nature portfolio

# Peer Review File

# Enamel proteins from six Homo erectus specimens across China

Corresponding Author: Dr Qiaomei Fu

Any redactions in this file are there to maintain patient confidentiality, the confidentiality of unpublished data, or to remove third-party material.

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

**Attachments originally included by the reviewers as part of their assessment can be found at the end of this file.**

Version 0:

Reviewer comments:

Referee #1

(Remarks to the Author)
Summary of key results

This study successfully recovers endogenous enamel proteomes from six Middle Pleistocene Homo erectus specimens (~0.4 Ma) from China. The recovered proteomes exhibit characteristic diagenetic alterations, particularly elevated deamidation, which authenticate their ancientness. Phylogenetically, the analysis relies on the reconstruction of Ameloblastin (AMBN) sequences, identifying two critical Single Amino Acid Polymorphisms (SAPs). First, a novel variant, AMBN-253G, is shared across all six individuals—unifying the Zhoukoudian, Hexian, and Sunjiadong specimens—but is absent in other hominins, marking it as a lineage-specific trait. Second, AMBN-273V is shared with Denisovans, providing proteomic evidence that the "super-archaic" introgression observed in Denisovan genomes could have originated from this East Asian H. erectus population. Additionally, the authors utilize the amelogenin Y isoform (AMELY) for sex determination, identifying five males and one female.

Originality and significance: if not novel, please include reference
I think this is a wonderful work that will be well-received by the academic community, with the potential to engage non-academics. It presents a wonderfully rich dataset to those in the palaeoproteomics field. The samples and their associated taxon are historically and culturally significant, both within the field of palaeoanthropology and beyond, and the data interpretations presented here appear reasonable.
Data & methodology: validity of approach, quality of data, quality of presentation
&
Appropriate use of statistics and treatment of uncertainties
While I am very supportive of publishing this, there is a methodological issue that requires attention. The conclusions of this paper are based on just 1-2 protein sequence mutations (single amino acid polymorphisms (SAPs)), and it appears the authors have made a methodological choice in data analysis that requires further elaboration. It is possible that the data presented here is only identified due to this methodological choice, so I think great care and detail must be directed here. The issue is that the FDR (false discovery rate) is inconsistently applied across samples. The standard is 1%, particularly with the tools applied and specifically in palaeoproteomics. A 1% FDR essentially means 1% of the reported identifications are expected to be false positives (and for context, 1%, while standard and reasonable in proteomics, is already quite high, when thinking about it in comparison to nucleotide sequencing, at least). For the Hexian samples, the authors choose to increase this FDR to 5% and 10%. This type of increase is very rare in our field and risks introducing false positives. Critically, I independently re-processed the authors' raw data using MaxQuant at a strict 1% FDR. My analysis confirmed that the phylogenetically informative variants in AMBN are indeed present even at this stricter threshold (though I did not have time to look closely at their spectra, or the quantity, in the Hexian samples). This indicates that the authors' results are robust, and the decision to relax the FDR is unnecessary. By using inconsistent thresholds (5-10% for some, 1% for others), the authors inadvertently undermine confidence in their results, creating an appearance of outcome-oriented data filtering. To ensure the study is viewed with the credibility it deserves, the authors should standardize the FDR to 1% across all samples.

Conclusions: robustness, validity, reliability
Currently, the conclusions do follow the results, and if the results remain consistent with a more strict FDR threshold, then they are appropriate.
Suggested improvements: experiments, data for possible revision
Recommendations
My recommendation is that the authors must run all analyses with a 1% FDR and report these results in the comparative tables. If the authors choose to relax the FDR, they need to explicitly state this in the main text, and at least a section of the main text and/or methods must be devoted to the theoretical underpinnings of this choice.
Furthermore, all downstream analyses should be rerun with the results of the 1% FDR analyses, alongside potentially the 5% FDR analyses (and 10% if authors choose to still include them). The relevant tables in the manuscript (e.g., Table 1, Supplementary Tables 1 and 2) should also be remade for each FDR threshold. In this case, perhaps the interpretation does not change substantially, but if it does, this should be integrated into the next revision.
Alternatively, if the authors believe the data is still robust with the 1% FDR threshold (as I found it to be), then they can just report those results and modify any interpretation if the informative peptides are not found in the Hexian samples.
Data & Figures
In addition, a higher number of supporting spectra need to be figured, complete with associated ion series coverages. I really would like to see at least a mirror plot for at least 1 specimen from each site, for both of the marker SAPs, and for the potential heterozygous sites.
I would also like to request the authors include all fasta databases they used in the PRIDE upload (perhaps they are uploaded somewhere, and I couldn't find them), and also output files. I see PEAKS output files are available, but I would also like to see the MQ output files and pFind output files, if possible, or at least the MSMS.txt from MQ and the pfind-Filtered.spectra from pFind.
In general, I also wonder if the authors have considered amino acid racemization analyses as a secondary measure of validation, and even just to contextualize these fossils and their degradative state. I am not recommending to do so here—I think the paper is complete without them—I just think it would be really nice to see how these samples (and the Harbin samples referenced herein) look from this perspective.
Line 134: I wouldn't say this is a new discovery—the false positive issue—but something that is 100% expected in proteomic experiments attempting sequence reconstruction or trying to discriminate between similar isoforms/variants. See Welker et al. (2018) for how pernicious this may be, particularly if a database is incomplete or when attempting to distinguish between isoforms. This line is particularly concerning for me because this issue goes well beyond just AMELY and sex-specific peptides. I am a little concerned the authors did not consider the possibility of this false positive rate for the rest of the proteome (though the strict acceptance criteria for sequence reconstruction should be sufficient, I think?). My recommendation is that Welker et al. (2018) and other relevant papers should be cited to contextualize this.
Line 157: I quite like this criterion. I think it follows the ethos of Cappellini et al. (2019) in attempting to establish the authenticity of the sequence residues themselves via fragmentation ions. However, I strongly encourage the authors to employ auxiliary tools for peptide-spectrum match prediction using AI-based tools. Frankly, I think both are important at this stage. I implore the authors to consider these alternative tools here. Perhaps they do not function perfectly with these data (granted, they are primarily trained on modern, tryptic data), but I think such an approach would remove any doubt that these peptide sequences could be misidentified. Even if these are not included in the main manuscript, I think it would be nice to see a couple figured in the response.
Line 597: I think this is the most pressing thing for this manuscript that requires clarification. I believe there are significant problems with FDR within database searches, and certainly for palaeoproteomic studies, but setting FDR >1% with MaxQuant is absolutely a methodological choice that requires more elaboration. Why only for specific samples? My assumption is because they are low-yield, but I still don't know if this justifies the choice. On my initial read, this creates the unintended appearance that the authors have chosen cutoffs to support a desired narrative, as opposed to presenting the data in an unbiased manner. I don't think this is the case—especially given my own re-analysis—but I think this is how other researchers might read this manuscript if the threshold remains inconsistent.
Line 606: I understand the rationale, but I think the counts for different samples cannot be reliably compared if the FDR is not consistent across samples. I think the results from each analysis must be more clearly reported using uniform thresholds, not just the combined total of PSMs across the different analyses.
Line 610: How are heterozygous sites identified? I do not think there is enough groundwork underlying the detection of heterozygous sites in the proteins we observe in modern enamel to assume heterozygosity without a degree of caution. Specifically, if identifying heterozygosity, what are the relative intensity ratios of the peptides representing the two alleles? If one is significantly more abundant than the other (e.g., >10:1), how do the authors rule out minor cross-contamination versus true heterozygosity?
Line 610: What is the specific criteria for choosing one potential allele over the other?
Line 692: PEAKS is not open access; it is proprietary commercial software, even if the results may be visualized freely on their online tool.

References: appropriate credit to previous work
Please see the point about line 134, and additional references must be included once the authors decide how to approach the FDR issues.
Clarity and context: lucidity of abstract/summary, appropriateness of abstract, introduction and conclusions
Everything flows logically in the main text.

Best,
Ryan Sinclair Paterson

Referee #2

(Remarks to the Author)
Fu et al., Enamel proteins from six Middle Pleistocene Homo erectus individuals across southern and northern China

Summary:

This paper investigates the enamel proteomes from three Pleistocene hominins, finding two novel peptide sequence variants in ameloblastin (AMBN). The authors conclude that one shared AMBN variant demonstrates a cohesive Homo erectus population in Eurasia, while the other, found at low frequencies among Denisovans and modern humans, provides evidence of H. erectus introgression into Denisovans.

The authors use a surface etching extraction and LC-MS/MS approach, and conduct triplicate DDA analyses in two laboratories. Peptide spectral matches (PSMs) must be at least two in number and unique to one protein. The authors report substantial PSM counts for most enamel proteins, good sequence coverage for areas of interest, and control blanks to test for contaminants and background.

This is a superb study and should be published: the use of triplicate analyses in two labs and the finding of variants relevant to hominin population history are striking. But some of the excellent data are poorly presented or hardly presented at all, and I suggest major revisions to correct these problems.

First: as noted below, some subset of spectra shown in supplementary figures 1 and 2 should be brought to the main text, with predicted spectra mirrored to provide support for the findings, and the multiple PSMs used to create the consensus sequences in those figures should be included as has been done in some papers, as in the supplementary figures of Paterson et al (2025), cited below.

Second: this work contains a wealth of PSM data on ancient peptides from 9 different enamel proteins, but these are mostly not presented: where do these peptides cluster on the larger protein sequences? What does this tell us about preservation characteristics?

Third: what systematic differences do the authors observe between fragment characteristics in the blank controls, which are essentially not discussed, and the Pleistocene teeth? Are there differences of length, acidity, PSM type and number? What about between sites? Are there differences in results between the two laboratories? These issues are not discussed.

Fourth: the available figures can be condensed and improved, see detailed comments below.

Additional comments:

Introduction

Lines 51-52: It should be noted that stone tools of even greater antiquity have been in Shangchen, in Lantian county:

Zhu, Z., Dennell, R., Huang, W. et al. Hominin occupation of the Chinese Loess Plateau since about 2.1 million years ago. Nature 559, 608–612 (2018). https://doi.org/10.1038/s41586-018-0299-4

Results

Lines 98-104: The authors note both negative (dentin and bone) and positive (enamel) findings of protein signals from MALDI-TOF MS analysis of animal remains. But the authors show no data either in a main text or supplementary figure, nor in main text or supplementary tables. These data, positive and negative, should be shown, or the appropriate paper referenced if the data is already published.

Lines 119-220: Figure 2a and Supplementary Table 2 don't show or report any exogenous contamination at all. Instead, all reported products are from enamel. So the relative abundance of endogenous versus exogenous proteins are not demonstrated by these data. Can the authors be more clear about what they mean with this text? And, is there a location where likely endogenous versus exogenous peptide numbers and sequences are reported?

Lines 132-142: The author's approach to this problem is innovative and commendable.

Lines 149-182: Supplementary Figures 1 and 2 are really at the heart of this paper and need to be shown in the main text. Your y- and b- ion coverage of many of these peptides is quite good, but I strongly urge the authors to show how spectra align relative to predicted spectra for the same peptides. Furthermore, I'd urge the authors to adopt a strategy use by Paterson et al in their supplementary figures S1, S2, and S3, showing both how multiple fragments align, and also, from where your AMBN and AMEL peptides derive on the larger protein:

Paterson, R.S., Mackie, M., Capobianco, A., Heckeberg, N.S., Fraser, D., Demarchi, B., Munir, F., Patramanis, I., Ramos-Madrigal, J., Liu, S. and Ramsøe, A.D., 2025. Phylogenetically informative proteins from an Early Miocene rhinocerotid. Nature, 643(8072), pp.719-724.

Lines 222-239: I agree with the authors in their conclusion about proximity and introgression, and I have two suggestions.

First, I suggest that Fig. 4 and supplementary Fig. 3 be merged into two panels in a single figure in the main text. Second, I suggest that the authors note the frequency of the AMBN-273V variant among Denisovans and Modern Humans/East Asians/Oceanians on Fig. 4, since this is a central point of the paper.

Lines 485-490: The decision to run analyses in triplicate at two labs is superb. Readers would benefit from seeing differences in outputs from these two different labs in the supplementary material: a comparison of methods would benefit similar work by the community moving forward.

Lines 491-536: Can the authors please provide information about the sequence and repetition of blank runs within the larger dataset, in the supplementary information.

Lines 535-536: Can the authors please provide further characterization of the blank control findings in the supplementary information? Do the authors find systematic differences in peptide length, isoelectric point, or PSM frequency or type between blank controls and fossil specimens?

Lines 605-621: While I do not believe it will alter their results, it would be advantageous to report the frequency with which PSMs match microbial contaminants.

Fig. 1 - I strongly urge the authors to include three stratigraphic columns as one or several sub panels in this figure, to illustrate the sedimentary context and age control of the remains. The illustration does not make it especially clear which fossil belongs to which site, as the fossils images are floating above the map hundreds of kilometers from the three sites; please use boxes, circles, or arrows, connected to the sites, to show which is derived from which. The fossil images need scale bars and the fossil image contrast should be enhanced so that occlusal morphology is actually visible. The map needs a scale bar and latitude, longitude markers. Lastly I urge the authors to include several other crucial Pleistocene sites as marked locations on the map.

Fig. 2 & 3 - These figures can be easily combined, reducing white space and consolidating some of the methods and validation work into a single figure.

Fig. 4 - This figure should be combined with Supplementary Figure 3: the phylogenetic tree, so that each is its own panel. In Fig. 4, can you note the percent of AMBN-273V found in modern humans / oceanians and East Asians in the graph?

Supplementary Tables 1 and 2: Can the authors please provide information on the locations of peptide sequences from their 10 proteins mapped as probability density functions onto each protein? This is crucial information for other practitioners and will illustrate where preservation tends to be better or weaker in these proteomes.

Referee #3

(Remarks to the Author)
The manuscript describes the recovery of dental enamel protein sequences from 6 Homo erectus teeth found in China. The authors identify an amino acid variant that is unique to Homo erectus and another one shared with Denisovans. Based on this they conclude that the regions in the Denisovan genome attributed to super-archaic introgression, some of which later passed to modern humans, likely originated from H. erectus. The results are original and highly significant. The data were generated using state of the art methodologies. Their quality is high and they strongly support the conclusions. Their presentation however can be improved, as suggested below. The use of statistics is appropriate in most of the cases (see below for improvement suggestions), and uncertainties are treated carefully, without over interpretation. The conclusions are robust and reliable, significantly contributing to the advancement of knowledge about human evolution and genetic introgression events in East Asia. Some minor improvements and integrations are suggested below to improve clarity and better support the results. Importantly, as the authors briefly mention previously unpublished results from the analysis of the proteins from the enamel of the Harbin specimen, the full characterisation of this proteome should be added to this manuscript and the relative raw data should be made publicly available together with those from the 6 H. erectus. The references provided are exhaustive and pertinent. The abstract is sharp and clear, the introduction is detailed and accurate and the conclusions are solid. Globally the authors deliver new, highly valuable knowledge. I have no major revisions to suggest, however a few improvements and some integrations are required to increase the quality of the final product.

