nature portfolio

Peer Review File

Implementing N-terminomics and machine learning to probe Nt-arginylation

Corresponding Author: Dr Cheolju Lee

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This is a very interesting paper investigating global screening approaches to the identification of targets of Nt-arginylation. Such identification is currently a bottleneck in the emerging studies of this important biological problem, so the present work is timely and of high interest to the field. A reliable method of arginylation identification would definitely address a critical need in the field.

The authors perform a very impressive set of experiments, both biochemical and computational, to identify and validate a number of arginylated targets. This method is highly promising for the future of the field.

Here are the comments that I think need to be addressed before the paper proceeds:

1. The overall tone of the paper is a bit one-sided. The authors make an impression that arginylation is primarily a mechanism of protein removal and quality control and analyze arginylation upon induction of ER stress and inhibition of protein degradation. While this design is appropriate for the enrichment of arginylated proteins, it is intrinsically biased toward degradation targets, and this should be clearly explained in the text. The authors should put the writing into the general context of the field and acknowledge that other arginylation pathways also exist and their targets are likely underrepresented in the current analysis.

2. As expected from this experimental design, the putative arginylated targets identified in the present study are heavily enriched in proteolyzed proteins, generated by caspases and cleavage of signal peptides, along with other mechanisms. This bias affects the prediction of the consensus and seqlogo results. The authors should clearly acknowledge that this may not be the case for proteins arginylated under normal non-stress conditions, which are underrepresented in the current dataset.

3. If the present analysis includes, e.g. N-terminally arginylated proteins with N-termini generated by Met-aminopeptidases (proteins arginylated on residue 2 or 3 from the N-terminus), these targets should be separately discussed and highlighted. If no such targets were found, it should also be stated, since it represents an interesting biological observation.

4. Previous attempts to develop algorithms for arginylation predictions have been made, and the authors should comment on the approaches used in that prior study as well as compare the results to their current work. Also, previously a number of Nt-arginylated proteins have been reported, including putative screens from older work. Is there any overlap?

5. In section 1 of the results, it is unclear what were the limitations of the searches. If tryptic cleavage sites expected to produce Nt-R were removed from the searches in the first place, why did a lot of them still show up? Mass ambiguities related to amino acid combinations are also extensively characterized and routinely used for arginylation identification and discarding false positives, so it is unclear why this cannot be automated. This sections should be written clearer to enable understanding by a non-expert.

6. Section 2 of the results: Multiple previous mass spec studies, especially from Yates lab, emphasize the prevalence of b ions in mass spectra of N-terminally arginylated peptides, yet here the authors present it as their own finding. They should cross reference this to the earlier work where all the arginylated peptide spectra were specifically characterized by

prominent sets of b ions.

7. In the first paragraph of the discussion a misleading statement makes an impression that studies identifying arginylation by database searches call false positives based on missed trypsin cleavage. This is inaccurate. It is true that missed trypsin cleavages would be called as arginylation by automated search, however every published study involving mass spec identification of arginylation has performed extensive additional validation steps, including manual removal of mass abmbiguities of amino acid combinations, ambiguious PTMs, and missed trypsin cleavage. Prior methods papers included mass ambiguity tables for manual validation. Thus, these prior searches are very labor intensive, but not inaccurate. This should be correctly reflected in the current manuscript.

8. The authors should comment the comparison of manual validation and their automated algorithm from the perspective of others in the field, who may be forced to continue validating manually until the current algorithm be adapated for general use.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)
The submission by Ju et al. addresses the identification of N-terminally arginylated proteins by mass spectrometry. Nt-arginylation is a well-characterized signal for degradation, but Nt-arginylated proteoforms are short-lived and remain challenging to identify. The manuscript describes a new approach to tackle this challenge by combining enrichment of N-terminal peptides using iNRICH with an improved data analysis strategy that uses a transfer learning model to predict MS2 spectra and peptide RTs of Nt-arginylated peptides. The improved pipeline is applied to identify Nt-arginylated proteins during thapsigargin-induced ER stress on top of MG132 treatment to block degradation. New candidate Nt-arginylated peptides are validated in the same cell lines by time-resolved PRM. The rationale behind experiments, the methodology and the results are in most cases well documented and, in several cases, independently corroborated (including immunoblots). The source code for the LMM is available from github.

Overall, the manuscript describes a methodological advance that will be of considerable interest and applicability , especially for reseachers interested in N-terminal stability determinants and Nt-arginylation. The section on methodological results appears strong and provides convincing evidence for numerous new Nt-arginylation sites. However, several points need to be clarified or addressed in greater detail. In addition, the biological interpretation is rather limited and avoids touching on obvious questions. No biological follow-up on the functional significance of any of the identified sites is provided.

Major points:
1. Please clarify the generation of the training dataset. Line 102 indicates that the same dataset of enriched N-termini is searched, and Suppl. Fig 3 indicates that these are mostly D3-acetylated peptides arising from missed cleavage. How is this possible if labeling takes place before digestion (Fig. S3a)? Is there significant carry-over of labeling reagent? Ideal would be a second experiment, where proteins are first digested to peptides, followed by D3-ac labeling after digestion to ensure that all peptides with an N-terminal Arg are labeled.

2. The transfer learning adaptation of the AlphaPeptDeep LLM was trained on which set of Nt-Arg pepides (line 113)? Labeled or non-labeled peptides? Has the effect of N-terminal acetylation been tested?

3. The ML-based filtering of PSMs using a training set of Nt-Arg containing peptides from missed cleavages is a novel and substantial improvement of the methodology to reliably identify Nt-arginylated peptides in N-terminome data. However, the principle of chemical labeling to generate reporter fragments of an N-terminal Arg residue is not novel. MS2 fragment error distribution was previously used to distinguish false positives (e.g. Nt-V-G peptides) from true Nt-arginylated peptides (Hoernstein et al. 2016, https://doi.org/10.1074/mcp.M115.057190). Similarly, it has been previously reported that Nt-arginylation promotes early b-ion fragments (Xu et al. 2009, doi:10.1038/nprot.2008.248). Pprominated formation of b1-ions has also been reported for other N-terminal basic amino acids (Hiserodt 2007, https://doi.org/10.1016/j.jasms.2007.04.018) as well as Nt-acetylated peptides (Yalcin et al., 1995, https://doi.org/10.1016/1044-0305(95)00569-2; Medzihradszky et al. 2005, https://doi.org/10.1016/S0076-6879(05)02007-0). These earlier reports should be cited and discussed.

4. How many Nt-arginylated peptides were present in the HeLa dataset selected for training from unlabeled data in the PRM approach?

5. The sequence logos show a clear predominance of amino acids in P1' which are secondary or tertiary destabilizing amino acids (D|E|Q|N) in the context of the N-end rule pathway. Please show sequence logos for peptides within treatment groups (Mock, MG, MGTG) – do these treatments affect Nt-arginylation patterns?

6. Several mitochondrial or secretory proteins are identified as Nt-arginylated at the sites of MTS or SP cleavage, suggesting that this occurs after translocation across the respective membrane(s). The authors allude that this may be ATE1-dependent Nt-arginylation of mistargeted proteins (line 401 following). This would be more convincing if shown in an ATE knock-down cell line, for example with the established PRM assays.

7. The quantification of Nt-arginylated peptides and their counterparts is an interesting and important aspect that is only explored in the PRM assay, and surprisingly failed more frequently for the non-modified peptides that would be expected to be much more abundant. How many non-arginylated counterparts of Nt-arginylated peptides were identified in the enriched N-terminome, and if so, how does their intensity in this unbiased assay compared to their arginylated counterparts?

8. The manuscript must be edited for language, flow and clarity, and proof-read in detail. For example, the first paragraph of the results section (lines 69-94) starts with describing enrichment and analysis of N-terminal peptides (lines 69-76) before even explaining which samples were analysed (line 77). The important fact that two proteases are used in the N-termini enrichment experiments (Trypsin and chymotrypsin) is only mentioned later in line 123. Similar convoluted descriptions are found at several places throughout the manuscript.

