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

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Abstract

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 is an essential process that ensures mitochondrial homeostasis and consequently cell survival. The localization and import of most mitochondrial proteins are driven by N-terminal mitochondrial targeting sequences (MTS), 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 prediction tools into one harmonized R/Shiny database called MTSviewer, which combines MTS predictions, MPP cleavage sites, genetic variants, pathogenicity predictions, and N-terminomics data with structural visualization using AlphaFold models. Using this platform, we have generated a list of disease-linked variants in protein MTS's and their predicted consequences as a resource for their functional characterization. Overall, MTSviewer is a platform that can be used to interrogate MTS mutations and their potential effects on import and proteolysis across the mitochondrial proteome.

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Keywords

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

Background

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, the majority of 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 proteins, which rely on a combination of accessory machinery and/or MTS's for their insertion and sorting (Hansen and Herrmann 2019). Recently, it has also been shown that imported proteins can contain

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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). There is also evidence that some proteins which lack an N-MTS may still localize to and import into mitochondria via their iMTS's (Bykov et al. 2021; 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;

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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, the precise details as to 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 no resources for researchers 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).

Construction and content

The human mitochondrial proteome was downloaded from the MitoCarta 3.0 (Human.MitoCarta3.0.xlsx, 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). For data aggregation across prediction algorithms, Human.MitoCarta3.0.fasta (2254 protein sequences for 1136 genes) was 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

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(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 experimental cleavage sites derived from N-terminomics, mass spectrometry data was aggregated from two studies in human cells, one using SILAC-based MS-TAILS, and one using TMPP chemical labelling (Vaca Jacome et al. 2015; Marshall et al. 2018). For genetic variants and functional annotations, dbNSFP v4.2a was parsed using the previously described Java tool (Liu et al. 2020). Specifically, MitoCarta 3.0 UniProt identifiers were queried against GRCh38/hg38 coordinates in dbNSFP v4.2a. The resulting list was filtered using an in-house Python script to simplify each entry to its Ensembl canonical transcript ("VEP_canonical"), and into separate datasets for gnomAD v3.1 and ClinVar. Variants unique to the ExAC database were ignored in this version of MTSviewer. AlphaFold models for the *Homo sapiens* proteome (UP000005640; 21-July-2021) were downloaded as individual PDB files and were matched by amino acid sequence to their corresponding MitoCarta 3.0 FASTA entry (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. All data files and protein structures were integrated into an interactive R/Shiny application with downloadable outputs and data visualizations using Plotly. 3D visualization of protein structures was achieved using an adapted version of NGLViewer

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for R/Shiny (Rose et al. 2018). The full database is accessible via https://mtsviewer.shinyapps.io/MTSviewer/.

Utility and discussion

MTSviewer serves as a user-friendly platform for investigating mitochondrial targeting sequences on a protein-level from both genetic and structural perspectives. The database requires minimal bioinformatics knowledge, and 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 experimental evidence for proteolytic events in the cell; and (4) map non-synonymous variants from a variety of databases within these MTS or cleavage sites. 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.

User interface

The MTSviewer user interface is intuitive and begins by selecting or searching a gene of interest (Fig. 2). 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 confidence score (pLDDT). Users can investigate specific residues or variants by clicking on the XY plot or 3D structure, and the structure viewer will automatically highlight the wild type interactions (ie. polar contacts) and residues in close proximity (default 5 Å) to the

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variant of interest. The XY plot and structure viewer also contain toggleable visualizations to highlight cleavage site predictions from the various MTS predictors and/or the experimentally determined N-terminomics sites. Aggregated comparisons of all targeting predictors are pooled in table format, and all data frames are exportable as CSV files to facilitate downstream analyses.

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 for this aggregator, 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 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 any 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

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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. Disease-specific variant databases will also be appended on a case-by-case basis, with specific emphasis on degenerative and developmental disorders. Overall, MTSviewer will be updated with new MTS prediction algorithms, experimental proteolytic evidence, and updated AlphaFold models on a regular basis.

Conclusions

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

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193 user-friendly data aggregator and visualizer for those studying mitochondrial import and
194 proteolysis.