A few suggestions are provided below:

Line 112: remove "respectively". There is no reason to use it here.

Line 114: The numbers mentioned here "651 and 2809 peptides" don't match those reported in Supplementary Table 1.

Line 124: The value: "2720" does not seem to match the one for the highest number of recovered peptides mentioned in line 114 and in Supplementary Table 1.

Line 136: The sentence: "However, due to the restricted database [...] minor AMELY-specific peptides were detected." is not sufficiently clear and potentially misleading. In particular, it is not clear whether the authors refer to: (1) the incorrect assignment to females of low signal male samples due to drop of the AMELY signal below detection limits, or (2) the assignment of samples to female despite minor AMELY-specific peptides, caused by "the restricted database for enamel

and the high sensitivity of the newest searching software". The authors should improve clarity

Also the methodology used to address this issue needs improvement. The authors decided to calculate the ratio of peptides covering the AMELY-specific positions relative to all peptides from both AMELY and AMELX, however it is not clear what parameter was used for this calculation. Is it spectral count, spectral intensity, or possibly something else? Please clarify. Also the use of the "confirmed sex" samples from both ancient and modern sources in references 36 and 37, shown in fig. 3, should be better clarified. It is not immediately clear why only a subset of the male and female individuals described there have been included in figure 3, and what were the criteria used to include these individuals and exclude the others. It is also suboptimal that the statistics to call female individuals in this sample set are based only on confirmed sex samples that are modern or just a few thousand years old. Accordingly, the attribution of the SJD10A specimen to a female individual needs to be better supported using a second approach, like the one described in Madupe et al, (PMID: 40440366), integrating in the statistics the data not only from Eocene, but also from Pleistocene material, such as: the Penghu specimen (PMID: 40208980), the 4 Paranthropus specimens (PMID: 40440366) and the H. antecessor specimen (PMID: 32269345).

Line 178: Supplementary Fig. 3 appears in the text before Supplementary Fig. 2, they should be re-ordered correctly.

Line 201: The authors rapidly mention here, and briefly show in Table 1, partial results from the analysis of the dental enamel proteome from the Harbin specimen. This proteome is so far unpublished and currently not exhaustively described in this manuscript. Accordingly, if the authors want to show any result from the analysis of this proteome they should exhaustively describe here the results they obtained from its analysis with the same level of detail used for the proteomes of the 6 H. erectus specimens, covering, among other features: recoveries, damage patterns, sequence reconstruction, and sex-ID. To assure reproducibility and transparency, all the raw data generated in connection with the palaeoproteomic analysis of the dental enamel of the Harbin specimen should also be mandatorily shared and made publicly available when this manuscript is published. Currently, the dataset identified by the accession number PXD068897 in the PRIDE archive does not seem to include the raw files of the Harbin specimen.

Lines 437 and 444: the authors should specify, possibly in a supplementary table, how many milligrams of dentin/enamel powder were used as starting material for protein extraction

Line 455: I believe the use of acid etching is only mentioned here. As this is another point of strength of this work (in terms of sustainability of the approach used), the authors may consider mentioning the use of minimally invasive sampling in the main text.

Lines 499, 500, 512 and 514: usually resolution is expressed in relation to a specific m/z value, for example: "120k at m/z 200". Please provide the missing information accordingly.

Line 551: Please check whether citation 25 is correct, or if it should be 24 instead.

Line 560: the precursor mass tolerance, considering the instrumentation used, should be reduced to 5ppm and all the analyses, with all the 3 software tools used, should be repeated using this more stringent parameter, to check whether the final results change significantly.

Lines 563 and 595: Change (here and everywhere else needed) "pyro-Glu" to "N-terminal pyro-Glu".

Lines 587-88: The content of the sentence: "To avoid incorrect X residue identification, we set the mass of X as 6228.71 Da (Sm41)." should be better clarified providing more details and explanation.

Line 597: please provide a valid justification about why the PSM FDR for HX-S1 and for HX-S2 were set at 10% and 5%, respectively, instead of 1%, like for all the other samples.

I don't see the point of paragraph "AMELY-162Q and 156-167" under "Supplementary Note 4". The peptides that were possibly identified, by any of the software tools the authors used, as covering AMELY-162H should be parsimoniously interpreted as peptides covering AMELX-161. This would leave AMELY 156-167 uncovered, as the authors correctly point out.

Figure 1: the image resolution of specimen HX-S2 is very poor and it should be improved.

Supplementary Fig. 1 and 2: the resolution of both the two images is quite low and it should be improved. The representation of the identified ion fragments supporting the reconstructed peptide sequence are too small to be read comfortably.

Integration needed: for all the 6 H. erectus specimens, to better visualise the richness of the evidence supporting the identified of the two SAPs: AMBN-253G and AMBN-273V, the authors should provide supplementary figures showing the alignments of the peptide sequences covering these SAPs, including the positively identified fragment ions. A possible example of this representation is shown by Tsutaya et al. (PMID: 40208980) in fig. 2D. Similar supplementary figures should also be provided for the AMBN-273V/M alleles from the Harbin specimen.

Referee #4

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

Version 1:

Reviewer comments:

Referee #1

(Remarks to the Author)
The authors have appropriately revised the documents based on the recommendations, and I have no major recommendations, just a few possible recommendations that I record line-by-line below. I think the manuscript is publishable as is, with just very minor edits that would improve it.

Line 33: change “not been seen” to “not been recorded” or “not been identified”

Line 604: “After adding, SJD10A also contains SERPINC1 with high deamidation rates”. I would recommend that this can be removed, because the identification of SERPINC1 in the main text and in the extended data table, and this is more of a result than a methodological detail (I think the sentence above is very clear on why SERPINC1 was added to the database). Either way is fine though.

Line 804, Figure 3: This is very good, I just want to clarify: these are just a selection of the best-looking peptides covering these SAPs, in the different specimens, correct? If so, I think you could add in the figure caption that you are just presenting a small subset of the supporting peptides (in other words).

Line 841, Extended Data Fig. 2: I like these, but I noticed only the peaks that are informative for the peptide sequences are represented, in the experimental spectrum. Or potentially, these are only the highest-intensity peaks? Would it be possible to include all the peaks in the experimental spectrum? I am unsure if this is a standard or not, so I will defer to the authors if they choose to do this or not. I think it is okay without the additional peaks, as well.

Best,
Ryan Sinclair Paterson

Referee #2

(Remarks to the Author)
Fu et al. Nature, “Enamel proteins from six Middle 1 Pleistocene Homo erectus specimens across southern and northern China”, 2025-10-27803A

I am satisfied with the authors’ revisions and thank them for their careful diligence in response to all reviewers. Note that some basic age model is required for the three Zhoukoudian, Sunjiadong, and Hexian stratigraphic columns shown in Figure 1.

Referee #3

(Remarks to the Author)
The authors satisfactorily replied to all my comments, and, for what I can see, to the comments of all the other reviewers. I would like to sincerely congratulate them for the high competence they showed in generating and processing the data and for the relevance of the results they achieved. I still have just a few more, very minor, comments:

Line 44: Replace: "A broad definition of Homo erectus (H. erectus sensu lato) was widely distributed...", with: "Broadly defined Homo erectus (H. erectus sensu lato) was widely distributed...".

Line 91: After introducing the H. erectus material, the Harbin specimen should also be introduced, also motivating its inclusion in this study, by adding a sentence here.

Lines 93-6: "By examining protein preservation [...], we aimed to assess preservation conditions [...] and to gain initial insights into protein preservation [...]." The readability of this sentence should be improved avoiding repetitions.

Line 110: "from published archaic individuals": does "individuals" refers to humans? hominins? Please clarify.

Line 178: "The maximum y/b ion": is this supposed to be "maximum", or hear you mean "minimum" instead"?

Line 232: "which are not found": please check this phrasing. If I'm not wrong, the subject of this verb should be "mutation" in line 231. If so please rephrase as follows: "which is not found".

Line 489: Here the amount of dentine powder should be indicated. If this detail is provided as supplementary information,

this should be indicated at the end of the sentence.

Line 496: "The trypsinized peptides were desalted and purified.", please specify how the peptides were desalted and purified.

Lines 520-21: "The protein peptides were eluted into a solution of 0.1% trifluoroacetic acid (TFA) and 80% acetonitrile (ACN)." It looks like a sentence is missing before this one, most probably somenthing similar to what reported in the sentence in lines 598-500: "The acid solution...".

Line 522: "The peptide mixture was further divided into different aliquots...": please specify how many aliquots. Alternatively, please the volume of each aliquot.

Line 638: Replace:
- "Gln->pyro-Glu[AnyN-termE]", with "Gln->pyro-Glu[AnyN-termQ]"
- "Glu->pyro-Glu[AnyN-termQ]", with "Glu->pyro-Glu[AnyN-termE]"

Lines 638-39: "Pro->N-terminal pyro-Glu[P]": please check the accuracy of the definition of this modification, it does not look correct to me.

Line 655: "Dioxidation[P](Pro->Glu[P]).": please check the accuracy of the definition of this modification, it does not look correct to me.

FASTA File "534830_1_related_ms_5038698_t42c44.txt": In the FASTA sequence: ">COL17A1-Harbin" there is a portion of reconstructed sequence reading: "...XEPGX...". It is surprising to see the identification of a peptide that is only 3 amino acids-long, considering that the minimum length of the peptides identified is 7 amino acids. Is this an inconsistency, or the authors have a valid reason not to discard such a very short peptide? Please check whether any other very short (i.e. 3-4 amino acid long) peptide appears in the FASTA file.

Enrico Cappellini

**Referees' comments:**

Referee #1 (Remarks to the Author):

Summary of key results

This study successfully recovers endogenous enamel proteomes from six Middle Pleistocene Homo erectus specimens (~0.4 Ma) from China. The recovered proteomes exhibit characteristic diagenetic alterations, particularly elevated deamidation, which authenticate their ancientness. Phylogenetically, the analysis relies on the reconstruction of Ameloblastin (AMBN) sequences, identifying two critical Single Amino Acid Polymorphisms (SAPs). First, a novel variant, AMBN-253G, is shared across all six individuals—unifying the Zhoukoudian, Hexian, and Sunjiadong specimens—but is absent in other hominins, marking it as a lineage-specific trait. Second, AMBN-273V is shared with Denisovans, providing proteomic evidence that the "super-archaic" introgression observed in Denisovan genomes could have originated from this East Asian H. erectus population. Additionally, the authors utilize the amelogenin Y isoform (AMELY) for sex determination, identifying five males and one female.
Response: Thanks for helping to improve the paper. We have incorporated all your suggestions.

Originality and significance: if not novel, please include reference
I think this is a wonderful work that will be well-received by the academic community, with the potential to engage non-academics. It presents a wonderfully rich dataset to those in the palaeoproteomics field. The samples and their associated taxon are historically and culturally significant, both within the field of palaeoanthropology and beyond, and the data interpretations presented here appear reasonable.
Data & methodology: validity of approach, quality of data, quality of presentation
&
Appropriate use of statistics and treatment of uncertainties
While I am very supportive of publishing this, there is a methodological issue that requires attention. The conclusions of this paper are based on just 1-2 protein sequence mutations (single amino acid polymorphisms (SAPs)), and it appears the authors have made a methodological choice in data analysis that requires further elaboration. It is possible that the data presented here is only identified due to this methodological choice, so I think great care and detail must be directed here.
The issue is that the FDR (false discovery rate) is inconsistently applied across samples. The standard is 1%, particularly with the tools applied and specifically in palaeoproteomics. A 1% FDR essentially means 1% of the reported identifications are expected to be false positives (and for context, 1%, while standard and reasonable in proteomics, is already quite high, when thinking about it in comparison to nucleotide sequencing, at least). For the Hexian samples, the authors choose to increase this FDR to 5% and 10%. This type of increase is very rare in our field and risks introducing false positives.
Critically, I independently re-processed the authors' raw data using MaxQuant at a strict 1% FDR. My analysis confirmed that the phylogenetically informative variants in AMBN are indeed present even at this stricter threshold (though I did not have time to look closely at their spectra, or the quantity, in the Hexian samples). This indicates that the authors' results are robust, and the decision to relax the FDR is unnecessary. By using inconsistent thresholds (5-10% for some, 1% for others), the authors inadvertently undermine confidence in their results, creating an appearance of outcome-

oriented data filtering. To ensure the study is viewed with the credibility it deserves, the authors should standardize the FDR to 1% across all samples.
Response: As suggested, we now use a 1% FDR across all 7 samples in MaxQuant. And as the reviewer observed, the results do not change. Now we change the method to "PSM FDR was set at 1% for all seven samples."

Conclusions: robustness, validity, reliability
Currently, the conclusions do follow the results, and if the results remain consistent with a more strict FDR threshold, then they are appropriate.
Suggested improvements: experiments, data for possible revision
Recommendations
My recommendation is that the authors must run all analyses with a 1% FDR and report these results in the comparative tables. If the authors choose to relax the FDR, they need to explicitly state this in the main text, and at least a section of the main text and/or methods must be devoted to the theoretical underpinnings of this choice.
Furthermore, all downstream analyses should be rerun with the results of the 1% FDR analyses, alongside potentially the 5% FDR analyses (and 10% if authors choose to still include them). The relevant tables in the manuscript (e.g., Table 1, Supplementary Tables 1 and 2) should also be remade for each FDR threshold. In this case, perhaps the interpretation does not change substantially, but if it does, this should be integrated into the next revision.
Alternatively, if the authors believe the data is still robust with the 1% FDR threshold (as I found it to be), then they can just report those results and modify any interpretation if the informative peptides are not found in the Hexian samples.
Response: As suggested, we used a 1% FDR for all samples and updated all these results in the related tables (Table 1, Extended Data Tables 1 and 2). Finally, we do not use the relaxed FDR.

