesin-like protein:** Detected in Stuhr et al. (2018); **Annexin VI (Annexin 6):** Also detected in Stuhr et al. (2018) (Cluster 6, host-assigned Reticulomyxa sequence), though not prominently discussed. Therefore, the claim that the current study provides "the first" characterization of several of these proteins or pathways in foraminifera is not always supported. So, the authors are asked to conduct a thorough review of these and other (see refs therein) prior studies and to appropriately cite them throughout the manuscript, including in the Introduction, in Table 1, and wherever specific protein families or pathways are discussed. Claims of novelty should be re-evaluated in light of prior work.	reported here for the first time. Our intention was to emphasize the characterization of these proteins in the specific biological context and experimental framework of the present study, rather than to claim their first identification in foraminifera. In this way, the revised manuscript will clearly distinguish between previously reported proteins and the novel aspects of our work.
4. The fractionation approach is the genuine methodological contribution, so it should be presented as such: The most defensible novel contribution of this study is the development of a protocol that isolates membrane-associated proteins from soluble proteins in a small number of foraminiferal specimens. This is a meaningful technical advance, as most prior	Following the reviewer's recommendation, we will revise the manuscript to better emphasize the development and application of the fractionation protocol as the primary methodological advance of this work. We will clearly highlight that this approach enables the separation of soluble and membrane-associated protein fractions from a limited number of

foraminiferal proteomics work (including the studies above) used total protein extracts or shell organic matrix preparations. The fractionation approach has genuine potential for addressing questions about the subcellular localization of proteins. From my perspective, this contribution is currently obscured by over-interpretation of the proteomic data and by novelty claims that do not hold up against the existing literature. The authors are encouraged to restructure the draft to foreground the fractionation protocol as the central contribution, and to frame the protein identifications, including the annexin finding, as hypothesis-generating observations that motivate future targeted experiments, rather than as evidence of specific functional roles.	foraminiferal specimens, providing a useful framework for future investigations of subcellular protein distribution. Consistent with the reviewer's previous comments, we will also substantially revise the Discussion to present the identified proteins as exploratory, hypothesis-generating observations rather than as evidence of established functional roles in biomineralization. In particular, the discussion of Annexin and other candidate proteins will be rewritten using more cautious language, emphasizing that these proteins represent promising targets for future localization and functional studies rather than definitive components of the calcification machinery. We believe that these revisions better reflect both the scope and the principal contribution of the present study.
5. XANES-based interpretation of the POS origin should be framed as a hypothesis: The STXM/XANES analysis is technically sound and represents a genuinely informative dataset. The spectral signatures of the POS seem correctly described as consistent with lipidic compounds. The interpretation that this signature reflects a plasma membrane-derived origin for the POS is an interesting and testable hypothesis. Nevertheless, the current discussion sometimes presents this interpretation as if it were established: for example, "we propose that the POS represents an organic layer likely derived at least in part from the plasma membrane" appears in the discussion as a fairly firm conclusion, while later the authors acknowledge it as a hypothesis. The spectral data are consistent with a lipid-rich composition but cannot definitively distinguish plasma membrane origin from, for example, intracellular membrane remnants or secreted lipoproteins. This should be more clearly acknowledged.	We agree that the spectral data support a lipid-rich composition of the POS but do not, by themselves, allow us to unequivocally determine its origin. Following the reviewer's recommendation, we will revise the Discussion to consistently present the proposed plasma membrane origin of the POS as a hypothesis rather than as an established conclusion. Statements that could be interpreted as definitive will be rephrased using more cautious language (e.g., "is consistent with", "may derive from", or "supports the hypothesis that"), and we will explicitly acknowledge that alternative explanations, including contributions from intracellular membrane remnants or other membrane-derived or secreted lipid-rich structures, cannot be excluded based on the present data alone. We believe these revisions provide a more balanced interpretation of the STXM/XANES results while preserving the biological significance of the proposed hypothesis.
Dr. Marleen Stuhr Minor Comments	**Response**
The 90 µm sieve for removing juveniles is described as separating "adult specimens," but the developmental stage of individuals post-sieving is not confirmed by any measurement. This should be stated more carefully.	We agree that the 90 µm sieve does not unequivocally distinguish adults from juvenile individuals. We will therefore revise the text to avoid referring to the retained specimens as "adult" and instead describe them as specimens larger than 90 µm, which were selected to minimize the inclusion of early juvenile stages.