Minor points
9. Line 141 and following: It would be helpful if the identity of the arginylated amino acid was added to the combination of protein name and position number, e.g instead of MTHFD2|36 write MTHFD2|36E.

10. Line 76: Rephrase “Before LC-MS/MS, we treated…” This is ambiguous, cells are treated also before any other critical sample handling steps such as enrichment, not directly before LC-MS/MS.

11. Line 272-274: what are the P1’ amino acids of the Nt-arginylated proteins identified in the mock samples? Please show a sequence logo or e.g. iceLogo of this subset and discuss the assumption of “a mechanism where Nt-arginylation may not induce protein degradation” in the context of the most frequent P1’ amino acids.

12. Line 282: Is this difference statistically significant? Is this a p-value in Fig. 4e? Please specify more precisely in the text and in the figure legend.

13. Line 290-292: “Since these proteins are essential for cancer cell survival and proliferation, the discovery of Nt-arginylation of these proteins may provide a novel tool for controlling these potential therapeutic targets”. Please describe more precisely how this is meant or skip or change the statement.

14. Line 318: Rephrase the statement about antibody limitation. The company Agrisera (https://www.agrisera.com/) provides commercial antibodies against N-terminal R-E and R-D which are among the most abundant arginylation patterns observed in this study. Have these been tested by the authors?

15. Fig S1e-g: How is the low overlap between proteins with Nt-arginylation between replicates explained?

16. Fig S18a: The PCC of the PRCT standards look comparably bad. Could you please comment if your fine-tuned MS2 prediction models fail to predict MS spectra of this standard or why this is the case?

17. Line 353: isn´t this rather unexpected that unmodified peptides did not change? Shouldn´t they go down?

18. Figures should use more consistent font sizes, and all panels of the Supplementary Figures should be readable in print size.

19. Please proof-read figures and figure legends carefully. A few examples for spelling errors:
• Fig 2e: “perocolator” instead of “percolator”
• Fig S3: “Nt-arginylated peptides are tested their MS2 spectra and RT…” should be “Nt-arginylated peptides are tested for their MS2 spectra and RT…”? Please phrase it more precisely.
• Fig S9: “PSMs that are passed MS2 module…” should be “PSMs that passed MS2 module”
• Fig S12: “…e are depicted by color, wieht each …..” should be “...with each…”

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

(Remarks on code availability)

Reviewer #4

(Remarks to the Author)
Review of manuscript “Implementing N-terminomics and machine learning to probe in vivo Nt-arginylation” by Ju et al.

In the manuscript, Ju and co-workers describe an impressive series of experiments, including generating new data for machine learning, validation and biological interpretation of the results. There is definitely enough material in this manuscript for three of four separate papers, but the author should be commended for not “salami slicing”. The authors even went so far as to clone twelve of the identified Nt-arginylated proteins and perform pulldown assays. This is certainly above and beyond most proteomics studies.

Even though it is a massive paper to read, all data and figures presented are relevant, and advance the narrative. The results are in my view definitely noteworthy and sufficient for consideration by the journal.

And even though the authors modestly state that their approach is only an interim solution while waiting for direct Nt-arginylation enrichment methods, I believe much of the framework could be reused. Even the best direct enrichment method will not be 100% efficient.

The work is original, and gives due credit to prior work and the state-of-the-art, both in ML for retention time and MS2 prediction, and N-terminomics. The methods are described in detail, and the data as well as analysis scripts are shared in proper repositories, with redundancy for the data.

I have some minor comments, however, which I believe are relatively easy to address:

Line 375: “Proteomics can now reach single-cell depth” - this statement reads a bit odd. I think the authors mean that proteomes can now be probed to significant depths also in single cells. I associate “depth” as an indicator of the proteome measurement, i.e. how many proteins are detected or the lowest concentration/copy number that can be quantified, not the number of cells needed to measure a given number of proteins.

Was there a conscious strategy to first use Sequest (or SequestHT) and then MSFragger for the LFQ, rather than MSFragger (or FragPipe) from the beginning? Likewise, was there a rationale for why initially allowing a 10 ppm mass measurement error, and subsequently 5 ppm? Does the 5 ppm tolerance mentioned on line 651 refer to MS1 or MS2, or both? What was the resolution (setting) for the MS1 in the PRM measurements?

The code is available on GitHub, which is commendable, but the documentation in the main README is rather limited, and the English could also do with some improvement. There is no overview or explanation for what the program does, and the README is rather unstructured, barely using any Markdown features. Also the code itself has rather few comments, but many lines of code that are commented away. There should at least be a comment about why these chunks are commented, and what they could be used for, if they are to be kept in the code (though I confess I share this habit with the authors). In summary, the authors could tidy up the GitHub repo somewhat. I would also recommend the authors create a release corresponding to the versions of the code used in the (accepted) manuscript and get a DOI for this in Zenodo (this will also create a backup of the GitHub repo at this point in time in Zenodo as well as Software Heritage for redundancy). It is quite easy to do (and completely free of course).

(Remarks on code availability)
As mentioned in the comments to the authors, the code is available on GitHub, which is commendable, but the documentation in the main README is rather limited, and the English could also do with some improvement. There is no overview or explanation for what the program does, and the README is rather unstructured, barely using any Markdown features. Also the code itself has rather few comments, but many lines of code that are commented away. There should at least be a comment about why these chunks are commented, and what they could be used for, if they are to be kept in the code (though I confess I share this habit with the authors). In summary, the authors could tidy up the GitHub repo somewhat. I would also recommend the authors create a release corresponding to the versions of the code used in the (accepted) manuscript and get a DOI for this in Zenodo (this will also create a backup of the GitHub repo at this point in time in Zenodo as well as Software Heritage for redundancy).

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have addressed all my comments.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)
The authors have extensively addressed all comments raised in the first round, further clarified their procedure and added

convincing additional experiments. This will be a valuable contribution to the field and I have only few minor comments that can easily be edited before publication.

Minor comments:
Precision of statements should be improved for some citations:

1. P6 line 215: “These findings suggest that b-ion mass errors likely stemmed from mis-annotation of the N-terminal modification, which may arise from near-isobaric unknown modifications that have not yet been characterized, as well as those extensively catalogued in previous reports (e.g., VG dipeptide)30, 33”

Please clarify. The cited studies did not only catalogue N-terminal modifications, but further propose that this can be used to distinguish false positives. The results given in the present study built on this and further confirm this.

2. P6 line 218: “Indeed, we have proposed that the observed error discrepancy could serve as a confidence metric for assessing the correctness of N-terminal modifications, which we termed the MET33, 34.”

Positioning matters. Please change to: “Indeed it has been proposed that the observed error discrepancy could serve as a confidence metric for assessing the correctness of N-terminal modifications33, 34, which we here termed the MET.”

3. Ref. 34, Lee H. et al. is now published.

4. P6 line 240: “Indeed, when comparing MS2 spectra of an Arg-starting peptide with those of the same peptides lacking Nt-Arg, the b1-ion of D3-acetylated Arg is typically observed at 202.138±0.005 m/z (Fig. 3c and Supplementary Fig. 10a-c)37”

Hiserodt et al. did not work with D3 labeled acetylation, but with deuterated amino acids and the 202 m/z fragment has not been reported in their study. Please rephrase, e.g.: “… and indeed, the b1 ion has been observed when MS2 spectra of an Arg-starting peptide were compared with those of the same peptides lacking Nt-Arg37. Here, we observed the signature b1 ion of D3-acetylated Arg at 202.138±0.005 m/z (Fig. 3c and Supplementary Fig. 10a-c).”