Data & Figures
In addition, a higher number of supporting spectra need to be figured, complete with associated ion series coverages. I really would like to see at least a mirror plot for at least 1 specimen from each site, for both of the marker SAPs, and for the potential heterozygous sites.
Response: We added mirror plots for at least 1 specimen from each site for both marker SAPs in Extended Data Figs. 2 and 4, including the heterozygous site for Harbin.

I would also like to request the authors include all fasta databases they used in the PRIDE upload (perhaps they are uploaded somewhere, and I couldn't find them), and also output files. I see PEAKS output files are available, but I would also like to see the MQ output files and pFind output files, if possible, or at least the MSMS.txt from MQ and the pfind-Filtered.spectra from pFind.
Response: Sorry for this. We have now included all fasta databases used in the PRIDE upload and added the MQ and pFind output files to the PRIDE upload.

In general, I also wonder if the authors have considered amino acid racemization analyses as a secondary measure of validation, and even just to contextualize these fossils and their degradative state. I am not recommending to do so here—I think the paper is complete without them—I just think it would be really nice to see how these samples (and the Harbin samples referenced herein) look from this perspective.

Response: Thank you for your insightful suggestion. The Homo erectus specimens came from the Hexian Museum, the IVPP collection room, and the Luanchuan County Culture, Radio, Television, and Tourism Bureau. They did not permit us to damage the specimens, which is why we use a minimally invasive enamel etching method to reduce damage, preserve morphology, and access the tooth. This is also why we do not perform amino acid racemization analysis in parallel, as it requires a substantial amount of enamel, which would deplete a large portion of the enamel in a human tooth.

Line 134: I wouldn't say this is a new discovery—the false positive issue—but something that is 100% expected in proteomic experiments attempting sequence reconstruction or trying to discriminate between similar isoforms/variants. See Welker et al. (2018) for how pernicious this may be, particularly if a database is incomplete or when attempting to distinguish between isoforms. This line is particularly concerning for me because this issue goes well beyond just AMELY and sex-specific peptides. I am a little concerned the authors did not consider the possibility of this false positive rate for the rest of the proteome (though the strict acceptance criteria for sequence reconstruction should be sufficient, I think?). My recommendation is that Welker et al. (2018) and other relevant papers should be cited to contextualize this.
Response: As suggested, we now cite Welker et al. (2018) and Presslee et al. (2019) papers and contextualize this in the main text. "This outcome is not unexpected, as previous research has already demonstrated cross-species proteomic effects resulting from search database bias [40,41]. To address this false positive issue, we have employed rigorous filtering criteria to ensure the reliability of all identified single amino acid polymorphisms (SAPs) in the endogenous proteome (details provided in the following section), including those used to determine sex in amelogenin. "

Line 157: I quite like this criterion. I think it follows the ethos of Cappellini et al. (2019) in attempting to establish the authenticity of the sequence residues themselves via fragmentation ions. However, I strongly encourage the authors to employ auxiliary tools for peptide-spectrum match prediction using AI-based tools. Frankly, I think both are important at this stage. I implore the authors to consider these alternative tools here. Perhaps they do not function perfectly with these data (granted, they are primarily trained on modern, tryptic data), but I think such an approach would remove any doubt that these peptide sequences could be misidentified. Even if these are not included in the main manuscript, I think it would be nice to see a couple figured in the response.
Response: As suggested, we utilize auxiliary tools for peptide-spectrum match prediction with PEAKS, as shown in the Extended data Figs. 2 and 4.

Line 597: I think this is the most pressing thing for this manuscript that requires clarification. I believe there are significant problems with FDR within database searches, and certainly for palaeoproteomic studies, but setting FDR >1% with MaxQuant is absolutely a methodological choice that requires more elaboration. Why only for specific samples? My assumption is because they are low-yield, but I still don't know if this justifies the choice. On my initial read, this creates the unintended appearance that the authors have chosen cutoffs to support a desired narrative, as opposed to presenting the data in an unbiased manner. I don't think this is the case—especially given my own re-analysis—but I think this is how other researchers might read this manuscript if the threshold remains inconsistent.
Response: As suggested, we used a 1% FDR for all samples to maintain consistency. The updated results are included in Table 1 and Extended Data Tables 1 and 2.

Line 606: I understand the rationale, but I think the counts for different samples cannot be reliably compared if the FDR is not consistent across samples. I think the results from each analysis must be more clearly reported using uniform thresholds, not just the combined total of PSMs across the different analyses.

Response: As suggested, we used a 1% FDR and uniform thresholds across all samples, and the consensus is based on overlapping data from three software programs (MaxQuant, PEAKS, and pFind). The updated consensuses are in Supplementary Data 2.

Line 610: How are heterozygous sites identified? I do not think there is enough groundwork underlying the detection of heterozygous sites in the proteins we observe in modern enamel to assume heterozygosity without a degree of caution. Specifically, if identifying heterozygosity, what are the relative intensity ratios of the peptides representing the two alleles? If one is significantly more abundant than the other (e.g., >10:1), how do the authors rule out minor cross-contamination versus true heterozygosity?

Response: For heterozygous sites, at least two peptides were required per allele. We have found only one potentially heterozygous position in one individual (AMBN-273 in Harbin). In this case, we directly counted the number of peptides representing the two alleles and identified 79 for the V allele and 38 for the M allele at AMBN-273 in Harbin. Both alleles are quite abundant and unlikely to be due to cross-contamination.

We changed the related part in the Methods: "For heterozygous sites, at least two peptides were required per allele. The heterozygous variant site, observed only in the Harbin specimen, was identified with 79 peptides for the V allele and 38 for the M allele at the AMBN-273 position (Table 1). We include both alleles of the Harbin consensus in Supplementary Data 2."

Line 610: What is the specific criteria for choosing one potential allele over the other?

Response: The related sample is the Harbin specimen for AMBN-273, and we no longer perform randomization. We present both alleles in the Harbin consensus in Supplementary Data 2.

Line 692: PEAKS is not open access; it is proprietary commercial software, even if the results may be visualized freely on their online tool.

Response: As suggested, we changed it to "All software used, except PEAKS, is open-access and available online." in the text.

References: appropriate credit to previous work

Please see the point about line 134, and additional references must be included once the authors decide how to approach the FDR issues.

Response: Thanks for the reminder. We have now added two references (Welker et al. (2018) and Presslee et al. (2019)) and also updated the related text. "However, due to the restricted database for enamel and the high sensitivity of the searching software, the assignment of samples to female may be a false positive issue, where minor AMELY-specific peptides are not detected. [39,40] This outcome is not unexpected, as previous research has demonstrated cross-species proteomic effects resulting from search database bias [41,42]. To address this false positive issue, we have employed rigorous filtering criteria to ensure the reliability of all identified single amino acid polymorphisms (SAPs) in the endogenous proteome (details provided in the following section), including those used to determine sex in amelogenin. "

Clarity and context: lucidity of abstract/summary, appropriateness of abstract, introduction and conclusions
Everything flows logically in the main text.
Response: Thanks, we make sure the flow and language are proper in the main text.

Best,
Ryan Sinclair Paterson

Referee #2 (Remarks to the Author):

Fu et al., Enamel proteins from six Middle Pleistocene Homo erectus individuals across southern and northern China

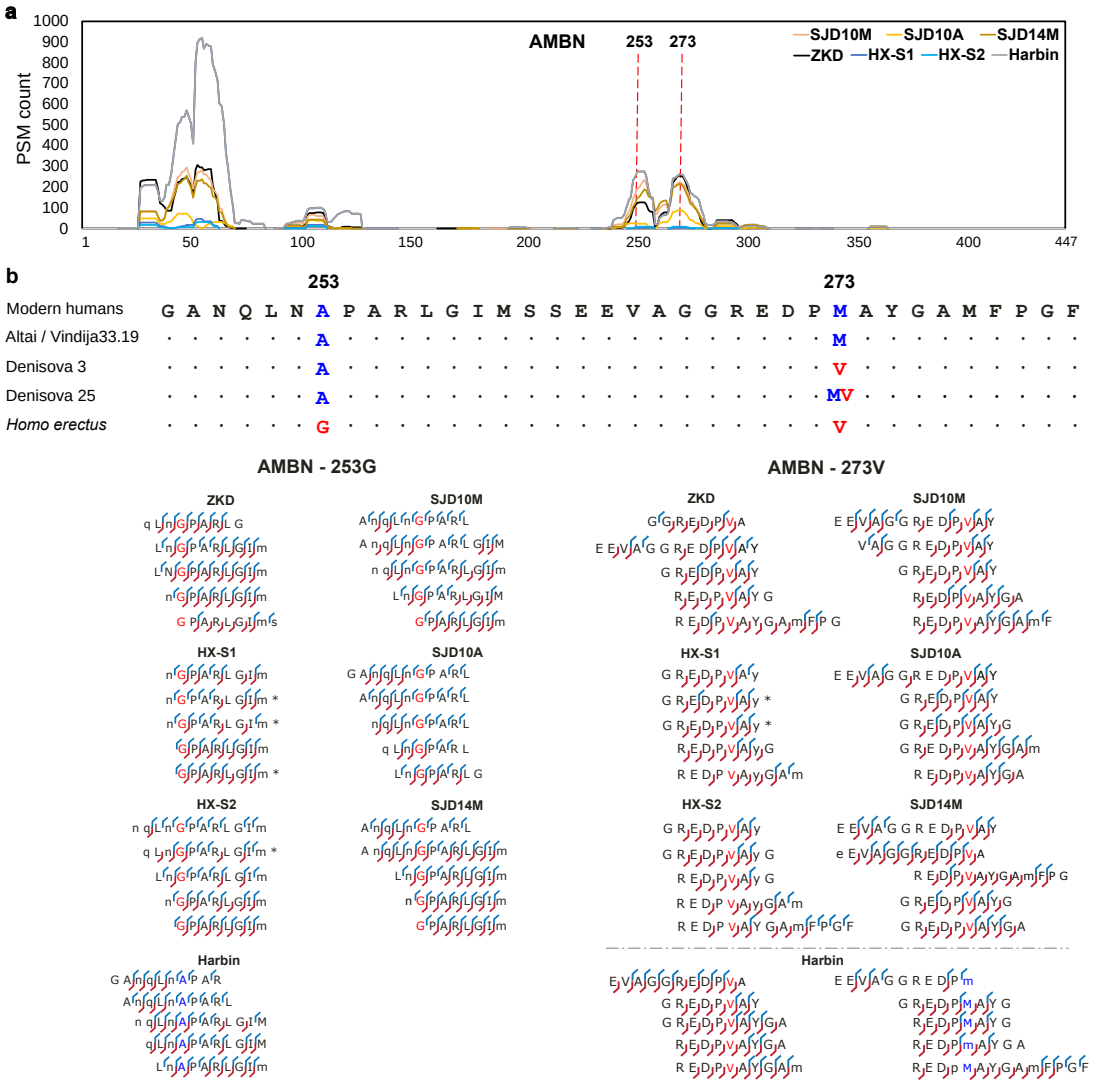
Summary:

This paper investigates the enamel proteomes from three Pleistocene hominins, finding two novel peptide sequence variants in ameloblastin (AMBN). The authors conclude that one shared AMBN variant demonstrates a cohesive Homo erectus population in Eurasia, while the other, found at low frequencies among Denisovans and modern humans, provides evidence of H. erectus introgression into Denisovans.

The authors use a surface etching extraction and LC-MS/MS approach, and conduct triplicate DDA analyses in two laboratories. Peptide spectral matches (PSMs) must be at least two in number and unique to one protein. The authors report substantial PSM counts for most enamel proteins, good sequence coverage for areas of interest, and control blanks to test for contaminants and background.

This is a superb study and should be published: the use of triplicate analyses in two labs and the finding of variants relevant to hominin population history are striking. But some of the excellent data are poorly presented or hardly presented at all, and I suggest major revisions to correct these problems.
Response: Thanks for helping to improve the paper. We have incorporated all your suggestions.

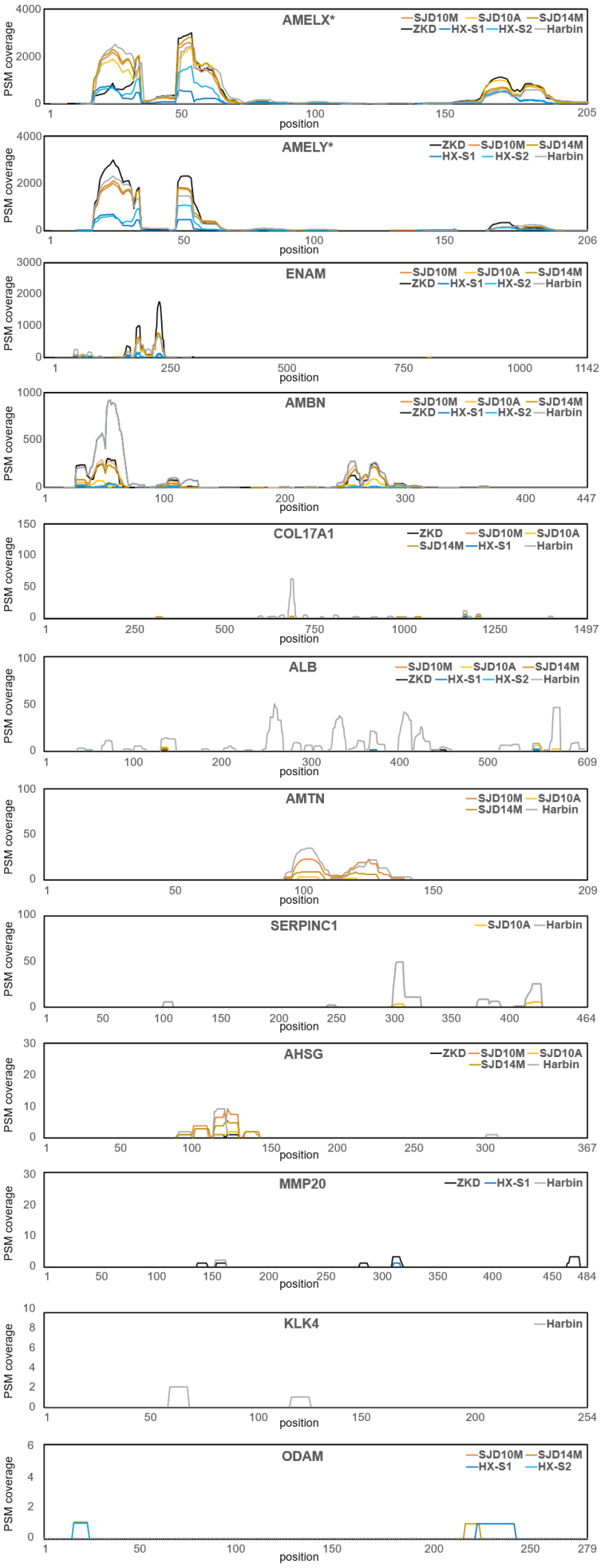
First: as noted below, some subset of spectra shown in supplementary figures 1 and 2 should be brought to the main text, with predicted spectra mirrored to provide support for the findings, and the multiple PSMs used to create the consensus sequences in those figures should be included as has been done in some papers, as in the supplementary figures of Paterson et al (2025), cited below.
Response: As suggested, we added a new Figure 3 showing the PSM coverage of AMBN, and peptide sequence alignments covering these SAPs, including the positively identified fragment ions, to support the main findings. Besides, supplementary figures 1 and 2, along with PSM coverage and peptide sequence alignments, are too large for Figure 3. To make these two figures easier to access, we moved them from the supplementary materials to Extended Data figures 2 and 4, and added the fragment ions of both the observed and predicted mirrors for SAP AMBN-253 and AMBN-273 in those figures.