The number of specimens used for Western blot (200) versus mass spectrometry (500) differs substantially. The impact of this on relative protein representation between fractions should be briefly addressed.	The different number of specimens reflects the distinct protein input requirements of the two analytical techniques. LC–MS analysis required a larger amount of starting material to obtain sufficient protein yield for comprehensive protein identification, whereas Western blot analysis required substantially less protein. Both sample sets were processed using the same fractionation protocol, as described in the Materials and Methods.
The discussion references Prada et al. (2024) extensively as a precedent for the proteomic framework, but this relationship should be described more precisely: is the present study replicating that framework, extending it, or comparing results... ? If it is used so excessively, it would be helpful to have more insights from that work than the one sentence in the intro.	We agree with the reviewer that the relationship between the present study and Prada et al. (2024) should be described more clearly. The study by Prada et al. (2024) was used primarily as a methodological reference, particularly with regard to sediment sampling and the general proteomic framework. As indicated in our response to the previous comment, adopting a broadly comparable approach was intended to provide a useful basis for comparison between the two datasets. At the same time, we consider the present study to extend the framework proposed by Prada et al. (2024), particularly through the use of a selective protein-extraction protocol. We chose a genus that does not host symbionts, in contrast to Prada's work, which analyzed a genus that does host symbionts. In addition to investigating a different foraminiferal species, our approach allowed the detected proteins to be assigned to operationally distinct soluble and membrane-associated fractions. This provides an additional level of cellular information that was not resolved in the study by Prada et al. (2024), in which these protein fractions were not distinguished.
The introduction states that "targeted proteomic analyses were performed", but I am not sure I understand what is meant by this. Targeted proteomics usually involve something like label-based quantitation of specific proteins, which is not describe in the methods (but might be an interesting approach to consider).	We agree that the term "targeted proteomic analyses" was inappropriate in this context and may cause confusion with targeted quantitative proteomic approaches. The text will be revised accordingly to describe the analyses as qualitative LC-MS proteomic analyses.
L103: what kind of eppendorff tubes were used? With such small amounts of proteins I highly recommend protein LoBind tubes, especially since some proteins may preferentially bind to the tube, getting underrepresented.	Standard microcentrifuge tubes were used throughout the study. We acknowledge that LoBind tubes may further reduce protein adsorption and represent a valuable consideration for future studies.
L106: which protease inhibitor cocktail was used? where was it purchased from? For such delicate techniques, all relevant materials and chemicals must be named with provider and country of origin (other missing below).	The manufacturer, composition and country of origin of the protease inhibitor cocktail will be included in the Materials and Methods.

L195-196: this reads as if the bubble formation is intended and improving the protocol, although I assume the opposite is the case. Please clarify,	We will revise the text accordingly to clarify that cavitation is not an unwanted phenomenon but rather a well-described effect of ultrasonic treatment that can promote solid–liquid extraction by generating localized mechanical forces and improving the disruption of biological material (Betti et al., 2021; Santos et al., 2008). We will also modify the wording to avoid any possible confusion between acoustic cavitation and the accidental formation of air bubbles during sample processing.
L202: spell genus name in full at first mention	We follow this recommendation and write "*Reticulomyxa filosa*" the first time it is mentioned.
L220: what were the thresholds and specifications for the database search, the reference taxa?	All missing parameters will be included in the revised manuscript.
L231: please write abbreviations in full at first mention, and what do you mean by foraminiferal walls? Cell walls? shells?	The terminology will be revised to refer consistently to the foraminiferal test (shell) rather than "wall", improving clarity for a broader readership.
Sometimes (e.g. L335, L362) functional implication statements do not have any references, which would strengthen your interpretation.	Additional bibliographic references and figure citations will be included, where appropriate, to support the functional interpretations presented in the Discussion. **Prada et al. (2024)**, *Proteomic characterization of a foraminiferal test's organic matrix* — DOI: **https://doi.org/10.1073/pnas.2417845121** **Stuhr et al. (2021)**, *Divergent proteomic responses offer insights into resistant physiological responses of a reef-foraminifera to climate change scenarios* — DOI: **https://doi.org/10.3390/oceans2020017** **Stuhr et al. (2018)**, *Disentangling thermal stress responses in a reef-calcifier and its photosymbionts by shotgun proteomics* — DOI: **https://doi.org/10.1038/s41598-018-21875-z**

