MTSviewer: a database to visualize mitochondrial targeting sequences, cleavage sites, and mutations on protein structures

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Abstract

Summary

Mitochondrial dysfunction is implicated in a wide array of human diseases ranging from neurodegenerative disorders to cardiovascular defects. The coordinated localization and import of proteins into mitochondria are essential processes that ensure mitochondrial homeostasis and consequently cell survival. The localization and import of most mitochondrial proteins are driven by N-terminal mitochondrial targeting sequences (MTS's), which interact with import machinery and are removed by the mitochondrial processing peptidase (MPP). The recent discovery of internal MTS's - those which are distributed throughout a protein and act as import regulators or secondary MPP cleavage sites – has expanded the role of both MTS's and MPP beyond conventional N-terminal regulatory pathways. Still, the global mutational landscape of MTS's remains poorly characterized, both from genetic and structural perspectives. To this end, we have integrated a variety of tools into one harmonized R/Shiny database called MTSviewer (https://neurobioinfo.github.io/MTSvieweR/) which combines MTS predictions, cleavage sites, genetic variants, pathogenicity predictions, and N-terminomics data with structural visualization using AlphaFold models of human and yeast mitochondrial proteomes.

Availability and Implementation

MTSviewer is freely available on the web at https://neurobioinfo.github.io/MTSvieweR/. Source code is available at https://github.com/neurobioinfo/MTSvieweR.

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Keywords

MTSviewer, variant database, structure visualization, mitochondrial targeting signal, mitochondrial import, cleavage site, MTS

1. Introduction

Mitochondria are central to organismal health and regulate a diverse array of cellular processes, ranging from energy generation to immunity, proteostasis, and more (Mills et al. 2017; Ruan et al. 2017; Spinelli and Haigis 2018; Pfanner et al. 2019). Even though mitochondria contain their own genome, most mitochondrial proteins are nuclear encoded, translated in the cytosol, and imported into mitochondria (Wiedemann and Pfanner 2017). Consequently, mitochondria have evolved an intricate system of targeting and translocation to import these proteins through translocases of the outer (TOM) and inner (TIM) mitochondrial membranes, and sort them into their correct subcompartment (Neupert 2015). The most common targeting mechanism for matrix-localized proteins utilizes N-terminal mitochondrial targeting sequences (N-MTS), which form amphipathic helices and engage with TOM receptors before being passed through the TIM23 complex into the matrix (Callegari et al. 2020). In the matrix, N-MTS are cleaved off by the mitochondrial processing peptidase (MPP), which acts as a gatekeeper between import and overall mitochondrial quality control (Poveda-Huertes et al. 2017). The breadth of import mechanisms expands considerably when considering proteins localized to the intermembrane space (IMS), which typically lack an N-MTS and rely on disulfide trapping, or transmembrane (TM) proteins, which rely on a combination of accessory machinery and/or MTS's for their insertion and sorting (Hansen and Herrmann 2019). It recently emerged that imported proteins can contain internal MTS's

(iMTS), which bind to TOM70 to regulate import rates and may also contain secondary MPP cleavage sites (Backes et al. 2018; Friedl et al. 2020). Furthermore, some proteins lacking an N-MTS still localize to and import into mitochondria via their iMTS's (Bykov et al. 2022; Rahbani et al. 2021).

Mitochondrial targeting and import are innately linked to proteolysis, as mitochondria contain more than 40 proteases, coined "mitoproteases", which regulate proteostasis, MTS removal, stress responses, signaling, and more (Deshwal et al. 2020). While MPP is the main protease implicated in N-MTS processing, other proteases act sequentially after MPP cleavage, including MIP, which removes an octapeptide, and XPNPEP3, which removes a single amino acid (Gomez-Fabra Gala and Vögtle 2021). In specialized cases, other mitoproteases can regulate distal cleavages to drive signaling events, including PARL, a rhomboid protease which cleaves TM domains within the inner membrane (Spinazzi and de Strooper 2016; Lysyk et al. 2021). One example of a tandem MPP/PARL-cleaved protein is PINK1, a mitochondrial kinase that relies on its import and processing to either initiate or avoid the mitophagic cascade (Jin et al. 2010; Meissner et al. 2011; Bayne and Trempe 2019).