Second: this work contains a wealth of PSM data on ancient peptides from 9 different enamel proteins, but these are mostly not presented: where do these peptides cluster on the larger protein sequences? What does this tell us about preservation characteristics?

Response: As suggested, we added Supplementary Fig. 4 of sequence-position to PSM-coverage for all proteins. Including Harbin, we have now identified 12 distinct enamel proteins across the seven individuals; some are enamel-specific, while others are also present in bones or dentine. As suggested, we calculated PSM coverage for each protein, and the results are shown in Supplementary Fig. 4. Based on these results, we found that the peptide distributions of some major proteins showed a highly consistent pattern across individuals, which may reflect preservation characteristics of the enamel proteome. (1) Amelogenin, including AMELX and AMELY, is the most abundant protein in enamel. We detected different isoforms of these two proteins, and their PSM counts were combined. The peptides clustered in the tyrosine-rich amelogenin polypeptide (TRAP) region[1,2], i.e., positions 17-74/75 in the figure, with a low-lying area in the middle at positions 35-48, which only derived from the longest isoforms (Q99217-3 for AMELX and Q99218-2 for AMELY). This pattern is consistent with previous observations[3,4]. (2) The peptides of AMBN protein clustered in the 15-kDa cleavage product[5-7] (a main cleavage product, positions 27-155 in the figure), with the most abundant peptides at positions 27-69. Another region with abundant peptides is positions 244-~280, which may be another cleavage product[6,7]. (3) For ENAM, the peptides clustered in the 32-kDa cleavage product[8,9] (a main cleavage product, position 173-280 in the figure), with the most abundant peptides at

positions 210-236. (4) The peptides of AMTN clustered around two phosphorylation sites, 115S and 116S, and ranged across 97-134. The peptides of AHSG clustered at 101-145, where regularly spaced acidic amino acid residues form suitable motifs for binding to basic calcium phosphate lattices. These patterns were also observed in previous research[3,10,11]. (5) The other six proteins, i.e., ODAM, MMP20, KLK4, ALB, COL17A1, and SERPINC1, were too degraded in the *H. erectus* samples to support an informative discussion of their preservation characteristics.


AMELX*
SJD10M SJD10A SJD14M
ZKD HX-S1 HX-S2 Harbin
PSM coverage
position
AMELY*
ZKD SJD10M SJD14M
HX-S1 HX-S2 Harbin
ENAM
AMBN
COL17A1
ZKD SJD10M SJD10A
SJD14M HX-S1 Harbin
ALB
AMTN
SJD10M SJD10A
SJD14M Harbin
SERPINC1
SJD10A Harbin
AHSG
ZKD SJD10M SJD10A
SJD14M Harbin
MMP20
ZKD HX-S1 Harbin
KLK4
Harbin
ODAM
SJD10M SJD14M
HX-S1 HX-S2

Third: what systematic differences do the authors observe between fragment characteristics in the blank controls, which are essentially not discussed, and the Pleistocene teeth? Are there differences of length, acidity, PSM type and number? What about between sites? Are there differences in results between the two laboratories? These issues are not discussed.

Response: As suggested, we added a series of comparisons of our Pleistocene teeth and their blank controls (BKs) as a new Supplementary Note 2.

Supplementary materials: "

**Supplementary Note 2. The comparisons of Pleistocene teeth and blank controls.**

Our ancient samples showed significantly different characteristics from blank controls, including fragment patterns, peptide length distributions, PSM counts and assignments, amino acid composition and peptide acidity, and PTMs. Among the sites, samples from Zhoukoudian, Sunjiadong, and Harbin were similar. They had similar average peptide pI values (6.32-6.56), and preserved higher PSM counts than the Hexian samples (HX-S1, HX-S2), indicating better preservation (Supplementary Table 1). The additional PTM on Y residues in the 2 Hexian samples also suggested a higher level of degradation and a distinct taphonomic process. Although Hexian samples differed slightly, the proteomes of all samples from the 3 sites showed clear ancient enamel features, including elevated deamidation levels of N/Q residues and a higher percentage of P residues (Supplementary Table 2).

**(1) Fragment characteristics, PSM number, and assignment**

Many more PSMs were identified in the sample fractions than in the blank controls (Supplementary Table 1). No PSM or peptide matched common microbial contaminants. And no PSM or peptide from enamel protein was detected in blank controls using either the "Hominidae enamel database" or a larger database search (SwissProt). Notably, all blank controls showed very high tryptic rates, a fragment characteristic of lab contaminants. On the contrary, all our ancient samples showed similar fragment characteristics, with high non-tryptic rates (85.1-87.1%), close to the random (unspecific) cleavage rate during degradation.

**(2) Peptide length distribution:**

The new ancient samples showed different peptide-length distributions from the blank controls. Both the percentage and count of peptides in our ancient samples decreased with increasing peptide length, while the blank controls showed a count peak at 11-16, with no clear pattern in peptide percentage (Supplementary Figs. 1 and 2). And this pattern was quite similar in all sites.

**(3) Amino acid composition and peptide isoelectric point (pI, Acidity):**

All samples had an average peptide isoelectric point (pI) above 6.32 and fell within the range of ancient and modern human enamels (5.90-7.41), while all blank controls were below 5.73 (5.15-5.73). Additionally, all enamel samples exhibited the same amino acid composition pattern, such as the significantly elevated level of P in enamel samples (>19.3% and >20.0% in new samples) compared to all blank controls, which were less than 3.6% (Supplementary Table 2). Lower levels of Q and higher percentages of A, D, T, and V were also observed in all blank controls (Supplementary Table 2). The new samples showed a lower G percentage than the modern enamel samples, consistent with the two published ancient samples, suggesting an ancient origin.

**(4) PTMs:**

All ancient samples had elevated deamidation N/Q levels (>91%), while all blank controls were nearly 0, and the modern enamel sample was between 40% and 50%. (Supplementary Table 3). Other PTMs, such as biological-related Phosphorylation S/Y, high-level Oxidation/Dioxidation M, and degradation-related PTMs on W and R residues, further authenticate the ancient enamel proteome.

**(5) Comparison of the results of two different labs:**

Across two labs, the PSMs identified from the Orbitrap Exploris 480 at Fudan University (FD) fractions were a subset of those identified from the Orbitrap Fusion Lumos at Capital Medical University (SY) fractions (Supplementary Table 4), except for a few contaminant PSMs and 1 PSM from SERPINC1. This is expected since we used the same peptide extraction but different data-acquisition gradients and repetitive runs: 65 minutes at FD for one run and 120 minutes at SY for two or three runs. Results from the two labs also showed similar fragment characteristics (Supplementary Table 5), peptide length distributions (Supplementary Fig. 3), and deamidation N/Q rates (Supplementary Table 6), and only showed a systematic difference in average peptide acidity (Supplementary Table 7, $pI_{FD}$ - $pI_{SY}$ = 0.20-0.70), possibly due to different chromatographic conditions, as SY used a relatively longer gradient at the lower organic buffer ratio (11-28% B for 88 min). Thus, a longer data acquisition time with repetitive runs could help acquire more PSM identifications while maintaining stability in most peptide characteristics."

Fourth: the available figures can be condensed and improved, see detailed comments below.

Additional comments:

Introduction

Lines 51-52: It should be noted that stone tools of even greater antiquity have been in Shangchen, in Lantian county: Zhu, Z., Dennell, R., Huang, W. et al. Hominin occupation of the Chinese Loess Plateau since about 2.1 million years ago. Nature 559, 608–612 (2018). https://doi.org/10.1038/s41586-018-0299-4

Response: As suggested, we added the reference and changed the text "the record of *H. erectus* dates back to about 2.1–1.6 Ma [7-9]"

Lines 98-104: The authors note both negative (dentin and bone) and positive (enamel) findings of protein signals from MALDI-TOF MS analysis of animal remains. But the authors show no data either in a main text or supplementary figure, nor in main text or supplementary tables. These data, positive and negative, should be shown, or the appropriate paper referenced if the data is already published.

Response: As suggested, we have added the Dentin and enamel results for the animals from the Zhoukoudian, Sunjiadong, and Hexian sites to Supplementary Data 1 and uploaded the related MALDI-TOF MS data to PRIDE.

Lines 119-220: Figure 2a and Supplementary Table 2 don't show or report any exogenous contamination at all. Instead, all reported products are from enamel. So, the relative abundance of endogenous versus exogenous proteins are not demonstrated by these data. Can the authors be clearer about what they mean by this text? And, is there a location where likely endogenous versus exogenous peptide numbers and sequences are reported?

Response: As suggested, we added a column for contamination and rows for their blank controls in Supplementary Table 2, and most of them had very few exogenous peptides or proteins. And this is an update in Figure 2a. We also added a new Supplementary Table 1 to show endogenous versus exogenous peptides and PSM numbers.

**Supplementary Table 1.** PSM and peptide counts of our ancient samples and their blank controls.

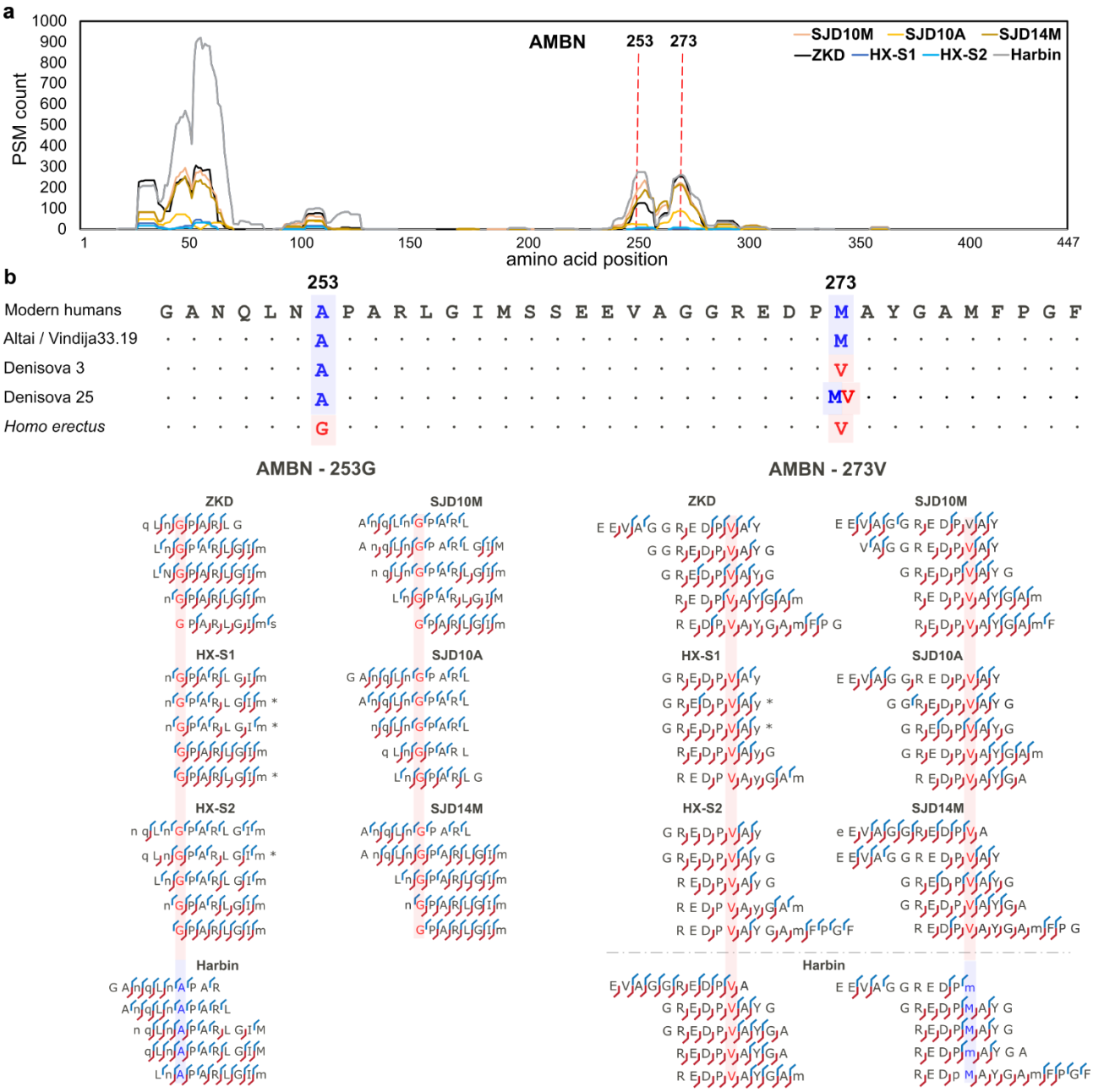
| Sample | PSM | | | Peptide | | | Non-tryptic peptides (%, C-terminal) |
|---|---|---|---|---|---|---|---|
| | All | Endogenous | Exogenous | All | Endogenous | Exogenous | |
| **ZKD** | 22107 | 21865 | 242 | 2201 | 2134 | 67 | 87.1 |
| **SJD10M** | 15467 | 15466 | 1 | 2809 | 2808 | 1 | 87 |
| **SJD10A** | 12520 | 12509 | 11 | 2015 | 2013 | 2 | 87.1 |
| **SJD14M** | 15547 | 15541 | 6 | 2721 | 2720 | 1 | 87 |
| **HX-S1** | 4097 | 4094 | 3 | 652 | 650 | 2 | 87.3 |
| **HX-S2** | 5182 | 5181 | 1 | 821 | 820 | 1 | 85.1 |
| **Harbin** | 17852 | 17280 | 572 | 3476 | 3457 | 19 | 85.5 |
| **ZKD_BK** | 642 | 0 | 642 | 148 | 0 | 148 | 0.7 |
| **SJD_BK** | 26 | 0 | 26 | 21 | 0 | 21 | 28.6 |
| **HX_BK** | 29 | 0 | 29 | 22 | 0 | 22 | 0 |
| **ZKD-BK-SP** | 9362 | 0 | 9362 | 2029 | 0 | 2029 | 0 |
| **SJD-BK-SP** | 75 | 0 | 75 | 73 | 0 | 73 | 4.1 |
| **HX-BK-SP** | 1389 | 0 | 1389 | 989 | 0 | 989 | 0 |

*SP=SwissProt database.