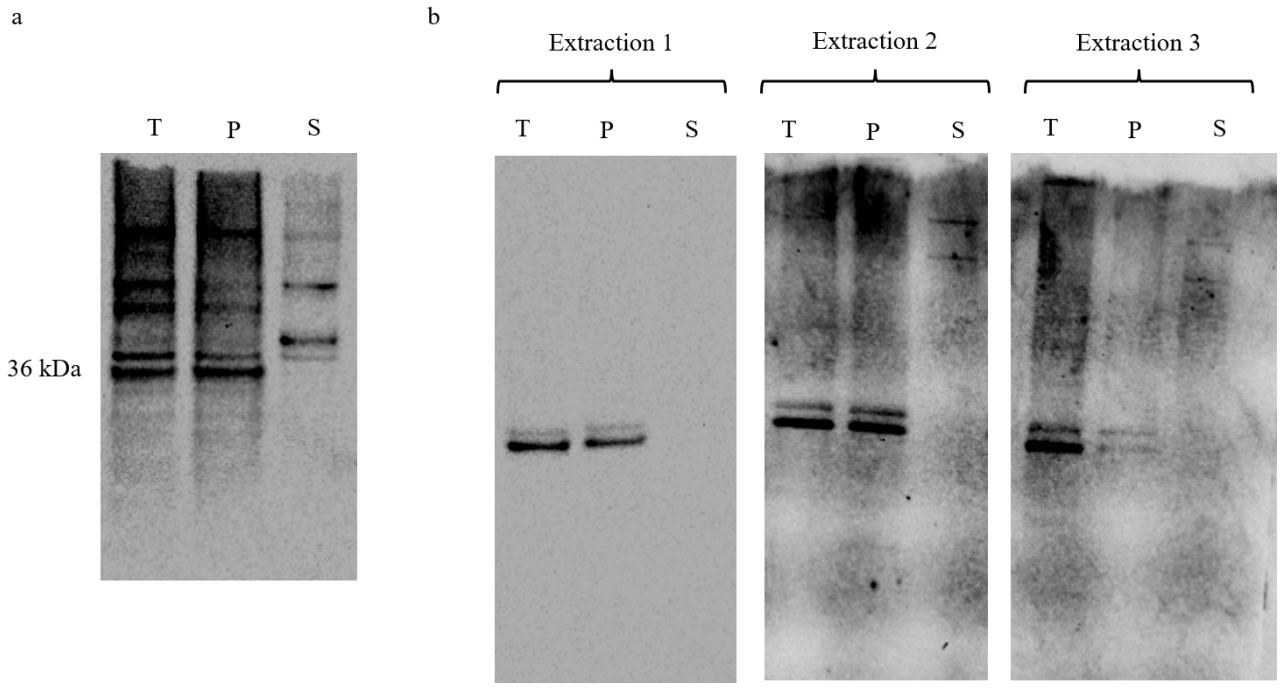

Figure R1. (a) Western blot probed with anti-Annexin-6 antibody showing total, soluble, and plasma membrane-associated protein fractions extracted from Ammonia spp. performed on 07/03/2024. (b) Western blot probed with anti-Annexin-13 antibody using total, soluble, and plasma membrane-associated protein fractions extracted from Ammonia spp. (Extraction 1, performed on 07/03/2024; Extractions 2 and 3, performed on 19/03/2024), showing a single immunoreactive band at approximately 36 kDa in the total and plasma membrane-associated protein fractions. T, total proteins; P, plasma membrane-associated proteins; S, soluble proteins.