To facilitate the combined study of mitochondrial import and proteolysis, various tools have emerged, namely databases of mitochondrially localized proteins and prediction algorithms for sorting, MTS/iMTS propensity, and cleavage sites. In terms of mitoproteases, mass spectrometry experiments optimized for the labelling and enrichment of newly generated N-termini (neo-N-termini) have provided evidence for both canonical (ie. MTS removal) and non-canonical (ie. distal sites or N-terminal ragging) cleavage events within mitochondria (Calvo et al. 2017; Kleifeld et al. 2011;

Vögtle et al. 2009). From a structural perspective, recent work has revealed the structures of human TOM and TIM complexes (Wang et al. 2020b; Qi et al. 2021), and of an iMTS-TOM70 complex between human TOM70 and the SARS-CoV2 protein ORF9b (Jiang et al. 2020). Still, how human MTS's engage with and are passed across the other translocase subunits remain unclear. The structure of human MPP in complex with MTS substrates also remains unknown, which makes it difficult to confidently predict the consequences of MTS variants on MPP processing. From a genetic perspective, comparing the phenotypes of non-synonymous mutations within MTS's, iMTS's, or near cleavage sites may provide key insight into both areas, yet there is no database to facilitate this kind of analysis. There are also currently no resources to rapidly compare the outputs of the numerous mitochondrial prediction algorithms or to visualize MTS's within 3D protein structures. To this end, we hope to expedite the genetic and structural interrogation of human mitochondrial proteins and their MTS's with a novel database: MTSviewer (Fig. 1).