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

(Remarks on code availability)

Reviewer #4

(Remarks to the Author)
The authors have addressed my comments and suggestions. The GitHub page is also much improved, and it is now much easier to understand what the tool does, and what is available in the repository.

(Remarks on code availability)
The documentation has been significantly improved, and new notebooks provided. There are still some lines of code here and there that are commented out, without a reason why. I _understand_ why (the library or line of code was not necessary, but may be useful in the future. While not a gold standard, the code is "good enough", in that it is generally clear what it does and why.

Reviewer #1 (Remarks to the Author):

This is a very interesting paper investigating global screening approaches to the identification of targets of Nt-arginylation. Such identification is currently a bottleneck in the emerging studies of this important biological problem, so the present work is timely and of high interest to the field. A reliable method of arginylation identification would definitely address a critical need in the field.

The authors perform a very impressive set of experiments, both biochemical and computational, to identify and validate a number of arginylated targets. This method is highly promising for the future of the field.

Here are the comments that I think need to be addressed before the paper proceeds:

1. The overall tone of the paper is a bit one-sided. The authors make an impression that arginylation is primarily a mechanism of protein removal and quality control and analyze arginylation upon induction of ER stress and inhibition of protein degradation. While this design is appropriate for the enrichment of arginylated proteins, it is intrinsically biased toward degradation targets, and this should be clearly explained in the text. The authors should put the writing into the general context of the field and acknowledge that other arginylation pathways also exist and their targets are likely underrepresented in the current analysis.

We thank the reviewer for this important point. We agree that our experimental design, based on ER stress induction and proteasome inhibition, enriches primarily for degradation-associated Nt-arginylation and therefore underrepresents other classes of arginylation substrates. We have clarified this limitation and broadened the context of our study in the revised manuscript.
In the Abstract, at line 21 on page 1, the original sentence “N-terminal arginylation (Nt-arginylation) is a degradation signal in the ubiquitin-proteasome and autophagy-lysosomal pathways” is replaced with “… is a multifunctional post-translational modification (PTM) with roles in protein quality control, organelle homeostasis and stress signaling.”
In the Discussion, at line 472 on page 11 we included a new paragraph emphasizing that our study design is intrinsically biased toward degradation targets and other arginylation are likely underrepresented in the dataset.
These changes ensure that the manuscript now places our findings in the broader biological context and transparently communicates the limitations of our experimental approach.

2. As expected from this experimental design, the putative arginylated targets identified in the present study are heavily enriched in proteolyzed proteins, generated by caspases and cleavage of signal peptides, along with other mechanisms. This bias affects the prediction of the consensus and seqlogo results. The authors should clearly acknowledge that this may not be the case for proteins arginylated under normal non-stress conditions, which are underrepresented in the current dataset.

We agree that our design enriches protease-generated N-termini, which biases consensus/seqlogo results toward degradation-linked substrates. To address baseline conditions, we included a non-stress MOCK condition in HeLa and processed it identically. As expected, the number of high-confidence Nt-arginylation PSMs was substantially lower in MOCK, and motif signals were correspondingly weaker; thus, the consensus is largely driven by the stress conditions.
We acknowledge this does not fully represent non-stress related arginylation. A comprehensive baseline will require broader profiling across multiple cell lines and tissues and in various physiological

conditions. We have revised the Results at line 264 on page 7 to explicitly state that the consensus/seqlogo primarily reflects stress-biased data

3. If the present analysis includes, e.g. N-terminally arginylated proteins with N-termini generated by Met-aminopeptidases (proteins arginylated on residue 2 or 3 from the N-terminus), these targets should be separately discussed and highlighted. If no such targets were found, it should also be stated, since it represents an interesting biological observation.

We thank the reviewer for raising this important point. With respect to Met-aminopeptidase–generated N-termini (i.e., residue 2 or 3), we did not detect such Nt-arginylated sites with high confidence in this dataset. Although we identified a putative isoform-specific Nt-arginylation at SHC1 (UniProt P29353) at position 112, which corresponds to the annotated p52SHC (P29353-2) N-terminus rather than the canonical p66 N-terminus. This peptide passed all ML filters (MS2 similarity, RT agreement within the prediction interval, and b–y mass-error symmetry), but we have not yet performed orthogonal validation specific to this isoform context. We therefore report it at line 301 on page 8 as a candidate isoform-derived Nt-arginylation site and highlight it separately in the Discussion at line 454 on page 11.

4. Previous attempts to develop algorithms for arginylation predictions have been made, and the authors should comment on the approaches used in that prior study as well as compare the results to their current work. Also, previously a number of Nt-arginylated proteins have been reported, including putative screens from older work. Is there any overlap?

We thank the reviewer for raising this important point. To address it, we systematically compared our in vivo arginylation dataset (Supplementary Data 5) with previously reported arginylation sites compiled from the literature (Wong et al., 2007; Xu et al., 2009; Lin et al., 2025; MacTaggart et al., 2023). Our understanding is that earlier studies primarily relied on rule-based heuristics coupled with careful manual curation—for example, screening for pronounced b-ion series, checking precursor/fragment mass inaccuracies, and consulting mass-ambiguity tables to eliminate near-isobaric substitutions and missed trypsin cleavages. These approaches were rigorous but labor-intensive and largely non-automated.
By contrast, our contribution is an automated scoring/triage pipeline that formalizes these criteria: we use transfer-learned MS2 and RT prediction together with a fragment mass-error symmetry test (MET) to rescore putative Nt-Arg PSMs and control FDR. To our knowledge, we are not aware of a previously published, arginylation-specific automated algorithm that integrates these orthogonal features into a unified rescoring framework; prior work emphasized manual post-processing or general search-engine scoring.
Regarding overlap with previously reported arginylomes, we compiled a literature set and compared it with our dataset. In the revised Discussion at line 462 on page 11 and corresponding Supplementary Table 3, we clarify that we found four positional matches, confirming that our workflow recovers bona fide sites while also expanding the arginylome under stress-enriched conditions.

5. In section 1 of the results, it is unclear what were the limitations of the searches. If tryptic cleavage sites expected to produce Nt-R were removed from the searches in the first place, why did a lot of them still show up? Mass ambiguities related to amino acid combinations are also extensively characterized and routinely used for arginylation identification and discarding false positives, so it is unclear why this cannot be automated. This sections should be written clearer to enable understanding by a non-expert.

We thank the reviewer for pointing this out. We agree that the rationale and limitations of the tandem search strategy were not described with sufficient clarity. We have now revised Section 1 of the Results to more explicitly explain why ambiguous peptides persist after the first search, and why mass ambiguities

cannot be resolved solely by automated database filtering.
Specifically, we clarify that:
The first-pass search (without Nt-Arg PTM) removes spectra that confidently match Arg-starting tryptic peptides, but a fraction of near-boundary matches may not be filtered at 1% PSM-level FDR and leak into the second search. Those residual spectra can then be mis-annotated as Nt-Arg when the modification is allowed.
For MS1 tolerance, we used 10 ppm absolute window scales (e.g., ~±0.01 Da at 1000 Da vs ~±0.02 Da at 2000 Da). Our N-terminomics enrichment tends to yield longer/heavier N-terminal peptides due to blockage of lysine side chain, which increases the number of plausible matches that can slip through the first pass.
While certain mass combinations (e.g., Nt-Arg vs. GV/VG) are catalogued, they often appear within the noise of experimental error and cannot always be disambiguated by mass shift alone. In addition, while amino acid combinations present in the sequence can be readily identified, cases involving unknown modifications combined with amino acids are more difficult to systematically tabulate and detect. This is why we introduced ML-based RT and MS2 prediction filters.
These clarifications have been added to the first section of Results at line 91 on page 3 to make the limitations of the tandem database search strategy transparent.