Lines 132-142: The author's approach to this problem is innovative and commendable.
Response: Thanks for seeing the value of our new approach.

Lines 149-182: Supplementary Figures 1 and 2 are really at the heart of this paper and need to be shown in the main text. Your y- and b- ion coverage of many of these peptides is quite good, but I strongly urge the authors to show how spectra align relative to predicted spectra for the same peptides. Furthermore, I'd urge the authors to adopt a strategy use by Paterson et al in their supplementary figures S1, S2, and S3, showing both how multiple fragments align, and also, from where your AMBN and AMEL peptides derive on the larger protein:
Paterson, R.S., Mackie, M., Capobianco, A., Heckeberg, N.S., Fraser, D., Demarchi, B., Munir, F., Patramanis, I., Ramos-Madrigal, J., Liu, S. and Ramsøe, A.D., 2025. Phylogenetically informative proteins from an Early Miocene rhinocerotid. Nature, 643(8072), pp.719-724.
Response: As suggested, we added a new Figure 3 showing the PSM coverage of AMBN, and peptide sequence alignments covering these SAPs, including the positively identified fragment ions, to support the main finding. Besides, supplementary figures 1 and 2, along with PSM coverage and peptide sequence alignments, are too large for Figure 3. To make these two figures easier to access,

we moved them from the supplementary materials to Extended Data figures 2 and 4, and added the fragment ions of both the observed and predicted mirrors for SAP AMBN-253 and AMBN-273.

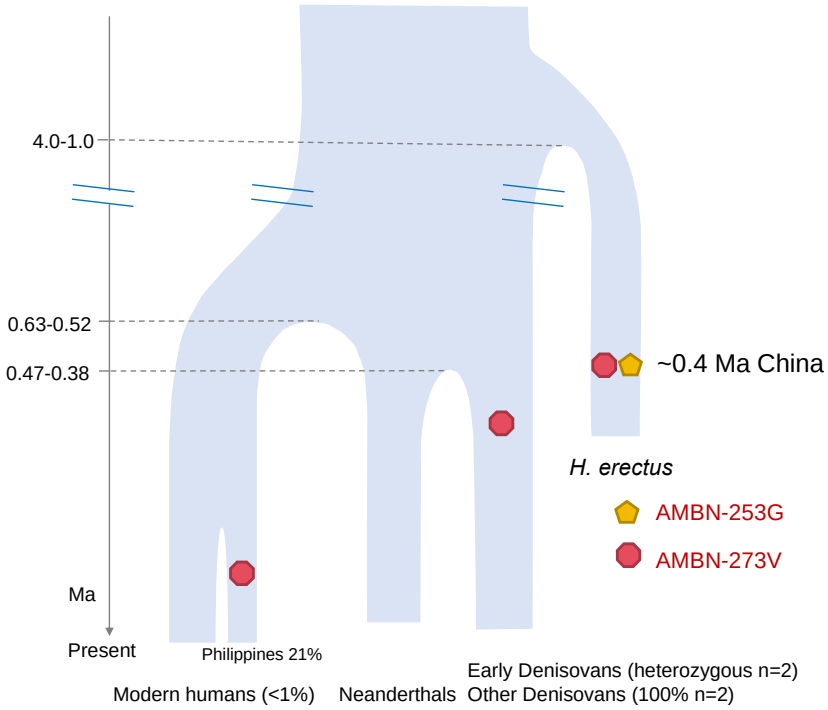



Lines 222-239: I agree with the authors in their conclusion about proximity and introgression, and I have two suggestions. First, I suggest that Fig. 4 and supplementary Fig. 3 be merged into two panels in a single figure in the main text. Second, I suggest that the authors note the frequency of the AMBN-273V variant among Denisovans and Modern Humans/East Asians/Oceanians on Fig. 4, since this is a central point of the paper.

Response: Thanks for the suggestion. Supplementary Figure 3 primarily supports the conclusion that all six Middle Pleistocene *H. erectus* samples from Zhoukoudian, Hexian, and Sunjiadong form a strongly supported monophyletic group. Previously, we planned to put it in the main Figure, but this could confuse the reader, since the AMBN-273V mutation disrupts the Homo group, as mentioned in Supplementary Note 5. Phylogenetic reconstruction "Further phylogenetic relationships within the *Homo* group are unable to be resolved due to the limited number of informative sites and the fact that

most of the branches are supported by one or several SAPs, especially the AMBN-273V mutation, which is present in all six Middle Pleistocene *H. erectus* samples, as well as in *Denisova 3* and a modern human from Oceania (posterior probabilities of 43%, 43%, and 54%, respectively, Supplementary Fig. 3). Although this allele is likely shared through introgression, due to the limited amount of data, it is not possible to directly incorporate introgression signals into the phylogenetic tree using the nuclear genomic method." Because of this, we put it into SI. To make it easier to see, we put it in the extended Fig. 3. Hope this is reasonable for you.

And we note the AMBN-273V frequency among Denisovans and Modern Humans/East Asians/Oceanians in Fig.4.




Lines 485-490: The decision to run analyses in triplicate at two labs is superb. Readers would benefit from seeing differences in outputs from these two different labs in the supplementary material: a comparison of methods would benefit similar work by the community moving forward.

Response: As suggested, we added a subsection comparing the similarities and differences between 2 labs in Supplementary Note 2.

Supplementary materials: " **(6) Comparison of the results of two different labs:**

Across two labs, the PSMs identified from the Orbitrap Exploris 480 at Fudan University (FD) fractions were a subset of those identified from the Orbitrap Fusion Lumos at Capital Medical University (SY) fractions (Supplementary Table 4), except for a few contaminant PSMs and 1 PSM from SERPINC1. This is expected since we used the same peptide extraction but different data-acquisition gradients and repetitive runs: 65 minutes at FD for one run and 120 minutes at SY for two or three runs. Results from the two labs also showed similar fragment characteristics (Supplementary Table 5), peptide length distributions (Supplementary Fig. 3), and deamidation N/Q rates (Supplementary Table 6), and only showed a systematic difference in average peptide acidity (Supplementary Table 7, $pI_{FD}$ - $pI_{SY}$ = 0.20-0.70), possibly due to different chromatographic conditions, as SY used a relatively longer gradient at the lower organic buffer ratio (11-28% B for 88

min). Thus, a longer data acquisition time with repetitive runs could help acquire more PSM identifications while maintaining stability in most peptide characteristics."

Lines 491-536: Can the authors please provide information about the sequence and repetition of blank runs within the larger dataset, in the supplementary information.
Response: As suggested, we re-searched the blank runs across the entire SwissProt database (-SP), and all identified contaminant sequences are listed in an online Table in our PRIDE folder, as it is too long for the SI. Although the SP search identified many more contaminant proteins and peptides, they shared characteristics similar to those in our original blank control search, and both were distinct from our ancient enamel samples (see replies on "Third…").

Lines 535-536: Can the authors please provide further characterization of the blank control findings in the supplementary information? Do the authors find systematic differences in peptide length, isoelectric point, or PSM frequency or type between blank controls and fossil specimens?
Response: As suggested, we have added a new note (Supplementary Note 2), and have described the details in the above response under "Third".

Lines 605-621: While I do not believe it will alter their results, it would be advantageous to report the frequency with which PSMs match microbial contaminants.
Response: Thanks for the suggestion. We double-checked, as no PSM matched common microbial contaminants in our database search of seven archaic hominins, the frequency of matching microbial contaminants would be zero. Thus, no conclusion was altered. We added "No PSM or peptide matched common microbial contaminants." in Supplementary Note 2.

Fig. 1 - I strongly urge the authors to include three stratigraphic columns as one or several sub panels in this figure, to illustrate the sedimentary context and age control of the remains. The illustration does not make it especially clear which fossil belongs to which site, as the fossils images are floating above the map hundreds of kilometers from the three sites; please use boxes, circles, or arrows, connected to the sites, to show which is derived from which. The fossil images need scale bars and the fossil image contrast should be enhanced so that occlusal morphology is actually visible. The map needs a scale bar and latitude, longitude markers. Lastly, I urge the authors to include several other crucial Pleistocene sites as marked locations on the map.
Response: As suggested, we include three stratigraphic columns, use symbols to connect fossils to their sites, add scale bars for the fossils, and enhance the contrast in the fossil images. For the map, we added a scale bar, latitude and longitude markers, and several other crucial Pleistocene sites.

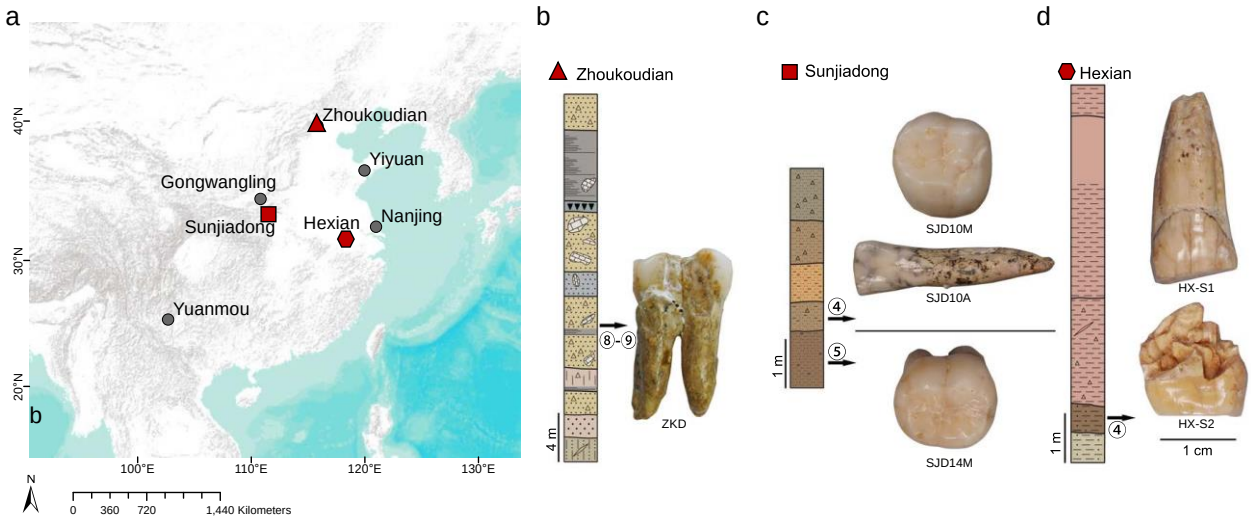



Fig. 2 & 3 - These figures can be easily combined, reducing white space and consolidating some of the methods and validation work into a single figure.

Response: As suggested, we combined Fig. 2 & 3 as Fig. 2.