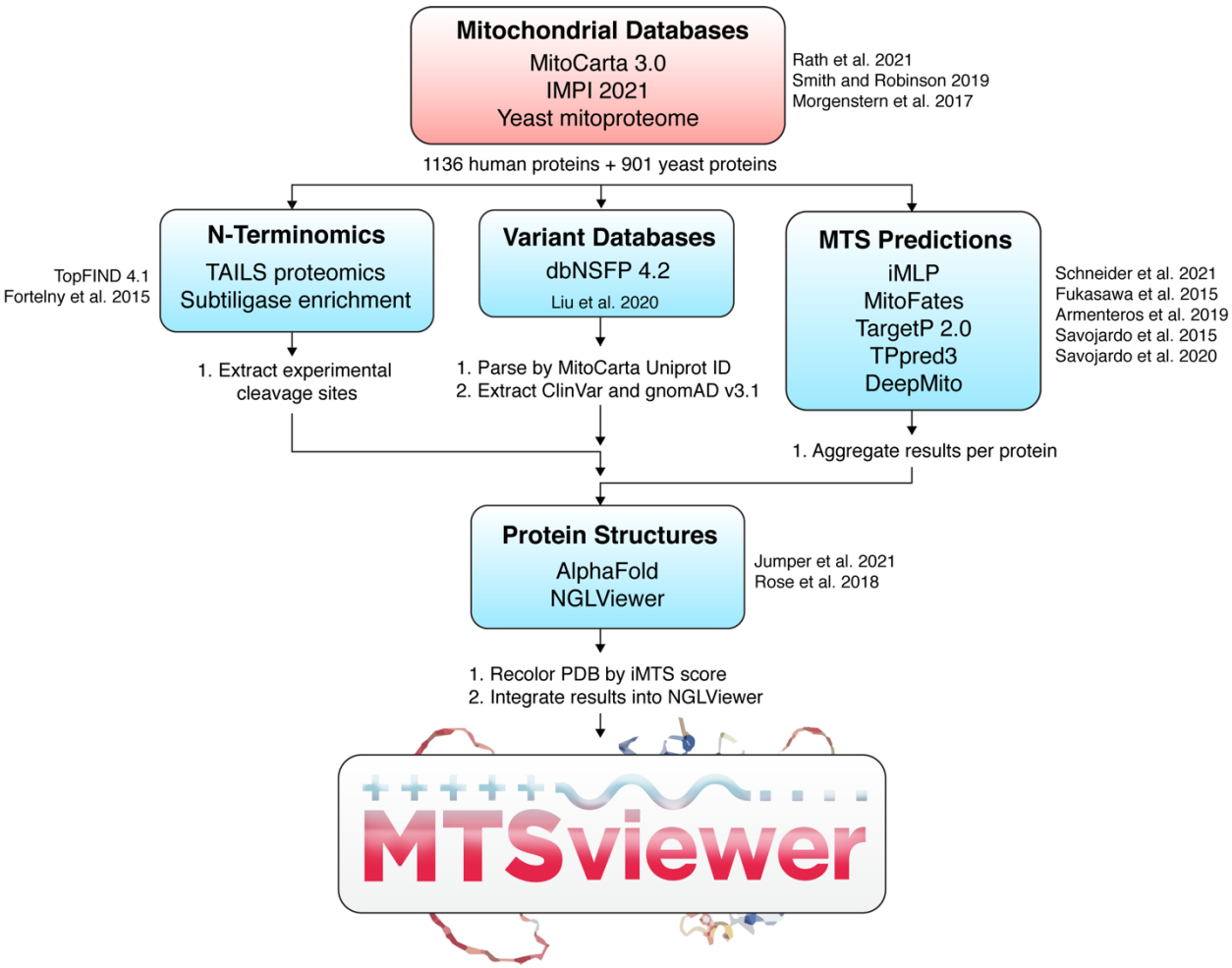

Figure 1. Workflow of MTSviewer. The database construction of MTSviewer, from initial mitochondrial databases to data integration and visualization.

2. Construction and content

The human mitochondrial proteome was downloaded from the MitoCarta 3.0 (1136 proteins) (Rath et al. 2021). Additional annotations for the MitoCarta protein list were appended from the Integrated Mitochondrial Protein Index (Q4pre-2021) (Smith and Robinson 2019). The yeast (*Sacchromyces cerevisiae*) mitochondrial proteome was derived from a high confidence dataset (901 proteins) (Morgenstern et al. 2017). Protein sequences were queried by UniProt ID and were submitted to: (1) iMLP – an internal

MTS predictor using long short-term memory (LSTM) recurrent neural network architecture (Schneider et al. 2021); (2) TargetP2.0 – a presequence and cleavage site predictor using deep learning and bidirectional LSTM (Almagro Armenteros et al. 2019); (3) MitoFates – a presequence and cleavage site predictor using support vector machine (SVM) classifiers (Fukasawa et al. 2015); (4) TPpred3 – a targeting and cleavage site predictor using Grammatical Restrained Hidden Conditional Random Fields (Savojardo et al. 2015); (5) DeepMito – a sub-mitochondrial localization predictor using deep learning and convoluted neural networks (Savojardo et al. 2020). For cleavage sites derived from N-terminomics, mass spectrometry data were aggregated from TopFIND 4.1 by Uniprot ID of both human and yeast proteins (Fortelny et al. 2015). For variants and functional annotations of human proteins, dbNSFP v4.2a was parsed by Uniprot ID against GRCh38/hg38 coordinates (Liu et al. 2020). The resulting list was filtered using an in-house Python script into separate datasets for gnomAD v3.1 and ClinVar. Variants unique to the ExAC database were ignored. AlphaFold models for the *Homo sapiens* proteome (UP000005640) and *Sacchromyces cerevisiae* (UP000002311) were downloaded and matched by Uniprot ID (Jumper et al. 2021). An in-house Python script based on BioPandas (Raschka 2017) was used to parse the PDB files and re-color B-factors according to iMTS scores via iMLP. 3D visualization of protein structures was achieved using an adapted version of NGLViewer integrated into our R/Shiny application (Rose et al. 2018).