6. Section 2 of the results: Multiple previous mass spec studies, especially from Yates lab, emphasize the prevalence of b ions in mass spectra of N-terminally arginylated peptides, yet here the authors present it as their own finding. They should cross reference this to the earlier work where all the arginylated peptide spectra were specifically characterized by prominent sets of b ions.

We thank the reviewer for pointing this out. Indeed, earlier studies, particularly from the Yates laboratory (Xu et al., Nat Protoc 2009; Wong et al., PLoS Biol 2007), characterized arginylated peptides as exhibiting strong b-ion series. Although we cited that paper, our description unintentionally gave the impression that we were reporting the phenomenon for the first time. We have now revised the text to remove that phrasing and instead refer explicitly to the previous finding at line 110 on page 4.

7. In the first paragraph of the discussion a misleading statement makes an impression that studies identifying arginylation by database searches call false positives based on missed trypsin cleavage. This is inaccurate. It is true that missed trypsin cleavages would be called as arginylation by automated search, however every published study involving mass spec identification of arginylation has performed extensive additional validation steps, including manual removal of mass abmbiguities of amino acid combinations, ambiguious PTMs, and missed trypsin cleavage. Prior methods papers included mass ambiguity tables for manual validation. Thus, these prior searches are very labor intensive, but not inaccurate. This should be correctly reflected in the current manuscript.

We thank the reviewer for highlighting this important point. We agree that our initial phrasing in the Discussion could give the impression that prior mass spectrometry studies of arginylation reported inaccurate results. This was not our intent. We fully acknowledge that previous work (e.g., Xu et al., 2009; Wong et al., 2007) performed extensive manual validation, including the removal of mass ambiguities and filtering of missed cleavages. These efforts produced reliable datasets, but at the cost of being extremely labor-intensive.
We have revised the Discussion at line 416 on page 10 to clarify that the main limitation of prior methods was not inaccuracy, but rather the heavy reliance on manual curation. Our contribution is to automate and formalize these validation steps through ML-based spectral prediction and statistical filters, thereby reducing the need for manual intervention while maintaining accuracy.

8. The authors should comment the comparison of manual validation and their automated algorithm from the perspective of others in the field, who may be forced to continue validating manually until the current algorithm be adapated for general use.

We thank the reviewer for this valuable suggestion. We agree that while our ML-based algorithm reduces the need for manual validation, researchers in the field may still need to rely on traditional manual approaches until such computational tools are broadly validated, standardized, and integrated into existing proteomics pipelines. We have now added text in the Discussion to emphasize that our algorithm should be viewed as a complement to, rather than an immediate replacement for, manual validation.
We have revised the Discussion at line 415 on page 10 to address this comment, together with Comment 7.

Reviewer #2 (Remarks to the Author):

The submission by Ju et al. addresses the identification of N-terminally arginylated proteins by mass spectrometry. Nt-arginylation is a well-characterized signal for degradation, but Nt-arginylated proteoforms are short-lived and remain challenging to identify. The manuscript describes a new approach to tackle this challenge by combining enrichment of N-terminal peptides using iNRICH with an improved data analysis strategy that uses a transfer learning model to predict MS2 spectra and peptide RTs of Nt-arginylated peptides. The improved pipeline is applied to identify Nt-arginylated proteins during thapsigargin-induced ER stress on top of MG132 treatment to block degradation. New candidate Nt-arginylated peptides are validated in the same cell lines by time-resolved PRM. The rationale behind experiments, the methodology and the results are in most cases well documented and, in several cases, independently corroborated (including immunoblots). The source code for the LMM is available from github.

Overall, the manuscript describes a methodological advance that will be of considerable interest and applicability , especially for reseachers interested in N-terminal stability determinants and Nt-arginylation. The section on methodological results appears strong and provides convincing evidence for numerous new Nt-arginylation sites. However, several points need to be clarified or addressed in greater detail. In addition, the biological interpretation is rather limited and avoids touching on obvious questions. No biological follow-up on the functional significance of any of the identified sites is provided.

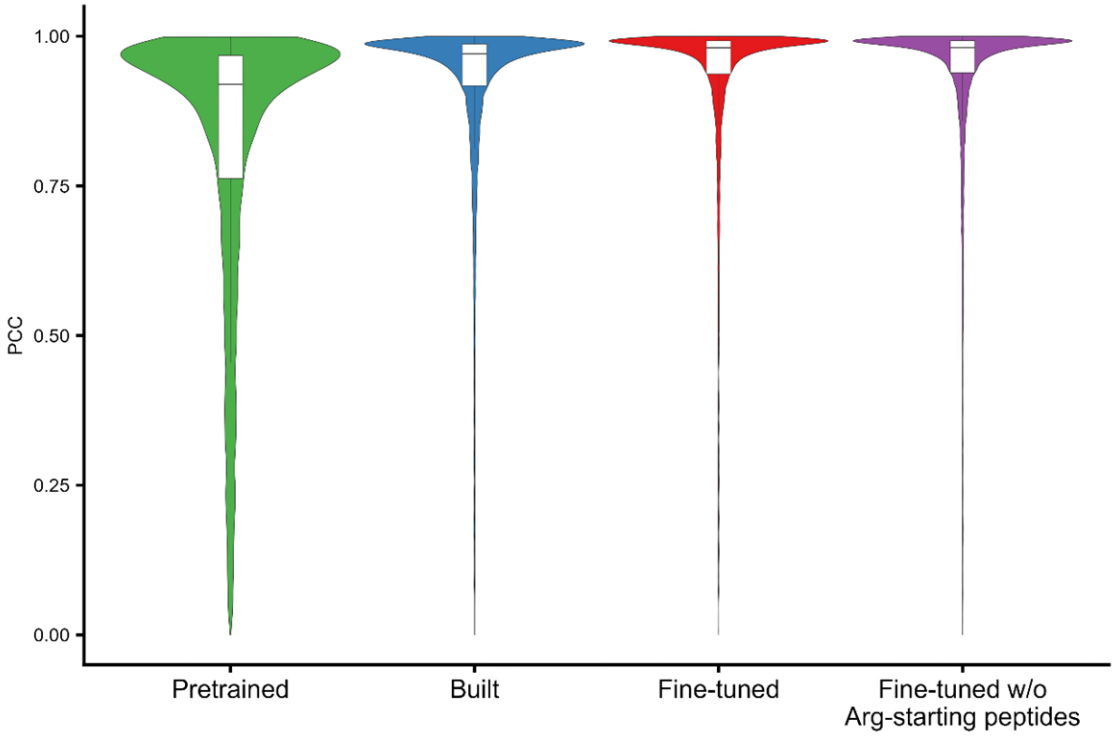
Major points:

1.Please clarify the generation of the training dataset. Line 102 indicates that the same dataset of enriched N-termini is searched, and Suppl. Fig 3 indicates that these are mostly D3-acetylated peptides arising from missed cleavage. How is this possible if labeling takes place before digestion (Fig. S3a)? Is there significant carry-over of labeling reagent? Ideal would be a second experiment, where proteins are first digested to peptides, followed by D3-ac labeling after digestion to ensure that all peptides with an N-terminal Arg are labeled.

We appreciate this comment and have clarified the trade-offs and controls. iNrich that we utilized to enrich N-terminome is a single-stage, encapsulated reactor workflow where protein primary amines are labeled with acetic anhydride-d6 before digestion, then newly created peptide N-termini are depleted with NHS chemistry; the single-reactor format boosts throughput/yield. To avoid stage changes and competitive amines, iNrich does not add a primary-amine quencher; instead it uses pyridine to catalyze hydrolysis of excess anhydride, minimizing carry-over without extra wash steps. Nevertheless, carryover does occur, and d3-acetylation of the remaining internal peptides (including missed cleavage R-starting peptide) is observed. Details regarding this are well described in our previous paper (Ju, S. et al, Anal. Chem. 92, 6462–6469 (2020), 10.1021/acs.analchem.9b05653). We used the spectra of such peptides for training.
I agree that your strategy would be better where proteins are first digested to peptides, followed by D3-ac labeling after digestion to ensure that all peptides with an N-terminal Arg are labeled. However, we had deep down analyzed iNrich samples, which provided us enough Arg-starting peptides to use for ML-training.