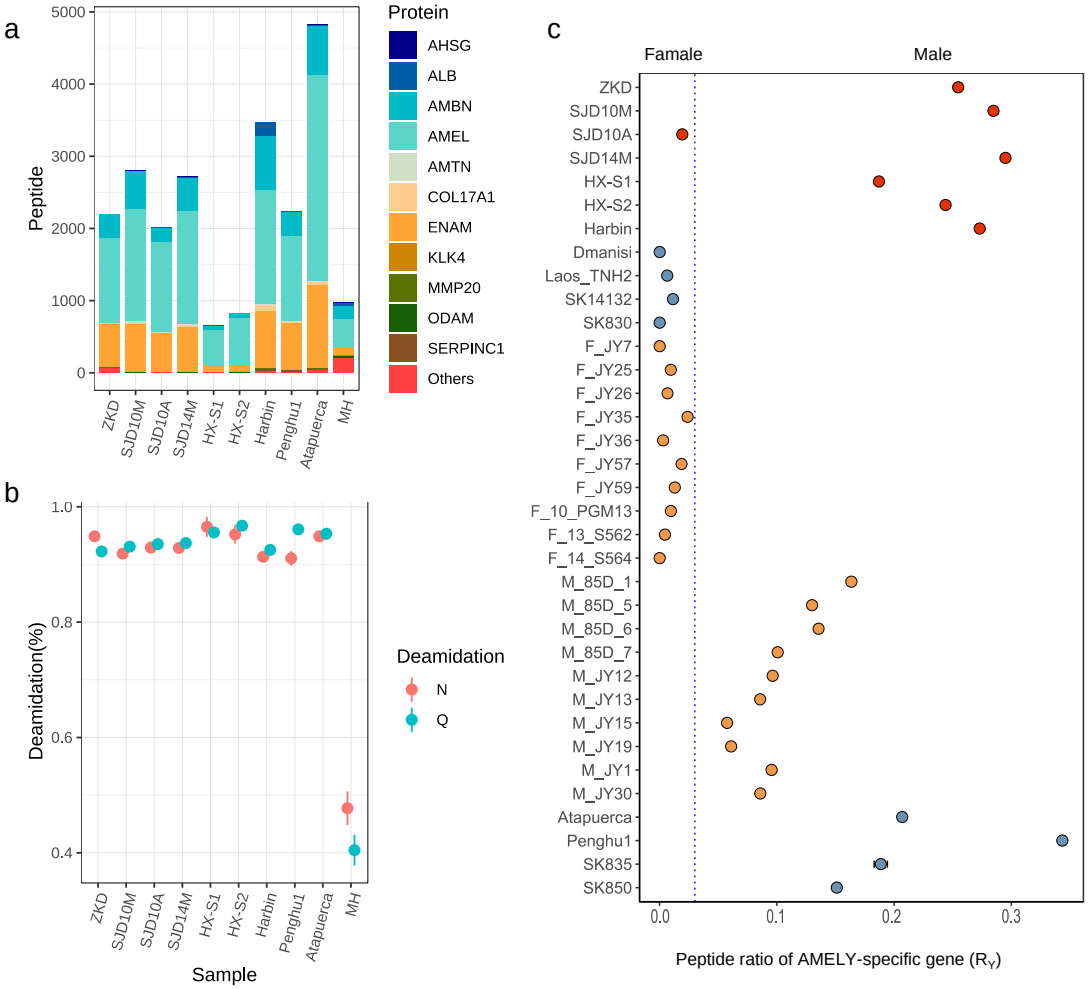


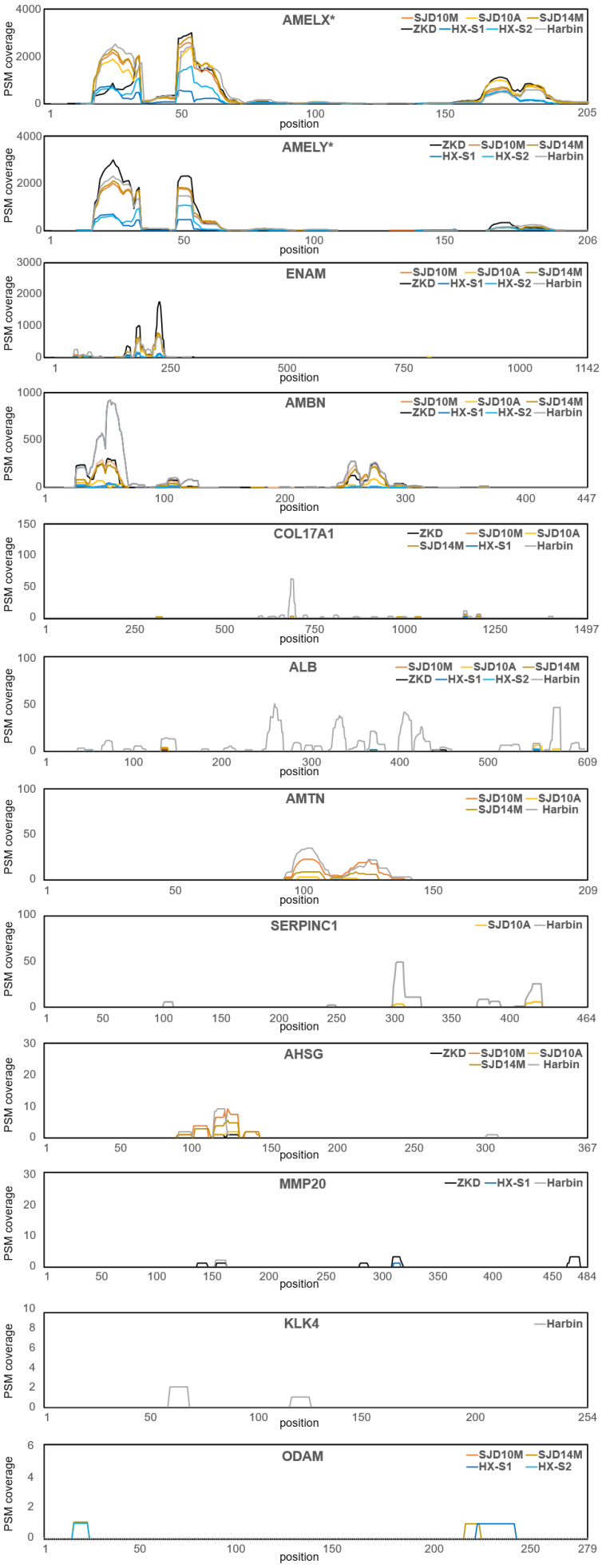

Fig. 4 - This figure should be combined with Supplementary Figure 3: the phylogenetic tree, so that each is its own panel. In Fig. 4, can you note the percent of AMBN-273V found in modern humans / oceanians and East Asians in the graph?

Response: Thanks for the suggestion. Supplementary Figure 3 primarily supports the conclusion that all six Middle Pleistocene *H. erectus* samples from Zhoukoudian, Hexian, and Sunjiadong form a strongly supported monophyletic group. Previously, we planned to include it in the main Figure, but

this could confuse the reader, since the AMBN-273V mutation occurs in present-day humans (Bougainville1027), Denisovans, and H. erectus within the Homo group, as mentioned in Supplementary Note 5. Phylogenetic reconstruction： "Further phylogenetic relationships within the *Homo* group are unable to be resolved due to the limited number of informative sites and the fact that most of the branches are supported by one or several SAPs, especially the AMBN-273V mutation, which is present in all six Middle Pleistocene *H. erectus* samples, as well as in *Denisova 3* and a modern human from Oceania (posterior probabilities of 43%, 43%, and 54%, respectively, Supplementary Fig. 3). Although this allele is likely shared through introgression, due to the limited amount of data, it is not possible to directly incorporate introgression signals into the phylogenetic tree using the nuclear genomic method." Because of this, we had put the tree into the SI. But now, to make it more visible, we have featured it as an extended figure. We hope that you will find this reasonable.

Supplementary Tables 1 and 2: Can the authors please provide information on the locations of peptide sequences from their 10 proteins mapped as probability density functions onto each protein? This is crucial information for other practitioners and will illustrate where preservation tends to be better or weaker in these proteomes.

Response: As suggested, we added a sequence-position figure to PSM coverage for all proteins in Supplementary Fig. 4. Including Harbin, we have now identified 12 distinct enamel proteins across the seven individuals; some are enamel-specific, while others are also present in bones or dentine. As suggested, we calculated PSM coverage for each protein, and the results are shown in Supplementary Fig. 4. Based on these results, we found that the peptide distributions of some major proteins showed a highly consistent pattern across individuals, which may reflect preservation characteristics of the enamel proteome. (1) Amelogenin, including AMELX and AMELY, is the most abundant protein in enamel. We detected different isoforms of these two proteins, and their PSM counts were combined. The peptides clustered in the tyrosine-rich amelogenin polypeptide (TRAP) region[1,2], i.e., positions 17-74/75 in the figure, with a low-lying area in the middle at positions 35-48, which only derived from the longest isoforms (Q99217-3 for AMELX and Q99218-2 for AMELY). This pattern is consistent with previous observations[3,4]. (2) The peptides of AMBN protein clustered in the 15-kDa cleavage product[5-7] (a main cleavage product, positions 27-155 in the figure), with the most abundant peptides at positions 27-69. Another region with abundant peptides is positions 244-~280, which may be another cleavage product[6,7]. (3) For ENAM, the peptides clustered in the 32-kDa cleavage product[8,9] (a main cleavage product, position 173-280 in the figure), with the most abundant peptides at positions 210-236. (4) The peptides of AMTN clustered around two phosphorylation sites, 115S and 116S, and ranged across 97-134. The peptides of AHSG clustered at 101-145, where regularly spaced acidic amino acid residues form suitable motifs for binding to basic calcium phosphate lattices. These patterns were also observed in previous research[3,10,11]. (5) The other six proteins, i.e., ODAM, MMP20, KLK4, ALB, COL17A1, and SERPINC1, were too degraded in the *H. erectus* samples to support an informative discussion of their preservation characteristics.


AMELX*
SJD10M SJD10A SJD14M
ZKD HX-S1 HX-S2 Harbin
PSM coverage
position
AMELY*
ZKD SJD10M SJD14M
HX-S1 HX-S2 Harbin
ENAM
AMBN
COL17A1
ZKD SJD10M SJD10A
SJD14M HX-S1 Harbin
ALB
AMTN
SJD10M SJD10A
SJD14M Harbin
SERPINC1
SJD10A Harbin
AHSG
ZKD SJD10M SJD10A
SJD14M Harbin
MMP20
ZKD HX-S1 Harbin
KLK4
Harbin
ODAM
SJD10M SJD14M
HX-S1 HX-S2

Referee #3 (Remarks to the Author):

The manuscript describes the recovery of dental enamel protein sequences from 6 Homo erectus teeth found in China. The authors identify an amino acid variant that is unique to Homo erectus and another one shared with Denisovans. Based on this they conclude that the regions in the Denisovan genome attributed to super-archaic introgression, some of which later passed to modern humans, likely originated from H. erectus. The results are original and highly significant. The data were generated using state of the art methodologies. Their quality is high and they strongly support the conclusions. Their presentation however can be improved, as suggested below. The use of statistics is appropriate in most of the cases (see below for improvement suggestions), and uncertainties are treated carefully, without over interpretation. The conclusions are robust and reliable, significantly contributing to the advancement of knowledge about human evolution and genetic introgression events in East Asia. Some minor improvements and integrations are suggested below to improve clarity and better support the results. Importantly, as the authors briefly mention previously unpublished results from the analysis of the proteins from the enamel of the Harbin specimen, the full characterisation of this proteome should be added to this manuscript, and the relative raw data should be made publicly available together with those from the 6 H. erectus. The references provided are exhaustive and pertinent. The abstract is sharp and clear, the introduction is detailed and accurate and the conclusions are solid. Globally the authors deliver new, highly valuable knowledge. I have no major revisions to suggest, however a few improvements and some integrations are required to increase the quality of the final product.

A few suggestions are provided below:

Line 112: remove "respectively". There is no reason to use it here.
Response: As suggested, we have removed "respectively".

Line 114: The numbers mentioned here "651 and 2809 peptides" don't match those reported in Supplementary Table 1.
Response: We have updated the number with the latest Supplementary Table 1. “and between 652 and 3476 peptides (Extended Data Table 1) across seven specimens.”

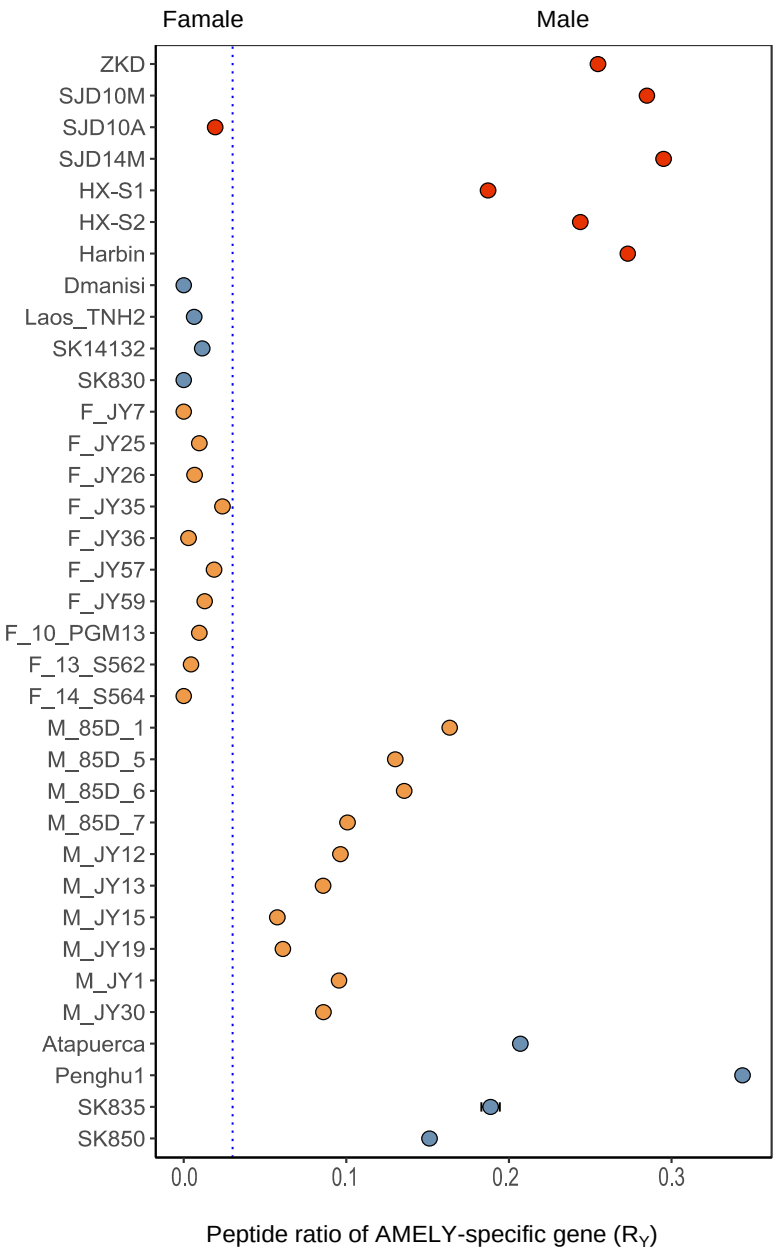
Line 124: The value: "2720" does not seem to match the one for the highest number of recovered peptides mentioned in line 114 and in Supplementary Table 1.
Response: We apologize for the mistakes, and have updated the numbers: “These criteria resulted in 650-3457 peptides recovered from 6-11 enamel-related proteins”

Line 136: The sentence: "However, due to the restricted database [...] minor AMELY-specific peptides were detected." is not sufficiently clear and potentially misleading. In particular, it is not clear whether the authors refer to: (1) the incorrect assignment to females of low signal male samples due to drop of the AMELY signal below detection limits, or (2) the assignment of samples to female despite minor AMELY-specific peptides, caused by "the restricted database for enamel and the high sensitivity of the newest searching software". The authors should improve clarity

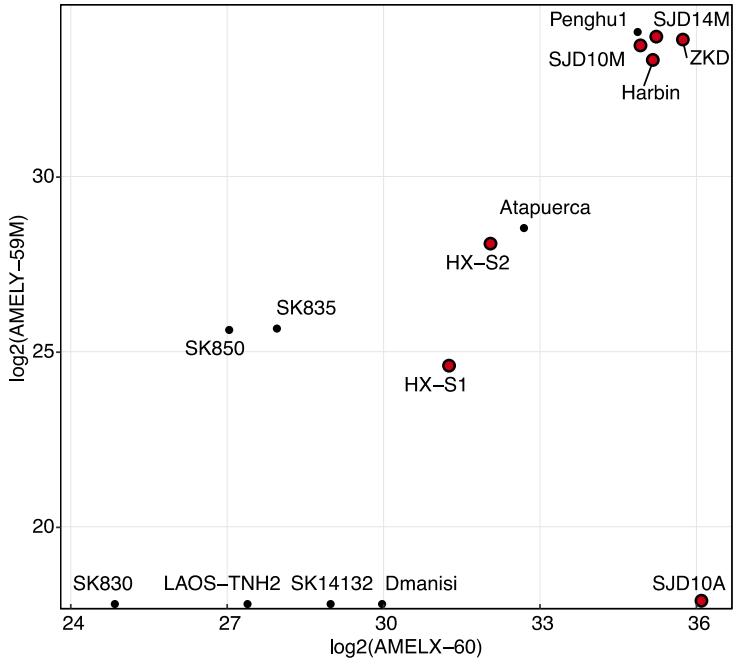
Response: We apologize for the confusion. Our meaning was your second suggestion, and we have reworded this to make it clear. “However, due to the restricted database for enamel and the high sensitivity of the searching software, the assignment of samples to female may be a false positive issue, where minor AMELY-specific peptides are not detected. [39,40] This outcome is not unexpected, as previous research has demonstrated cross-species proteomic effects resulting from search database bias [41,42]. To address this false positive issue, we have employed rigorous filtering criteria to ensure the reliability of all identified single amino acid polymorphisms (SAPs) in the endogenous proteome (details provided in the following section), including those used to determine sex in amelogenin.”