3. Utility and discussion

MTSviewer serves as a user-friendly platform for investigating MTS's from both genetic and structural perspectives. The database requires minimal bioinformatics knowledge

and features both human and yeast mitochondrial proteomes. With MTSviewer, users are able to: (1) compare mitochondrial prediction outputs from a variety of algorithms; (2) visualize MTS likelihood on a folded protein structure; (3) compare experimentally identified and predicted proteolytic events; (4) map non-synonymous variants (gnomAD, ClinVar, or user uploaded) within these MTS's and cleavage sites. Using this platform, we have also curated a list of disease-linked variants within human MTS's as a resource for their functional characterization.

User interface

The MTSviewer user interface is intuitive and begins by selecting or searching a gene of interest. Users specify the desired database for variant visualization (currently gnomAD v3.1 or ClinVar), and variants are overlaid onto an XY plot with the iMTS probability from protein N- to C-terminus. Hovering over a variant reveals cursory details which are fully expanded in the variant table. For the structure viewer, two coloring schemes are toggleable: the iMTS score, or the AlphaFold per-residue predicted local distance difference test (pLDDT) confidence score. Users can investigate specific residues or variants by clicking on the iMTS plot or 3D structure, and the structure viewer will automatically highlight the interactions (ie. polar contacts) and residues in proximity (5 Å) to the residue of interest (Fig. 2). Users can also upload custom variant lists for their proteins of interest in CSV format, which will be added to the iMTS propensity curve, populated into the variant list data tables, and become visualizable on the 3D protein structure. This feature allows users to compare where their variants lie in terms of MTS propensity, cleavage sites and other pathogenic variants on primary sequence and structural levels.