195 **List of abbreviations**

196 MTS – mitochondrial targeting sequence

197 iMTS – internal mitochondrial/matrix targeting sequence

198 N-MTS – N-terminal mitochondrial targeting sequence

199 LSTM – long-term short-term memory

200 TOM – translocase of outer mitochondrial membrane

201 TIM – translocase of inner mitochondrial membrane

202 MPP – mitochondrial processing peptidase

203 TAILS - terminal amine isotopic labeling of substrate

204 SILAC – stable isotope labeling by amino acids

205 MS-TAILS – mitochondrial SILAC TAILS

206 TMPP - trimethoxyphenyl phosphonium

207 **Declarations**

208 **Ethics approval and consent to participate**

209 Not applicable

210 **Consent for publication**

211 Not applicable

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Availability of data and materials

The MTSviewer database is freely accessible via

https://mtsviewer.shinyapps.io/MTSviewer/.

Competing interests

Not applicable.

Funding Authors' contributions

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Figures

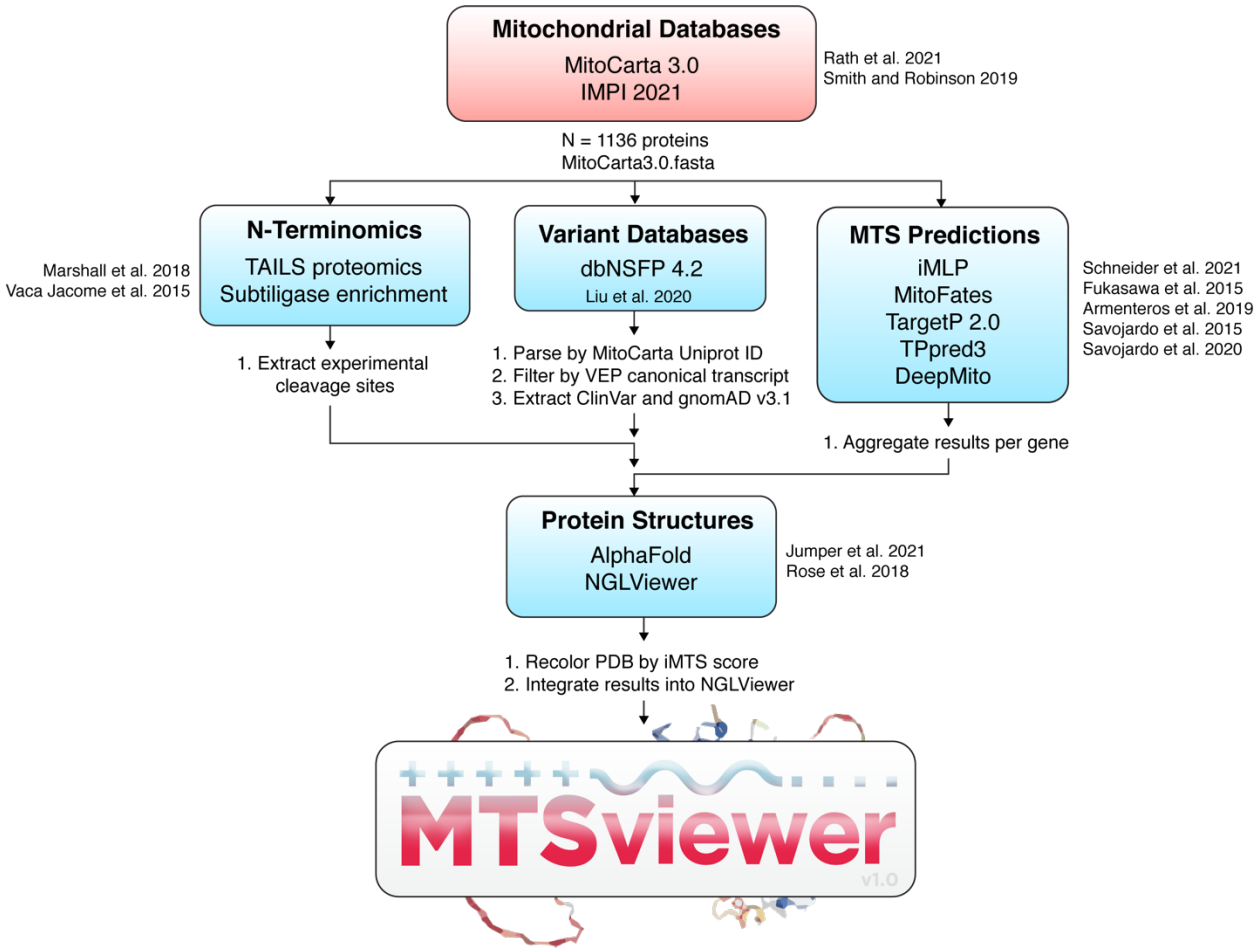

Figure 1. Workflow of MTSviewer. The database construction of MTSviewer, from initial mitochondrial databases (MitoCarta and IMPI) to data integration and visualization.