2.The transfer learning adaptation of the AlphaPeptDeep LLM was trained on which set of Nt-Arg pepides (line 113)? Labeled or non-labeled peptides? Has the effect of N-terminal acetylation been tested?

We fine-tuned the AlphaPeptDeep MS2/RT models on all available PSMs from the same N-terminomics runs, spanning both labeled protein N-termini (D3-acetylated at the α-amine) and labeled internal peptide generated due to reagent carry-over, non-labeled internal peptides generated due to incomplete depletion during enrichment, as well as common PTMs (e.g., Met oxidation). And we explicitly evaluated model performance on an endogenous Nt-acetylated subset. The fine-tuned MS2 model achieved an average PCC = 0.946 across 388,316 PSMs overall, and average PCC = 0.920 on the Nt-acetylated subset (n = 15,530). This small decrease is expected given altered charge localization/fragmentation at acetylated N-termini, but performance remained high, indicating that the transfer-learned model generalizes across both labeled and non-labeled N-termini and across Nt-acetyl chemistry. We clarified which peptides utilized in training set in the Result at line 129 on page 4.
For the effect on N-terminal acetylation, median PCC of Nt-acetylated peptides were; Pretrained, 0.919. From scratch model, 0.970. Finetuned, 0.981. Finetuned without Arg-starting peptides, 0.981. Concordantly, transfer learning on pretrained model could improve Nt-acetylated peptides as well as Nt-arginylated peptides. And for Nt-acetylation, finetuning without Arg-starting peptides did not affect on performance of models predicting Nt-acetylation, as expected. (Figure below)

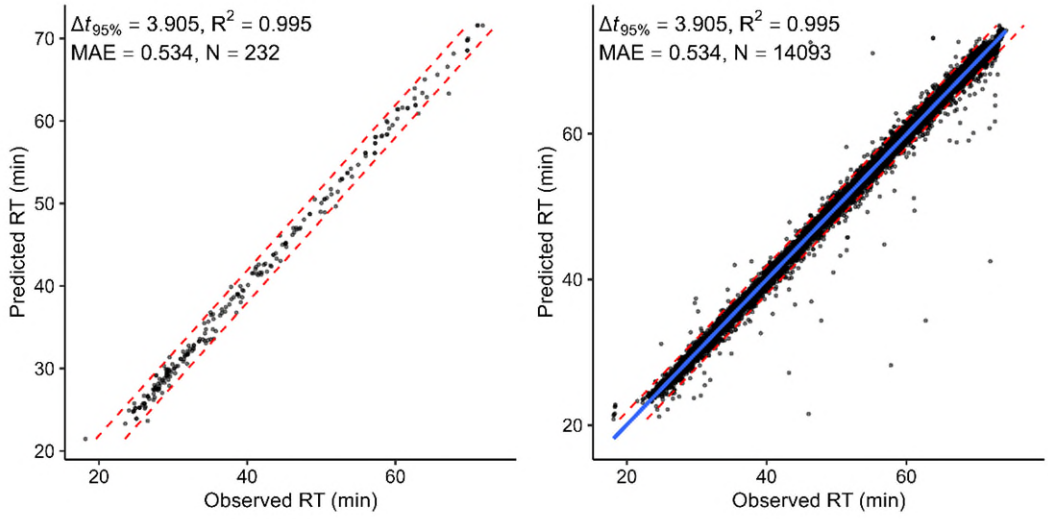


3.The ML-based filtering of PSMs using a training set of Nt-Arg containing peptides from missed cleavages is a novel and substantial improvement of the methodology to reliably identify Nt-arginylated peptides in N-terminome data. However, the principle of chemical labeling to generate reporter fragments of an N-terminal Arg residue is not novel. MS2 fragment error distribution was previously used to distinguish false positives (e.g. Nt-V-G peptides) from true Nt-arginylated peptides (Hoernstein et al. 2016, https://doi.org/10.1074/mcp.M115.057190). Similarly, it has been previously reported that Nt-arginylation promotes early b-ion fragments (Xu et al. 2009, doi:10.1038/nprot.2008.248). Pprominated formation of b1-ions has also been reported for other N-terminal basic amino acids (Hiserodt 2007, https://doi.org/10.1016/j.jasms.2007.04.018) as well as Nt-acetylated peptides (Yalcin et al., 1995, https://doi.org/10.1016/1044-0305(95)00569-2; Medzihradszky et al. 2005, https://doi.org/10.1016/S0076-6879(05)02007-0). These earlier reports should be cited and discussed.

Thank you for highlighting prior works. We have added the requested citations and clarified novelty. Our study does not claim novelty for: (i) chemical labeling to generate reporter fragments at N-terminal Arg; (ii) the use of MS2 mass-error distributions to flag false positives (e.g., Nt-VG vs Nt-Arg) (Hoernstein et al., 2016); or (iii) the observation that Nt-Arg promotes early/strong b-ions (Xu et al., 2009), that N-terminal basic residues favor b1 formation (Hiserodt, 2007), and that Nt-acetylated peptides produce b1 reporter ions (Yalcin et al., 1995; Medzihradszky et al., 2005).
Our advance is to automate and unify these principles for N-terminomics-scale discovery: we (a) train transfer-learned MS2 and RT predictors on abundant Arg-starting proxies, (b) implement a generalized fragment mass-error symmetry test (MET) inspired by the mass-error logic of Hoernstein et al., and (c) combine these orthogonal signals in a single rescoring pipeline that reduces manual curation while maintaining specificity. We have revised the Results and Discussion to cite and discuss these precedents explicitly (Hoernstein et al., 2016; line 218 and 220 on page 6. Xu et al., 2009; line 110 on page 4 and line 422 on page 10. Hiserodt et al., 2007, Yalcin et al., 1995 and Medzihradszky et al., 2005; line 239 on page 6)

4.How many Nt-arginylated peptides were present in the HeLa dataset selected for training from unlabeled data in the PRM approach?

In PRM experiments, a mixture of HeLa digest and PRTC (Thermo Fisher Scientific) as internal standards was analyzed by LC-MS/MS in DDA mode before every six PRM experiments. The dataset collected in this DDA mode was used to build an RT prediction model, which was used to analyze the



following six PRM data. We include this information in Method section at line 640 on page 15 and clarify the workflow in the Result at line 357 on page 9. Although the number of Arg-starting peptides varies with each calibration, in general case, for example, 232 PSMs of Arg-starting peptides (Figure, left) were included in total 14,093 PSMs of HeLa digest 1 µg (Figure, right).

5.The sequence logos show a clear predominance of amino acids in P1' which are secondary or tertiary destabilizing amino acids (D|E|Q|N) in the context of the N-end rule pathway. Please show sequence logos for peptides within treatment groups (Mock, MG, MGTG) – do these treatments affect Nt-arginylation patterns?