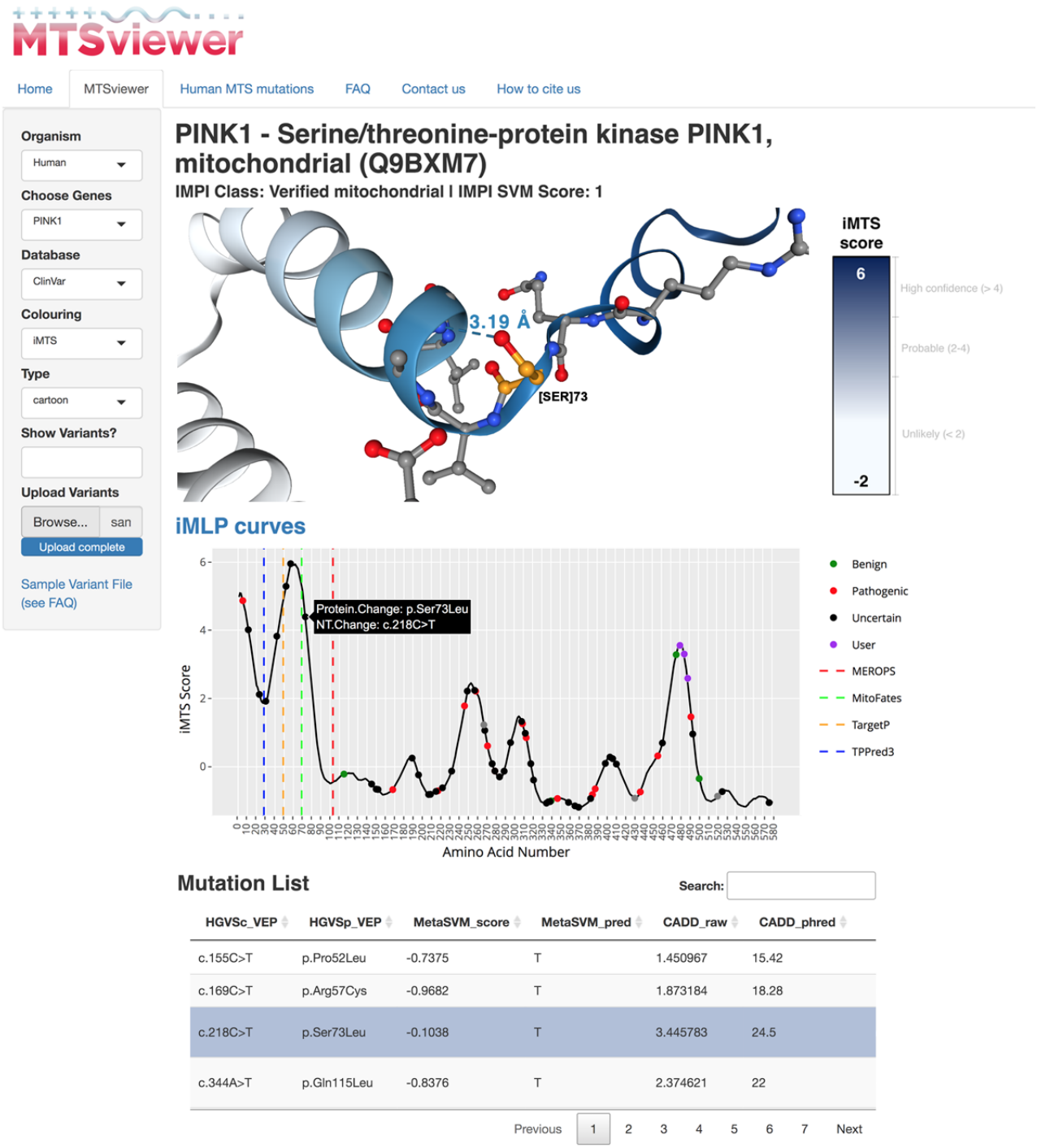

Figure 2. MTSviewer output for PINK1. A sample output from MTSviewer investigating human PINK1, a mitochondrial kinase with a uniquely long MTS and multiple predicted cleavage sites. Protein sequence, prediction algorithms, and N-

terminomics data tables have been omitted for clarity but are available in full on the interactive MTSviewer web server. Ser73Leu has been highlighted as a variant of interest, as Ser73 is found within a region of high MTS propensity near the predicted MitoFates cleavage site.

The iMTS plot and structure viewer also contain toggleable visualizations to highlight cleavage site predictions from the various MTS predictors and/or experimentally determined N-terminomics sites. Aggregated comparisons of targeting predictors are pooled in table format, and data frames are exportable to facilitate downstream analyses. Taken together, these features enable users to rapidly generate protein-level hypotheses to test *in vitro*, or to rationalize previous *in vitro* findings with import- or protease-specific context.

PINK1 as a case study

To highlight the utility of MTSviewer we have chosen PINK1 as a case study, given its cryptic N-MTS and the innate coupling of its import and processing to gate its accumulation on the TOM complex. Briefly, PINK1 is known to be cleaved by the rhomboid protease PARL in the IMM at Ala103, which is validated by the N-terminomics outputs seen in MTSviewer. The precise MPP cleavage site within the PINK1 N-MTS remains unknown, though an MPP-cleaved PINK1 fragment accumulates upon PARL knockdown (Greene et al. 2012). Based on the MTSviewer output for PINK1, there are many possibilities for the N-MTS MPP cleavage site, which will be critical to validate using *in vitro* assays, along with the effects of N-MTS variants (eg. Gly30Arg, Pro52Leu, Arg57Cys, and Ser73Leu). While some of these PINK1 N-MTS variants are still cleaved by PARL in healthy mitochondria and accumulate following mitochondrial damage

(Sekine et al. 2019), their import rates and effects on MPP processing remain unstudied. Experiments which swap the PINK1 N-MTS with those from other mitochondrial proteins have shown that PINK1 can still be imported into mitochondria with chimeric N-MTS's, though PINK1 accumulation is prevented (Kakade et al. 2022). While many of these N-MTS PINK1 chimeras can still be imported, their specific rates of import have also yet to be measured. This suggests that distal N-MTS elements of mitochondrial proteins (and variants within these regions) will be critical to study beyond the context of binary import success or blockage. Another useful feature of MTSviewer is the ability to gauge the length of a protein's N-MTS by looking at the iMTS propensity plots. For reference, it has been estimated that MTS's are usually 15-50 amino acids long (Wiedemann and Pfanner 2017), yet the PINK1 N-terminus exhibits high MTS propensity across its first 90 amino acids. As all of the MTSviewer iMTS data is available to download, users will be able to analyze global trends in MTS length and propensity across protein families to investigate the downstream consequences of longer or atypical N-MTS's within mitochondrial proteins. Beyond the PINK1 N-MTS, the PINK1 iMTS plot within MTSviewer reveals a putative iMTS within the PINK1 C-terminus (a.a. 460-500), which could regulate PINK1 import or processing rates at the mitochondrial surface. It is known that PINK1 mRNA is co-transported with mitochondria (Harbauer et al. 2022), so it will be important to investigate the role of TOM70 binding to PINK1 and this putative iMTS during translation and import. The MTSviewer output for PINK1 also highlights the need to consider the oligomerization status of proteins when investigating their monomeric AlphaFold structures. PINK1 is known to dimerize on the OMM following depolarization which could occlude its iMTS in the folded dimeric state