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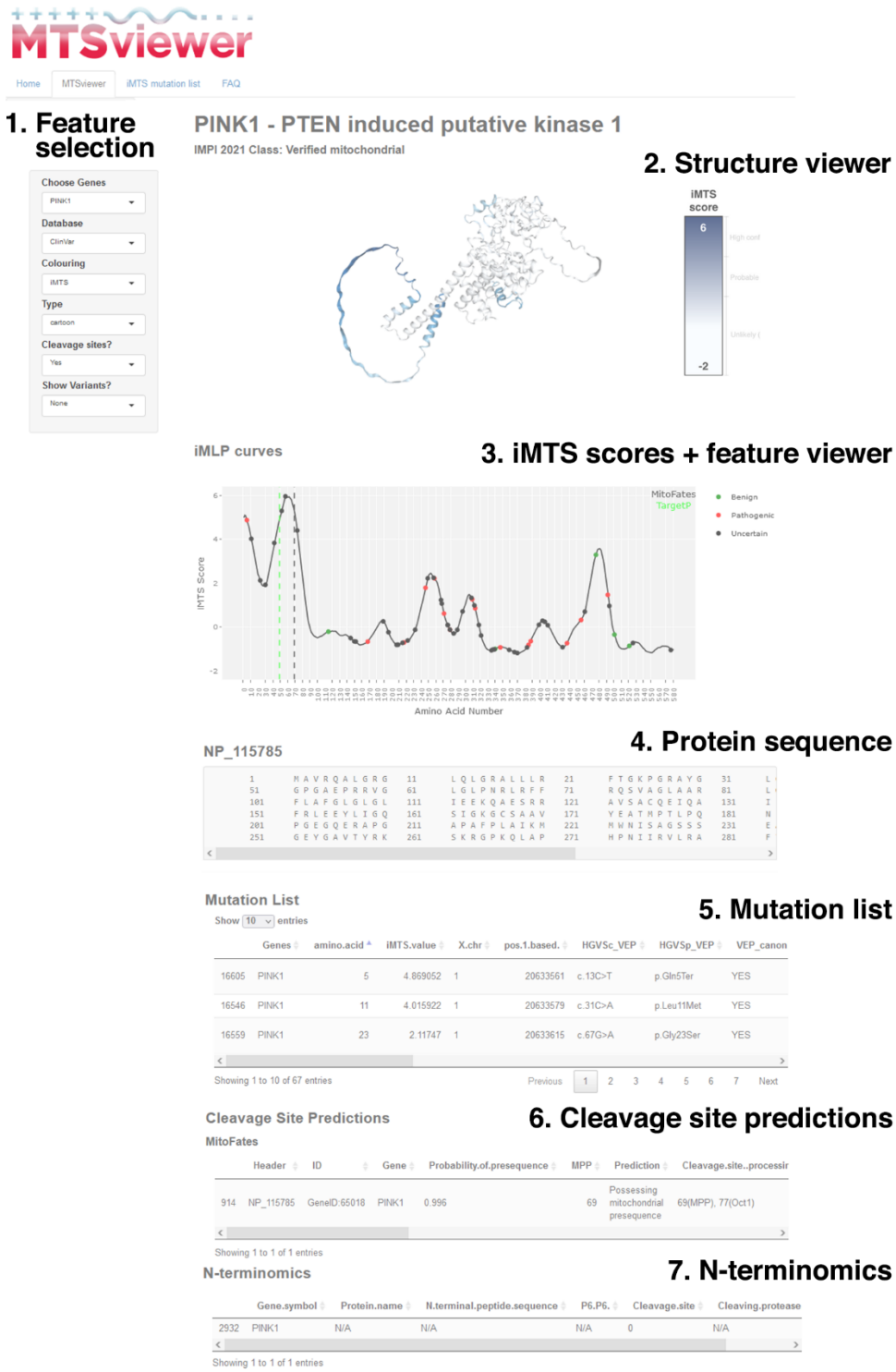

Figure 2. Annotated MTSviewer user interface.