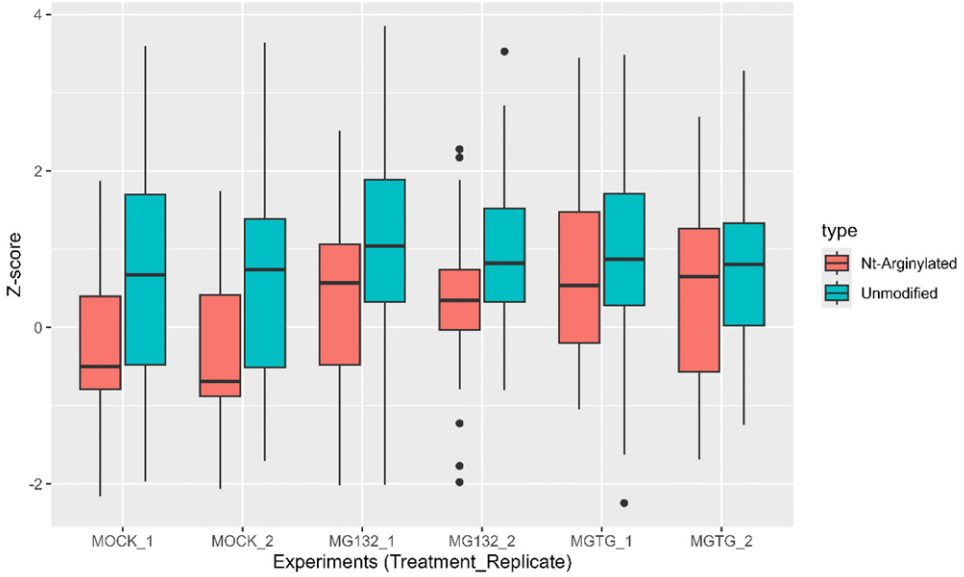
We agree and have now stratified the sequence-logo analyses by treatment (MOCK, MG, MGTG). Across all conditions we observed enrichment of Arg/N-degron pathway secondary/tertiary destabilizing residues at P1' (D/E/Q/N). In MGTG, a clear caspase-like DXXD motif is evident. MG132 shows a weaker, heterogeneous pattern in P5-P1. Importantly, MOCK (non-stress) still shows a modest D enrichment at P1 (N = 16), consistent with low-level/basal caspase activity, but not the full DXXD consensus seen in MGTG; the effect size is limited by the small site count. Despite the applied treatments, the logos within P1' remained unaltered.
We have added per-treatment P1' logos and a cleavage-site logo panel (Supplementary Fig. 14a-c) and clarified in the result at line 291 on page 8 that the consensus in the main figure is dominated by stress-enriched conditions, particularly MGTG.

6.Several mitochondrial or secretory proteins are identified as Nt-arginylated at the sites of MTS or SP cleavage, suggesting that this occurs after translocation across the respective membrane(s). The authors allude that this may be ATE1-dependent Nt-arginylation of mistargeted proteins (line 401 following). This would be more convincing if shown in an ATE knock-down cell line, for example with the established PRM assays.

We thank the reviewer for their profound remark regarding our supposition concerning the localization of Nt-arginylated proteins. We agree and performed the requested validation. We combined ATE1 knock-down (shATE1) with mitochondrial/cytosolic fractionation and targeted PRM assays for four mitochondrial transit-peptide cleavage sites (MTHFD2|36E , SSBP1|17E , UQCRHL|15D , SHMT2|27Q). For three targets (MTHFD2|36E , SSBP1|17E , UQCRHL|15D) we quantified both the Nt-arginylated peptide and its unmodified counterpart as a ratio (Nt-Arg/unmodified); the SHMT2 Nt-Arg form was not detected in PRM (unmodified was detected), so it is reported as not monitored. Across the three quantified targets:
MGTG (MG132 and thapsigargin) increased the Nt-Arg/unmodified ratio in the mitochondrial fraction.
shATE1 decreased the Nt-Arg/unmodified ratio and blunted the MGTG-induced increase.
In cytosolic fractions, Nt-Arg/unmodified ratios were weak relative to mitochondria.
These results support that the mitochondrial transit site Nt-arginylation events are ATE1-dependent and predominantly mitochondrial, consistent with processing after import and mitochondrial transit cleavage rather than in the cytosol. We have revised the text to Results at line 405 on page 10 and reflect the findings in Discussion at line 443 on page 11; also added figures (Fig. 6o and p, Supplementary Figure. 26) with PRM traces/ratios for MTHFD2|36E, SSBP1|17E, and UQCRHL|15D; added materials and methods in Methods at line 503 on page 12.

7.The quantification of Nt-arginylated peptides and their counterparts is an interesting and important aspect that is only explored in the PRM assay, and surprisingly failed more frequently for the non-modified peptides that would be expected to be much more abundant. How many non-arginylated counterparts of Nt-arginylated peptides were identified in the enriched N-terminome, and if so, how does their intensity in this unbiased assay compared to their arginylated counterparts?

We concur with the reviewer's observation concerning the anticipated higher abundance of the unmodified peptide compared to its Nt-arginylated counterpart. Our N-terminome analysis revealed a greater prevalence of unmodified peptides, evidenced by 1,399 PSMs, in contrast to 560 PSMs for the corresponding Nt-arginylated forms (Supplementary Data 5), aligning with the reviewer's assertion. Furthermore, the LFQ intensity data derived from the enriched N-terminome further substantiates this point (figure below). PRM results generally exhibited the same trend. However, for three proteins—EIF4B|46D, LSM3|7Q, and UQCRH|15D—the opposite pattern was observed: the modified forms were clearly detected, whereas the unmodified counterparts could not be monitored. This appears to be a protein-specific phenomenon limited to these cases, and may indicate that the arginylated forms are predominant in cells. While arginylation is often regarded as a signal for protein degradation, it cannot be excluded that, in certain contexts, arginylation may instead serve as a PTM required for proper cellular activity. In the case of UQCRH|15D, we further strengthened this possibility through deeper validation using mitochondrial fractionation and ATE1 knock-down (line 405 on page 10 and Figure 6p).



8.The manuscript must be edited for language, flow and clarity, and proof-read in detail. For example, the first paragraph of the results section (lines 69-94) starts with describing enrichment and analysis of N-terminal peptides (lines 69-76) before even explaining which samples were analysed (line 77). The important fact that two proteases are used in the N-termini enrichment experiments (Trypsin and chymotrypsin) is only mentioned later in line 123. Similar convoluted descriptions are found at several places throughout the manuscript.

We appreciate notifying us an example that impedes readability of the paper. We have restructured the Results for clarity and performed a full proof-reading pass including subtitles (language, flow, and consistency).
For the example the reviewer raised:
We moved the study design to the very start of the Results (conditions; MOCK, MG132, MGTG).

We introduced the use of two proteases (trypsin and chymotrypsin) in the first paragraph, rather than later.
We incorporate explicit demarcation points to distinguish between the design phase, the enrichment and analysis overview, and subsequent downstream analyses, concurrently unifying the nomenclature throughout this section.
These changes are reflected in the revised manuscript's opening of the Results section.

Minor points

9.Line 141 and following: It would be helpful if the identity of the arginylated amino acid was added to the combination of protein name and position number, e.g instead of MTHFD2|36 write MTHFD2|36E.

We appreciate reviewer's comment on clearer annotation. We amended all the annotations including in figures in accordance with the reviewer's recommendations.

10.Line 76: Rephrase "Before LC-MS/MS, we treated…" This is ambiguous, cells are treated also before any other critical sample handling steps such as enrichment, not directly before LC-MS/MS.

Thank you for indicating the ambiguity. We modified the paragraph at line 76, also prompted by major remark 8, to improve the clarity and tidiness of our points' presentation.

11. Line 272-274: what are the P1' amino acids of the Nt-arginylated proteins identified in the mock samples? Please show a sequence logo or e.g. iceLogo of this subset and discuss the assumption of "a mechanism where Nt-arginylation may not induce protein degradation" in the context of the most frequent P1' amino acids.

The sequence logo of the MOCK samples was included in Supplementary Figure 14a in the revised manuscript as a response of the major remark 5. As it shows that P1' amino acid frequency has not been altered through treatment, we suspect that these sites are homogeneous subset of those seen in MGTG treatment. Additionally, a DXXD motif of caspase was observed in MOCK still, which implies that low-level caspase processing can appear even without overt stress. Hence, we suggest the proteins in the MOCK samples are homeostatic remnant of the system behind Nt-arginylation involved, which appeared in stable samples of previous degradomics study (10.1152/ajplung.00175.2018).
We amended the content to deliver more explicit way including the citation in line 287 on page 7.