Also the methodology used to address this issue needs improvement. The authors decided to calculate the ratio of peptides covering the AMELY-specific positions relative to all peptides from both AMELY and AMELX, however it is not clear what parameter was used for this calculation. Is it spectral count, spectral intensity, or possibly something else? Please clarify.
Response: We used peptide-count here. We now clarify this in the main text “we established a method based on the ratio of peptide counts covering the AMELY-specific positions relative to all peptides from both AMELY and AMELX ($R_Y$) ”

Also the use of the "confirmed sex" samples from both ancient and modern sources in references 36 and 37, shown in fig. 3, should be better clarified. It is not immediately clear why only a subset of the male and female individuals described there have been included in figure 3, and what were the criteria used to include these individuals and exclude the others. It is also suboptimal that the statistics to call female individuals in this sample set are based only on confirmed sex samples that are modern or just a few thousand years old. Accordingly, the attribution of the SJD10A specimen to a female individual needs to be better supported using a second approach, like the one described in Madupe et al, (PMID: 40440366), integrating in the statistics the data not only from Eocene, but also from Pleistocene material, such as: the Penghu specimen (PMID: 40208980), the 4 Paranthropus specimens (PMID: 40440366) and the H. antecessor specimen (PMID: 32269345).
Response: Fig. 3 is a sampling mostly from ancient modern humans. As suggested, we have added 8 known archaic samples: the Penghu specimen (PMID: 40208980 Penghu1), the 4 Paranthropus specimens (PMID: 40440366 SK14132, SK830, SK835, and SK850), two individuals in the *H. antecessor* paper (PMID: 32269345 Dmanisi and Atapuerca), and the Lao specimen (PMID: 35581187 Laos_TNH2). We update the main text: “Using 16 ancient and 4 present-day modern humans [39,40], along with 4 *Paranthropus* specimens (SK14132, SK830, SK835, and SK850) [2], and 4 archaic hominins (including Penghu1 [34], two specimens (Dmanisi (D4163) and Atapuerca (ATD6-92)) [11], and Laos_TNH2 [43]), with confirmed sex, samples can be classified as male if the lower bound of the CI for RY exceeds 0.057, and female if the upper bound is below 0.024 (see Figure 2c).”




In addition, we used a second approach to validate these results and included the results in Extended Data Fig. 1. Both sets of results are consistent.




**Extended Data Fig. 1 | The intensity of AMELY-59M site as a function of the intensity of AMELX-60 site.** The Y-axis and X-axis represent the $\log_2$-transformed sum of intensities of all peptides covering the AMELY-59M and AMELX-60 sites, respectively. Black dots denote reference samples of known sex; red dots denote new samples.

Line 178: Supplementary Fig. 3 appears in the text before Supplementary Fig. 2, they should be re-ordered correctly.
Response: Sorry for this oversight. We have reordered them.

Line 201: The authors rapidly mention here, and briefly show in Table 1, partial results from the analysis of the dental enamel proteome from the Harbin specimen. This proteome is so far unpublished and currently not exhaustively described in this manuscript. Accordingly, if the authors want to show any result from the analysis of this proteome they should exhaustively describe here the results they obtained from its analysis with the same level of detail used for the proteomes of the 6 H. erectus specimens, covering, among other features: recoveries, damage patterns, sequence reconstruction, and sex-ID. To assure reproducibility and transparency, all the raw data generated in connection with the palaeoproteomic analysis of the dental enamel of the Harbin specimen should also be mandatorily shared and made publicly available when this manuscript is published. Currently, the dataset identified by the accession number PXD068897 in the PRIDE archive does not seem to include the raw files of the Harbin specimen.
Response: As suggested, we now add all parallel results for the Harbin sample alongside those of the other six *H. erectus* samples, including proteome results for Harbin enamel coverage, recoveries, damage patterns, sequence reconstruction, and sex-ID, across all related tables and figures. The raw files for the Harbin specimen have also been uploaded to the PRIDE archive.
In addition, we have found abundant peptides with nealy 100% deamidation for the SERPINC1 protein in Harbin. Considering this protein has also been reported in modern enamel samples, we have expanded our ‘Hominidae enamel database’ with this protein, which now includes 13 proteins. We have explained this in the Method.

Method： "The 'Hominidae enamel database' was composed of 13 selected common enamel proteins from Hominidae. Besides the commonly used 12 proteins (AHSG, ALB, AMBN, AMELX, AMELY, AMTN, COL17A1, ENAM, KLK4, MMP20, ODAM, and TUFT1), SERPINC1 was also added because we identified this protein in Harbin with abundant peptides and elevated deamidation rates (nearly 100%), and this protein was also reported in modern enamel [56,57]. After adding, SJD10A also contains SERPINC1 with high deamidation rates. "

Lines 437 and 444: the authors should specify, possibly in a supplementary table, how many milligrams of dentin/enamel powder were used as starting material for protein extraction
Response: As suggested, we included all animal protein extraction data from Zhoukoudian, Sunjiadong, Hexian sites in Supplementary Data 1, including the amount of dentin/enamel powder used in milligrams.

Line 455: I believe the use of acid etching is only mentioned here. As this is another point of strength of this work (in terms of sustainability of the approach used), the authors may consider mentioning the use of minimally invasive sampling in the main text.
Response: As suggested, we mentioned the minimally invasive sampling in the main text now "Ancient enamel proteins were successfully extracted using minimally invasive sampling techniques from six *H. erectus* teeth from Zhoukdoudian, Hexian, and Sunjiadong sites, and a tooth from an earlier-reported Denisovan skull from Harbin, China [25,26] (Methods)"

Lines 499, 500, 512 and 514: usually resolution is expressed in relation to a specific m/z value, for example: "120k at m/z 200". Please provide the missing information accordingly.
Response: All the resolutions are calculated at m/z 200. As suggested, we added the specific m/z value in the method

Line 551: Please check whether citation 25 is correct, or if it should be 24 instead.
Response: We double-checked the 25 citations; now updated to 28, Bergström, A. et al. Insights into human genetic variation and population history from 929 diverse genomes. Science 367, eaay5012, doi:10.1126/science.aay5012 (2020), which is the correct citation for the 929 high-coverage genome HGDP dataset.

Line 560: the precursor mass tolerance, considering the instrumentation used, should be reduced to 5ppm and all the analyses, with all the 3 software tools used, should be repeated using this more stringent parameter, to check whether the final results change significantly.
Response: Since the MaxQuant default precursor mass tolerance of 4.5 ppm is already strict, we mainly focus on PEAKS and pFinds. To observe the effects of PEAKS and pFind, we compare the results of 5 ppm and 10 ppm settings. The results were similar, and the two main SAPs (AMBN-253 and AMBN-273) within the *Homo* genus are consistently identified (Supplementary Table 8). As the final results are consistent, we focus here on the results from the commonly used 10 ppm search to make our results more comparable to previous studies.
Change in the Methods: "Considering the high resolution of the instrumentation used, we also reduced the precursor ion (MS1) mass tolerance to 5 ppm and repeated all analyses in PEAKS and pFind. The results were similar, and the two main SAPs (AMBN-253 and AMBN-273) within the *Homo genus* are consistently identified (Supplementary Table 8). As the final results do not change significantly,

we mainly focus on the results from the commonly used 10 ppm search to make our results more comparable to previous studies."

| SAP assignment | | | *Homo erectus* (New) | | Denisovan-related | | Confirmation | |
|---|---|---|---|---|---|---|---|---|
| Protein (UniProt Accession) | | | AMBN (Q9NP70) | | | | | |
| Position | | | 253 | | 273 | | PEAKS | pFind |
| Data | Sample | Age (Ma) | 5ppm | 10ppm | 5ppm | 10ppm | | |
| New | ZKD | 0.42 | G (109;17)* | G (124;21) | V (249;54) | V (252;52) | √ | √ |
| New | SJD10M | 0.4 | G (190;40) | G (191;39) | V (222;80) | V (219;75) | √ | √ |
| New | SJD10A | 0.4 | G (17;9) | G (24;9) | V (79;45) | V (87;46) | √ | √ |
| New | SJD14M | 0.4 | G (139;36) | G (145;35) | V (216;72) | V (211;69) | √ | √ |
| New | HX-S1 | 0.41 | G (3;2) | G (3;2) | V (2;2) | V (4;3) | √ | √ |
| New | HX-S2 | 0.41 | G (10;6) | G (9;4) | V (3;3) | V (8;6) | √ | √ |
| New | Harbin | >0.146 | A (275;58) | A (276;58) | V(216;79):M(46;38) | V(211;79):M(49;38) | √ | √ |

Lines 563 and 595: Change (here and everywhere else needed) "pyro-Glu" to "N-terminal pyro-Glu".
Response: We have now updated all instances to change "pyro-Glu" to "N-terminal pyro-Glu."

Lines 587-88: The content of the sentence: "To avoid incorrect X residue identification, we set the mass of X as 6228.71 Da (Sm41)." should be better clarified providing more details and explanation.
Response: Any large mass is acceptable as long as it can avoid potential false positives caused by the pFinds setting. Within pFind's modification configuration, the default mass for "X" is preset to that of isoleucine/leucine. To prevent this default setting from generating spurious peptide-spectrum matches (PSMs), we manually reconfigured the mass value for "X" to an arbitrarily large number (6228.71 Da, carrying 41 Samarium atoms), which significantly exceeds the mass of any natural amino acid. Thus, any in silico peptide containing an "X" yields an aberrant theoretical precursor and fragment ion mass, effectively preventing its match to experimental spectra during the database search and thereby minimizing false positive identifications originating from these ambiguous sequence regions. Now we added the explanation for this. "Within pFind's modification configuration, the default mass "X" is preset to that of isoleucine/leucine. To avoid incorrect identification of the X residue, we set its mass to 6228.71 Da (Sm41), which significantly exceeds the mass of any natural amino acid. Therefore, any *in silico* peptide with an "X" produces abnormal theoretical precursor and fragment ion masses, effectively preventing its matching to experimental spectra during database searches and thus reducing false positives from these ambiguous sequence regions."

Line 597: please provide a valid justification about why the PSM FDR for HX-S1 and for HX-S2 were set at 10% and 5%, respectively, instead of 1%, like for all the other samples.
Response: As mentioned by reviewer 1, we now use only 1% PSM FDR to remain consistent, and the results are not affected.

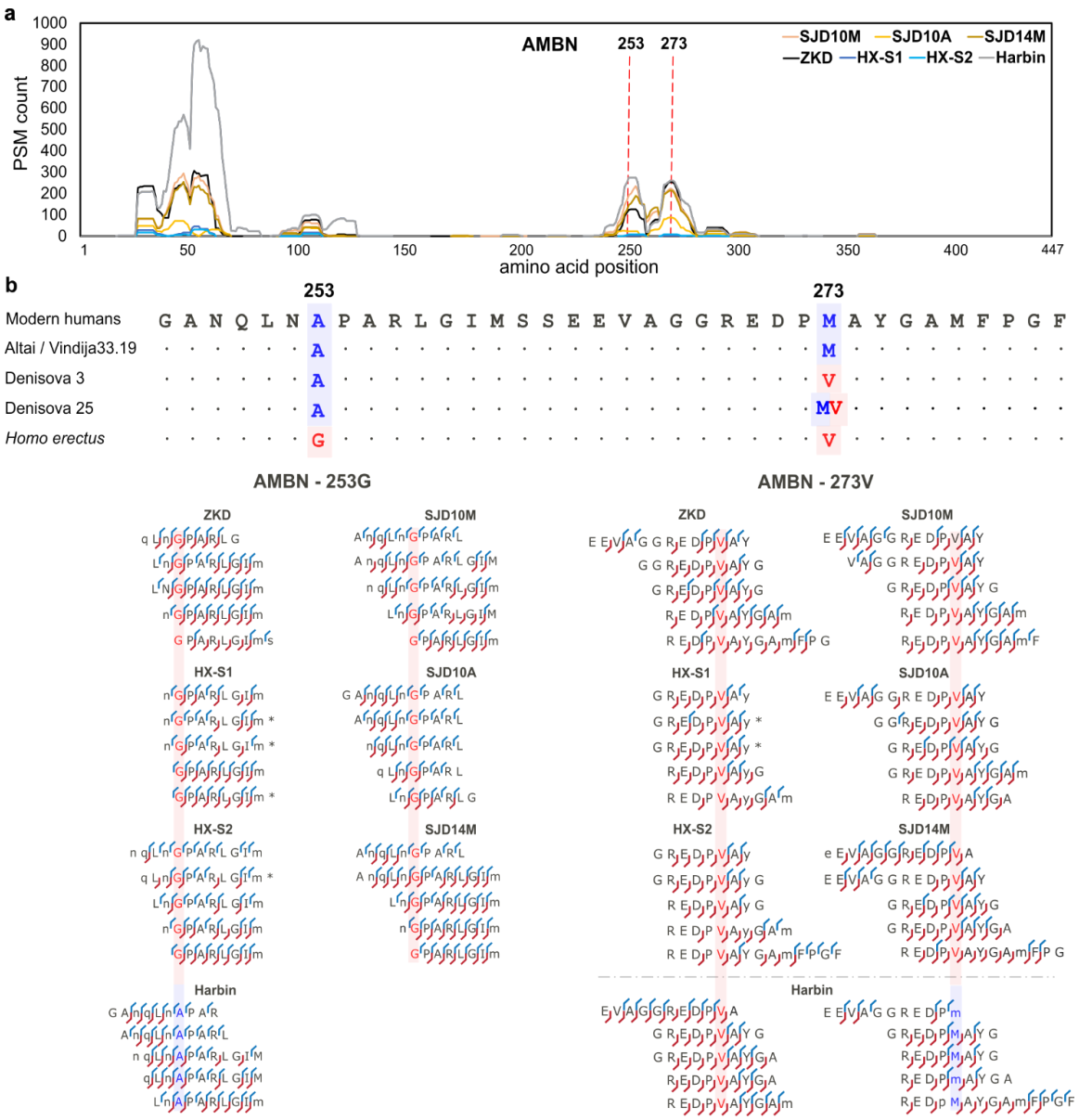
I don't see the point of paragraph "AMELY-162Q and 156-167" under "Supplementary Note 4". The peptides that were possibly identified, by any of the software tools the authors used, as covering

AMELY-162H should be parsimoniously interpreted as peptides covering AMELX-161. This would leave AMELY 156-167 uncovered, as the authors correctly point out.
Response: As suggested, we have removed this paragraph.