(Rasool et al. 2022; Okatsu et al. 2013), even if partially unfolded PINK1 monomers could bind to TOM70 upon import. Overall, MTSviewer will guide subsequent studies of atypically targeted proteins like PINK1 in the context of their MTS propensity, cleavage sites, and genetic variants.

Comparison to similar databases

MTSviewer is the first interactive database to bridge genetic variants with mitochondrial targeting predictions, proteolytic evidence, and 3D protein structures. As such, it is essential to highlight the tools and databases that laid the foundation, and to highlight the gaps that our database aims to address. For MTS and MPP cleavage site predictions, TargetP2.0, MitoFates, and TPpred3 utilize orthogonal and sophisticated approaches, yet there remains no harmonized resource to compare their results. Our database currently only features these three predictors, as they are the most recently developed and performed best in benchmarking studies (Imai and Nakai 2020). For raw N-terminomics mass spectrometry data, TopFIND represents the gold standard for data accessibility and cleavage evidence across studies, but it does not provide genetic variants nor structural context for these proteolytic events (Fortelny et al. 2015). In terms of similar 3D structure viewers, the AlphaFold database contains its own module for visualizing contacts of a specified protein but does not allow for significant customizability (Jumper et al. 2021). ICN3D provides another alternative for user uploaded PDB visualization and manipulation, similar in complexity to the standalone PyMOL interface (Wang et al. 2020a). In terms of overall construction, MTSviewer resembles COSMIC-3D, which provides structural visualization for cancer genetics, with a specific focus on the druggability of protein targets (Jubb et al. 2018). KinaseMD has

also taken a structural approach to the kinase mutational space, focusing on drug resistance, mutation hotspots, and network rewiring (Hu et al. 2021).

Future developments and limitations

The current construction of MTSviewer features the inherent limitation that N-terminal MTS's within AlphaFold predictions are typically low confidence and are depicted as unstructured. In the future, the inevitable structural determination of human MTS's in complexes with TOM/TIM and/or MPP will enable us to model N-MTS's more accurately and could be integrated as a scoring metric or docking module into later versions of MTSviewer. We will also implement a module for protease-specific exports (ie. variant lists near protease sites) to assess enrichment of pathogenic or uncharacterized variants near proteolytic sites. Overall, MTSviewer will be updated with new MTS prediction algorithms, experimental proteolytic evidence, and updated AlphaFold models on a regular basis.

4. Conclusions

MTSviewer is a novel R/Shiny database for investigating the mutational space, targeting sequences, proteolysis, and 3D structures of mitochondrial proteins. Users require minimal bioinformatics training and can rapidly generate variant lists, investigate structural consequences, compare the results of various mitochondrial prediction tools, and dissect potential cleavage sites.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The MTSviewer database is freely accessible via https://neurobioinfo.github.io/MTSvieweR/. Source code is available at https://github.com/neurobioinfo/MTSvieweR.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

A.N.B. and J.F.T conceptualized MTSviewer. A.N.B., J.D., and S.A. created the original R/Shiny and Python codes used in database construction. All authors contributed to feature development, troubleshooting, and optimization of the database functionalities. A.N.B and J.F.T wrote the manuscript with contributions and editing from J.D., S.A., and S.F. All authors read and approved the final manuscript.

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