12.Line 282: Is this difference statistically significant? Is this a p-value in Fig. 4e? Please specify more precisely in the text and in the figure legend.

We appreciate you clarifying a point we vaguely stated. We agree that our use of the word "significant" was misleading, as no hypothesis test was intended. The statement at line 300 on page 8 refers to a detection criterion (present vs. absent), not to statistical significance. We have therefore removed "significant" and rewritten the sentence to describe the observation descriptively.

14. Line 318: Rephrase the statement about antibody limitation. The company Agrisera (https://www.agrisera.com/) provides commercial antibodies against N-terminal R-E and R-D which are among the most abundant arginylation patterns observed in this study. Have these been tested by the authors?

We sincerely appreciate this valuable comment and the information regarding the Agrisera antibodies. We agree that these antibodies represent an important tool for studying Nt-arginylation and may be particularly useful for initial enrichment. For example, Wong et al.(PMID:17896865) performed immunoaffinity chromatography using such antibodies on whole-cell extracts from several mouse tissues

(fibroblasts, heart, lung, etc.) and initially identified about 300 proteins as being arginylated. However, after applying stringent criteria, including the removal of mass ambiguities that could mimic arginylation (e.g., GV/VG or carbamylated I/L), the final list was reduced to 43 substrates, highlighting the limitations of antibody-based approaches in terms of specificity and coverage.
Moreover, in our study, we aimed to identify Nt-arginylation substrates comprehensively. Antibodies against N-terminal R-E and R-D motifs are insufficient for this purpose because they recognize only the terminal dipeptide sequence rather than the specific proteins.
Nonetheless, we fully agree with the reviewer's suggestion, and we have revised the relevant section of the manuscript at line 346 on page 9 as follows;
(Previous) "As antibodies to Nt-arginylated proteins are limited, monitoring arginylation using antibodies has been possible only for HSPA5, CALR, and P4HB. We attempted PRM-MS to detect Nt-arginylated proteins whose antibodies were not available."
(Revised) "Although a commercial antibody recognizing the RE and RD motifs is available, the specific proteins targeted in this study have not been validated. To address this limitation, we employed PRM-MS to detect Nt-arginylated proteins, except in cases where antibodies recognizing individual arginylated proteins could be applied."

15.Fig S1e-g: How is the low overlap between proteins with Nt-arginylation between replicates explained?

Thank you for noting the lower replicate overlap for Nt-arginylated proteins. This subset shows markedly lower overlap (~20%) than the broader enriched N-terminome (~53%; Fig. S1a–c vs. S1e–g). Nt-arginylation events are low-stoichiometry and often arise on short-lived protease-generated neo-N-termini, making detection close to the limit of identification and more variable across replicates. Additionally, in discovery DDA runs, precursor selection is semi-stochastic, which increases missing values and reduces set overlap for rare PTM peptides (Nicolas et al., 10.1074/mcp.m112.026500).

16.Fig S18a: The PCC of the PRCT standards look comparably bad. Could you please comment if your fine-tuned MS2 prediction models fail to predict MS spectra of this standard or why this is the case?

Our fine-tuned MS2 prediction models do not fail on PRTC. In routine runs the PRTC standards show high agreement (median PCC > 0.90; Fig. S18b). The panel in Fig. S18a was acquired during an injection-time (IT) optimization when overall MS2 quality was temporarily reduced (lower fragment ion signal and broader mass error across all targets). This global acquisition degradation depresses PCC values for every peptide, including PRTC, and therefore S18a should be interpreted only for relative IT comparisons within that run, not as a benchmark of model accuracy. For benchmarking, please refer to S18b, which reflects typical performance.

17.Line 353: isn´t this rather unexpected that unmodified peptides did not change? Shouldn´t they go down?

We are thankful for the indication of a vital element that had not been sufficiently perceived by us. We agree that the reactivity observed in the unmodified peptides demands an explanation, given its unexpected nature. Based on the normalized intensities, which were calculated using internal standards as depicted in Figure S23, unmodified proteins demonstrated an increase as the treatment progressed in every treatment group. ERO1A|24E can be regarded as an ideal representation. Since unmodified peptides increased not only stressed conditions but also control, fold changes over MOCK were remain stable. Despite this, Nt-arginylated peptides did not exhibit an increase in MOCK samples, which accounts for the elevated fold changes observed within them.
While the unmodified peptides monitored in PRM follows the trend, ATF4 and SQSTM1 demonstrated no concordance with the trend within the MOCK group. The results on these proteins reflect that the result accurately represents endogenous protein concentration. And given that the targets were identified under the UPR stress model, the targets' response in MOCK sample may be elucidated by the potential

induction of starvation stress during our experimental setup, resulting from the absence of media exchange for a maximum of 48 hours.
The manuscript is revised to accommodate the reviewer's feedback and the potential rationales behind it at line 378 on page 9.

18.Figures should use more consistent font sizes, and all panels of the Supplementary Figures should be readable in print size.

Thank you for pointing out figures that are hard to read. We revised the figures to have consistent font sizes. Full changelogs are:

1. Supplementary Figure 4c-e, were made larger to enable better visibility.
2. Supplementary Figure 5a-c, were increased the text size.
3. Supplementary Figure 9a-b, were increased the text size.
4. Supplementary Figure 10b-c, were increased the text size.
5. Supplementary Figure 12b-d, were increased the text size and c was changed into heat map plot for better delivery.
6. Supplementary Figure 10f-g, their names were changed to e and f, respectively, which were assigned mistakenly in the first place.
7. Supplementary Figure 14a-d, their names were changed to d-g, respectively, a change necessitated by the sequential order and the associated context.
8. Supplementary Figure 14a-c, was inserted to address reviewer 2's remark 5.
9. Supplementary Figure 19, was increased the text size.
10. Supplementary Figure 20a-c, were made larger and increased the text size.
11. Supplementary Figure 22, the notations of the peptide were changed to address reviewer 2's remark 9.
12. Supplementary Figure 23, the notations of the peptide were changed to address reviewer 2's remark 9.
13. Supplementary Figure 26, was inserted to address reviewer 2's remark 6.
14. Supplementary Figure 27, they were renamed 26 and 27, a change necessitated by the sequential order and the associated context.

19.Please proof-read figures and figure legends carefully. A few examples for spelling errors:

• Fig 2e: “perocolator” instead of “percolator”
•Fig S3: “Nt-arginylated peptides are tested their MS2 spectra and RT…” should be “Nt-arginylated peptides are tested for their MS2 spectra and RT…”? Please phrase it more precisely.
•Fig S9: “PSMs that are passed MS2 module…” should be “PSMs that passed MS2 module”
•Fig S12: “…e are depicted by color, wieht each …..” should be “...with each…”

Thank you for taking your time to meticulously read through the manuscript. We thoroughly proof-read figures and their legends intensively, fixed any errors regarding to tense and typos including the suggestions the reviewer mentioned.

Reviewer #3 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4 (Remarks to the Author):

Review of manuscript “Implementing N-terminomics and machine learning to probe in vivo Nt-arginylation” by Ju et al.

In the manuscript, Ju and co-workers describe an impressive series of experiments, including generating new data for machine learning, validation and biological interpretation of the results. There is definitely enough material in this manuscript for three of four separate papers, but the author should be commended for not “salami slicing”. The authors even went so far as to clone twelve of the identified Nt-arginylated proteins and perform pulldown assays. This is certainly above and beyond most proteomics studies.

Even though it is a massive paper to read, all data and figures presented are relevant, and advance the narrative. The results are in my view definitely noteworthy and sufficient for consideration by the journal.

And even though the authors modestly state that their approach is only an interim solution while waiting for direct Nt-arginylation enrichment methods, I believe much of the framework could be reused. Even the best direct enrichment method will not be 100% efficient.