Figure 1: the image resolution of specimen HX-S2 is very poor and it should be improved.
Response: Thanks for pointing that out. We have improved the image resolution.

Supplementary Fig. 1 and 2: the resolution of both the two images is quite low and it should be improved. The representation of the identified ion fragments supporting the reconstructed peptide sequence are too small to be read comfortably.
Response: Thank you for pointing this out. We improved the image resolution, as well as added the results for the Haribin individual and have changed both Supplementary Figs. 1 and 2 to Extended Data Figs. 2 and 4.

Integration needed: for all the 6 H. erectus specimens, to better visualise the richness of the evidence supporting the identified of the two SAPs: AMBN-253G and AMBN-273V, the authors should provide supplementary figures showing the alignments of the peptide sequences covering these SAPs, including the positively identified fragment ions. A possible example of this representation is shown by Tsutaya et al. (PMID: 40208980) in fig. 2D. Similar supplementary figures should also be provided for the AMBN-273V/M alleles from the Harbin specimen.
Response: As suggested, we added a new Figure 3b showing the peptide sequence alignments covering these SAPs, including the positively identified fragment ions. To make these two supplementary figures easier to access, we moved them from the supplementary materials to Extended Data figures 2 and 4, and added the fragment ions of both the observed and predicted mirrors for SAP AMBN-253 and AMBN-273 in those figures for all seven new samples, including the Harbin individual.




Referee #4 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

Response: Thanks. We have replied to all three reviewers.

Referees' comments:

Referee #1 (Remarks to the Author):

The authors have appropriately revised the documents based on the recommendations, and I have no major recommendations, just a few possible recommendations that I record line-by-line below. I think the manuscript is publishable as is, with just very minor edits that would improve it.
Response: Thanks for your recommendation.

Line 33: change “not been seen” to “not been recorded” or “not been identified”
Response: Changed as suggested.

Line 604: “After adding, SJD10A also contains SERPINC1 with high deamidation rates”. I would recommend that this can be removed, because the identification of SERPINC1 in the main text and in the extended data table, and this is more of a result than a methodological detail (I think the sentence above is very clear on why SERPINC1 was added to the database). Either way is fine though.
Response: Deleted as suggested.

Line 804, Figure 3: This is very good, I just want to clarify: these are just a selection of the best-looking peptides covering these SAPs, in the different specimens, correct? If so, I think you could add in the figure caption that you are just presenting a small subset of the supporting peptides (in other words).
Response: Thanks. Yes, we only add a small subset of high-quality peptides. Now we indicate this in the figure caption. “The alignments of multiple high coverage human sequences, and the small subset of supporting peptides and fragment ions for SAP AMBN-253 and AMBN-273 in the new samples.”

Line 841, Extended Data Fig. 2: I like these, but I noticed only the peaks that are informative for the peptide sequences are represented, in the experimental spectrum. Or potentially, these are only the highest-intensity peaks? Would it be possible to include all the peaks in the experimental spectrum? I am unsure if this is a standard or not, so I will defer to the authors if they choose to do this or not. I think it is okay without the additional peaks, as well.
Response: In Extended Data Fig. 2, we are required to present both the experimental peaks and the predicted mirror for the other reviewer. If the figures include mirrors, they cannot present uninformative peaks. These are the reasons we only show the informative peaks.

Best,
Ryan Sinclair Paterson

Referee #2 (Remarks to the Author):

Fu et al. Nature, “Enamel proteins from six Middle 1 Pleistocene Homo erectus specimens across southern and northern China”, 2025-10-27803A

I am satisfied with the authors’ revisions and thank them for their careful diligence in response to all reviewers. Note that some basic age model is required for the three Zhoukoudian, Sunjiadong, and Hexian stratigraphic columns shown in Figure 1.
Response: As suggested, we have added the basic ages for the fossil-bearing layers in the stratigraphic columns in Figure 1.

Referee #3 (Remarks to the Author):

The authors satisfactorily replied to all my comments, and, for what I can see, to the comments of all the other reviewers. I would like to sincerely congratulate them for the high competence they showed in generating and processing the data and for the relevance of the results they achieved. I still have just a few more, very minor, comments:
Response: We thank you for the recognition of our efforts.

Line 44: Replace: "A broad definition of Homo erectus (H. erectus sensu lato) was widely distributed...", with: "Broadly defined Homo erectus (H. erectus sensu lato) was widely distributed...".
Response: Thanks, changed as suggested.

Line 91: After introducing the H. erectus material, the Harbin specimen should also be introduced, also motivating its inclusion in this study, by adding a sentence here.
Response: Thanks for the suggestion. We added a sentence for Harbin after introducing the H. erectus material. “Additionally, we included a tooth from an earlier-reported Denisovan skull (minimum age 0.15 Ma) from Harbin, China (Ji et al., 2021; Ni et al., 2021).”

Lines 93-6: "By examining protein preservation [...], we aimed to assess preservation conditions [...] and to gain initial insights into protein preservation [...]." The readability of this sentence should be improved avoiding repetitions.
Response: Now remove the repetitions and change to “By first examining protein preservation in animal fossils from Zhoukoudian, Hexian, and Sunjiadong, we gained initial insights into the feasibility of the analysis without damaging the ancient human fossils.”

Line 110: "from published archaic individuals": does "individuals" refers to humans? hominins? Please clarify.
Response: To avoid confusion, we now change this part to “enamel proteins from translated genomes and published proteomes of present-day humans and other

hominids.”

Line 178: "The maximum y/b ion": is this supposed to be "maximum", or hear you mean "minimum" instead"?
Response: Sorry for the misunderstanding. Here, 'maximum' refers to the highest quality peptide. We now specify it as “The highest-quality peptide for this SAP in each of the six samples has at least 3 y/b ions” to prevent confusion.

Line 232: "which are not found": please check this phrasing. If I'm not wrong, the subject of this verb should be "mutation" in line 231. If so please rephrase as follows: "which is not found".
Response: Yes, and to make it clearer, we change to “, and that this mutation is not found in Denisova 3”.

Line 489: Here the amount of dentine powder should be indicated. If this detail is provided as supplementary information, this should be indicated at the end of the sentence.
Response: As suggested, add Supplementary Data 1 (containing the amount of powder) to the text. “The powder was drilled from the dentin (Supplementary Data 1),”

Line 496: "The trypsinized peptides were desalted and purified.", please specify how the peptides were desalted and purified.
Response: Thanks for your suggestion. As with the enamel peptides, the trypsinized dentine peptides were also desalted and purified using C18 ZipTips. We have supplemented this detail in the Method.
"The trypsinized peptides were desalted and purified using C18 ZipTips."

Lines 520-21: "The protein peptides were eluted into a solution of 0.1% trifluoroacetic acid (TFA) and 80% acetonitrile (ACN)." It looks like a sentence is missing before this one, most probably somenthing similar to what reported in the sentence in lines 598-500: "The acid solution...".
Response: Thanks for pointing this out. We have reviewed the details of our methods and found that vacuum concentration was unnecessary for the enamel peptide extraction from hominin samples, as only 200 μL of acid extraction solution (two rounds of etch solution combined) was used for desalting and purification using C18 ZipTips. However, for peptide extraction from the animal fossils, more than 1mL of acid solution was used; thus, vacuum concentration was performed before desalting and purification. So nothing was missed here. But we have reordered some sentences to make it clear.
"This second etch was repeated, and the etch solutions were combined. After etching, the etched area was treated with 100 μL of 50 mM ammonium bicarbonate solution for 1 minute to neutralize the acid. It was then rinsed with ultrapure water for 30 seconds and dried. The combined etch solution was then desalted using C18 ZipTips (Thermo Fisher Scientific) and eluted into a solution of 0.1% trifluoroacetic acid (TFA) and 80%

acetonitrile (ACN). "

Line 522: "The peptide mixture was further divided into different aliquots...": please specify how many aliquots. Alternatively, please the volume of each aliquot.
Response:

Response: Thanks for your suggestion. We have added more details to the Method section.
"The peptide mixture (50 μL) was further divided into three aliquots; one aliquot was composed of 16 μL, among which 3 μL was used for the MALDI-TOF mass spectrometry test and 13 μL was retained as backup in our laboratory; two aliquots (each composed of 17 μL) were dried for Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis in two independent laboratories. "

Line 638: Replace:
- "Gln->pyro-Glu[AnyN-termE]", with "Gln->pyro-Glu[AnyN-termQ]"
- "Glu->pyro-Glu[AnyN-termQ]", with "Glu->pyro-Glu[AnyN-termE]"
Response: Replaced as suggested.

Lines 638-39: "Pro->N-terminal pyro-Glu[P]": please check the accuracy of the definition of this modification, it does not look correct to me.
Response: Thanks for your suggestion. "Pro->N-terminal pyro-Glu[P]" should be replaced with "Pro->pyro-Glu[P]," which is listed as UNIMOD:359 in the UNIMOD database (+13.98) (Creasy and Cottrell, 2004). We have now changed it to "Pro->pyro-Glu[P]."

Line 655: "Dioxidation[P](Pro->Glu[P])."Please check the accuracy of the definition of this modification; it does not look correct to me.
Response: “Dioxidation[P](Pro->Glu[P])” is correct and could be found as UNIMOD:425 and UNIMOD:1168 in the UNIMOD database. They have equal mass shifts (+31.98) and thus are combined in pConfig. First detected in ancient samples by pFind open-search, they were then added to the variable modification list and were considered possible further degradation PTMs on Pro besides Hydroxylation[P] (Berlett and Stadtman, 1997).

FASTA File "534830_1_related_ms_5038698_t42c44.txt": In the FASTA sequence: ">COL17A1-Harbin" there is a portion of reconstructed sequence reading: "...XEPGX...". It is surprising to see the identification of a peptide that is only 3 amino acids-long, considering that the minimum length of the peptides identified is 7 amino acids. Is this an inconsistency, or the authors have a valid reason not to discard such a

very short peptide? Please check whether any other very short (i.e. 3-4 amino acid long) peptide appears in the FASTA file.

Response: We discarded peptides shorter than 8 amino acids in SAP confirmation and consensus sequence reconstruction. Now we add this filter in the note of Table 1, "Note: Peptides shorter than 8 amino acids were discarded in SAP confirmation", and in the consensus part of the method: "Peptides shorter than 8 amino acids or with abnormal or artificial post-translational modifications (PTMs) were excluded for the consensus sequence reconstruction of each endogenous protein". The amino acid consensus in the FASTA sequence requires coverage by at least two peptides; this means that any position with single peptide coverage would be called as "X". When the reconstructed sequence read "...XEPGX...", it didn't mean the identification of a peptide with only 3 amino acids long. Instead, it meant that two peptides overlaped at "EPG" region, while both sides of "EPG" region were supported by only one peptide and represented by X. This occurrence is common in reports with similar sequence reconstruction strategies, especially in studies of collagens, such as the Harbin cranium (COL11A1) and Penghu1 mandible (JUP, LUM, LYZ, OMD, and nearly all collagens). We added some details in the legend of Supplementary Data 2. "Note that the amino acid consensus in the fasta sequence requires coverage by at least two peptides; thus， single-peptide supported positions were represented by X."

Enrico Cappellini

## References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Summary of the Paper

*Homo erectus* has been discovered in multiple locations around the world and is generally considered an ancestor of *Homo sapiens*. Thus, it holds significant implications for hominin evolution. Despite advances in molecular detection of Pleistocene hominins, we still lack robust data from *H. erectus*. This study aims to fill that gap and contribute to clarifying what has been termed “the muddle in the middle.” The authors employ palaeoproteomics methodology, first testing it on faunal specimens before applying it to hominin material.

All specimens originate from China; both northern and southern regions, across three sites: Zhoukoudian, Sunjiadong, and Hexian. For the *H. erectus* specimens, a minimally destructive extraction method (acid etching) was used. The number of specimens per site is as follows: Zhoukoudian (1), Sunjiadong (3), and Hexian (2), all dated to approximately 400,000 years ago (Middle Pleistocene).

The authors report determining the biological sex of all hominin specimens: one female and five males. Male sex determination was based on identifying peptides matching to amelogenin Y (amelY), while female identification was inferred using a ratio of peptides covering Y-specific positions relative to peptides from both X and Y, with a 95% confidence interval calculated for this ratio.

They also report two amino acid variants (SAPs) in the protein ameloblastin; one unique to this *H. erectus* sample set, and another previously identified as Denisovan-specific. They suggest that the Denisovan SAP may have originated from this *H. erectus* population.

This is great work and deserves to be published in **Nature**.

Recommended Improvements

- For the unique SAP, I suggest the authors validate the peptide by generating a synthetic version or, if not modified, use software-based validation such as ProteomicsDB (https://www.proteomicsdb.org/use/), which was used in the enamel *Paranthropus* paper.

- The methodology is mostly clear, but the following points could be expanded or clarified:

- The authors used both 1% and 10% FDR for PSM identification in MaxQuant for the Hexian individuals. Why was this dual threshold applied? And the final data which FDR was used?
- Regarding sex determination based on amelX and amelY peptide ratios:
    - What assumptions are required for these results to be valid to make sense to the reader?
    - Since the calculation is based on modern individuals, should we assume that *H. erectus* expressed amelogenin similarly to modern humans?
    - Is the ratio calculated based on the number of peptides or their intensity? If theres a reseason for one or the other, may you please include this in the methods
        - It would be helpful for the authors to elaborate on these points, perhaps in the supplementary materials.

- It would also be useful to include the amelY spectra used to identify the male individuals in the supplementary materials.
- For the spectra shared in the supplementary section, including scan numbers would allow readers to investigate further.
- Lastly, I think it would be useful to have a table with information on which raw files match which hominins and which fanua.

References

- The manuscript generally references previous literature appropriately. However, when discussing the widespread distribution of *H. erectus*, the authors could also mention the South African *H. erectus* from Drimolen (Herries et al., 2020: -> http://science.org/doi/10.1126/science.aaw7293).

Thank you kindly for your work,

Palesa P. Madupe