The work is original, and gives due credit to prior work and the state-of-the-art, both in ML for retention time and MS2 prediction, and N-terminomics. The methods are described in detail, and the data as well as analysis scripts are shared in proper repositories, with redundancy for the data.

I have some minor comments, however, which I believe are relatively easy to address:

Line 375: “Proteomics can now reach single-cell depth” - this statement reads a bit odd. I think the authors mean that proteomes can now be probed to significant depths also in single cells. I associate “depth” as an indicator of the proteome measurement, i.e. how many proteins are detected or the lowest concentration/copy number that can be quantified, not the number of cells needed to measure a given number of proteins.

Thank you for pointing this out. Our intention was to convey that mass-spectrometry workflows can now measure proteomes from individual cells, not to redefine “depth.” We’ve revised the sentence to avoid conflating sample input (single cell) with measurement depth (number/dynamic range of proteins quantified) at line 415 on page 10.

Was there a conscious strategy to first use Sequest (or SequestHT) and then MSFragger for the LFQ, rather than MSFragger (or FragPipe) from the beginning? Likewise, was there a rationale for why initially allowing a 10 ppm mass measurement error, and subsequently 5 ppm? Does the 5 ppm tolerance mentioned on line 651 refer to MS1 or MS2, or both? What was the resolution (setting) for the MS1 in the PRM measurements?

Our tandem search (two-stage search) for Nt-arginylation was implemented in Proteome Discoverer (PD) with SequestHT + Percolator including Precursor Quantifier for LFQ. Due to the comprehensive support

for the tandem search approach within Proteome Discoverer, a feature absents in MSFragger at that time, Proteome Discoverer was deemed suitable for the discovery of Nt-arginylation. We used MSFragger with IonQuant (equivalent of Precursor Quantifier in Proteome Discoverer) for quantification in the PRM/confirmatory analyses, because of its fast LFQ-MBR and easy export for downstream stats. Ultimately, no specific search engine choice stands out as preferred.
10 ppm was the MS1 precursor tolerance used in the database searches (DDA/tandem search). 5 ppm was used only for PRM spectral matching of MS2 fragment ions to the theoretical spectra. In PRM, we first gated candidates by MS1 m/z within 0.001 m/z, then required MS2 fragment matches within 5 ppm for spectral-similarity scoring.
We amended the Methods at line 716 on page 17 to state explicitly that 5 ppm refers to MS2 fragment matching in PRM, not to MS1.

The code is available on GitHub, which is commendable, but the documentation in the main README is rather limited, and the English could also do with some improvement. There is no overview or explanation for what the program does, and the README is rather unstructured, barely using any Markdown features. Also the code itself has rather few comments, but many lines of code that are commented away. There should at least be a comment about why these chunks are commented, and what they could be used for, if they are to be kept in the code (though I confess I share this habit with the authors). In summary, the authors could tidy up the GitHub repo somewhat. I would also recommend the authors create a release corresponding to the versions of the code used in the (accepted) manuscript and get a DOI for this in Zenodo (this will also create a backup of the GitHub repo at this point in time in Zenodo as well as Software Heritage for redundancy). It is quite easy to do (and completely free of course).

We thank the reviewer for the constructive suggestions. We have substantially improved the repository: Rewrote and restructured the README with: Overview, Installation, Core workflow, and Auxillary workflow.
We added Rmarkdown codes for N-terminome part and PRM part, edited English throughout and used Markdown headers, lists, and code blocks for clarity.
Added a versioned release corresponding to the manuscript and archived it on Zenodo (DOI: 10.5281/zenodo.17247948).

Reviewer #4 (Remarks on code availability):

As mentioned in the comments to the authors, the code is available on GitHub, which is commendable, but the documentation in the main README is rather limited, and the English could also do with some improvement. There is no overview or explanation for what the program does, and the README is rather unstructured, barely using any Markdown features. Also the code itself has rather few comments, but many lines of code that are commented away. There should at least be a comment about why these chunks are commented, and what they could be used for, if they are to be kept in the code (though I confess I share this habit with the authors). In summary, the authors could tidy up the GitHub repo somewhat. I would also recommend the authors create a release corresponding to the versions of the code used in the (accepted) manuscript and get a DOI for this in Zenodo (this will also create a backup of the GitHub repo at this point in time in Zenodo as well as Software Heritage for redundancy).

Given this comment appears identical to "Reviewer #4: Remarks to the Author", the reply for this segment is accordingly superseded by the aforementioned.

Reviewer #1 (Remarks to the Author):

The authors have addressed all my comments.

Reviewer #2 (Remarks to the Author):

The authors have extensively addressed all comments raised in the first round, further clarified their procedure and added convincing additional experiments. This will be a valuable contribution to the field and I have only few minor comments that can easily be edited before publication.

Minor comments:

Precision of statements should be improved for some citations:

1.P6 line 215: “These findings suggest that b-ion mass errors likely stemmed from mis-annotation of the N-terminal modification, which may arise from near-isobaric unknown modifications that have not yet been characterized, as well as those extensively catalogued in previous reports (e.g., VG dipeptide)30, 33”

Please clarify. The cited studies did not only catalogue N-terminal modifications, but further propose that this can be used to distinguish false positives. The results given in the present study built on this and further confirm this.

We thank the reviewer for pointing out a statement that could misguide the audiences. We clarified the sentence to convey that the references had catalogued the combinations which are near-isobaric to arginylation and modified to link with the following sentence that describing the proposition of utility to distinguish false positives.
We have revised the sentence as: “These findings suggest that b-ion mass errors likely stemmed from mis-annotation of the N-terminal modification, which may originate not only from near-isobaric unknown modifications that have not yet been characterized but also from those already catalogued in previous reports (e.g., VG dipeptide), where such unknown modifications were also suggested as potential contributors to these discrepancies[30, 33].”

2.P6 line 218: “Indeed, we have proposed that the observed error discrepancy could serve as a confidence metric for assessing the correctness of N-terminal modifications, which we termed the MET33, 34.”

Positioning matters. Please change to: “Indeed it has been proposed that the observed error discrepancy could serve as a confidence metric for assessing the correctness of N-terminal modifications33, 34, which we here termed the MET.”

We agree that the sentence needs reposition of the references. We addressed the issue as following the suggestion of the reviewer.

3.Ref. 34, Lee H. et al. is now published.

We thank the reviewer to recognize publication of the reference. We replaced the reference to show published information properly.

4.P6 line 240: “Indeed, when comparing MS2 spectra of an Arg-starting peptide with those of the same peptides lacking Nt-Arg, the b1-ion of D3-acetylated Arg is typically observed at 202.138±0.005 m/z (Fig. 3c and Supplementary Fig. 10a-c)37”

Hiserodt et al. did not work with D3 labeled acetylation, but with deuterated amino acids and the 202 m/z fragment has not been reported in their study. Please rephrase, e.g.: “… and indeed, the b1 ion has been observed when MS2 spectra of an Arg-starting peptide were compared with those of the same peptides lacking Nt-Arg37. Here, we observed the signature b1 ion of D3-acetylated Arg at 202.138±0.005 m/z (Fig. 3c and Supplementary Fig. 10a-c).”

We sincerely appreciate the reviewer’s point and suggestion. We rephrased the sentence as per the reviewer's recommendation.

Reviewer #3 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4 (Remarks to the Author):

The authors have addressed my comments and suggestions. The GitHub page is also much improved, and it is now much easier to understand what the tool does, and what is available in the repository.

Reviewer #4 (Remarks on code availability):

The documentation has been significantly improved, and new notebooks provided. There are still some lines of code here and there that are commented out, without a reason why. I _understand_ why (the library or line of code was not necessary, but may be useful in the future. While not a gold standard, the code is "good enough", in that it is generally clear what it does and why.