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Von Willebrand factor A domain-containing protein 8 (VWA8) Localizes to the Matrix Side of the Inner Mitochondrial Membrane

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Abstract

VWA8 is a poorly characterized mitochondrial AAA+ ATPase protein. However, the specific submitochondrial localization of VWA8 remains unclear. The purpose of this study was to determine the specific submitochondrial compartment within which VWA8 resides in order to provide more insight into the function of this protein. Bioinformatics analysis showed that VWA8 has a 34 amino acid N-terminal Matrix-Targeting Signal (MTS) that is similar to those in proteins known to localize to the mitochondrial matrix. Experiments in C2C12 mouse myoblasts using confocal microscopy showed that deletion of the VWA8 MTS (vMTS) resulted in cytosolic, rather than mitochondrial, localization of VWA8. Biochemical analysis using differential sub-fractionation of mitochondria isolated from rat liver showed that VWA8 localizes to the matrix side of inner mitochondrial membrane, similar to the inner mitochondrial membrane protein Electron Transfer Flavoprotein-ubiquinone Oxidoreductase (ETFDH). The results of these experiments show that the vMTS is essential for localization to the mitochondrial matrix and that once there, VWA8 localizes to the matrix side of inner mitochondrial membrane.

Keywords

VWA8; KIAA0564; ETFDH; Mitochondrion; Inner Mitochondrial Membrane

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1. Introduction

Von Willebrand A Domain Containing Protein 8 (VWA8) is a poorly characterized protein named after its C-terminal VWA domain. However, VWA8 also contains an AAA+ ATPase domain and Walker A and B motifs. It is present in many tissues as two isoforms in the mouse, VWA8 (1905 amino acids) and VWA8b (1038 amino acids). VWA8b lacks all of the C-terminus of VWA8 including the VWA domain. Although the precise function of VWA8 is not known, genome-wide association studies have shown that variation in the VWA8 gene is associated with serum calcium concentrations [1,2], autism [3], and bipolar disorder with comorbid migraine [4], as well as acute myeloid leukemia [5]. A study in mice showed the full-length VWA8a is highly expressed relative to the truncated VWA8b transcript across all developmental time points and tissues analyzed [6]. VWA8 protein is significantly downregulated in breast cancer brain metastases and the expression of VWA8 in brain metastases is significantly decreased [7]. Moreover, we showed that VWA8 abundance was higher in livers of mice fed a high fat diet [8]. Subsequently, we found that, VWA8, as suggested by its AAA+ ATPase domain, has ATPase activity in vitro and is localized to the mitochondrion [9]. Taken together, these last two observations suggest VWA8 plays a role in metabolism and bioenergetics. Although it is clear that VWA8 resides in the mitochondrion, the submitochondrial location of VWA8 remains unclear. More complete nowledge of the location of VWA8 within the mitochondrion might give more insight as to its function.

The mitochondrion has four compartments; the Outer Mitochondrial Membrane (OMM), Intermembrane Space (IMS), the Inner Mitochondrial Membrane (IMM) and Matrix. Here, we use several strategies to define the submitochondrial location of VWA8. First, a bioinformatics analysis was performed using the mitochondrial prediction software MitoProt II to analyze Matrix-Targeting Signals in VWA8 and other known mitochondrial proteins; second, we used various VWA8-GFP (Green Fluorescent Protein) fusion proteins to further localize VWA8; and third, using biochemical techniques, we sub-fractionated mitochondria isolated from rat liver to define the precise the sub-mitochondrial compartment containing VWA8.

2. Materials and Methods

2.1. Animals, mitochondrial isolation and sub-fractionation

All procedures were approved by the University of Arizona Animal Care and Use Committee. Adult male Sprague–Dawley rats (225–250 g; Envigo, Indianapolis, IN) were housed in a pathogen-free environment under controlled temperature (23±3°C) and light (12-hour light/12- hour dark cycle; lights on 07:00–19:00) conditions with standard rodent chow and water available *ad libitum*.

Mitochondria were isolated and sub-fractionated by a modification of a previously published protocol [10]. To isolate mitochondria, rat livers were washed in Mg_2^+ and Ca_2^+-free phosphate buffered saline (PBS), minced in homogenization buffer (250 mM sucrose, 10 mM MOPS-pH 7.4, 1 mM EGTA, 2 mM MgCl2, 0.1% bovine serum albumin) and briefly disrupted using a glass Dounce homogenizer. The homogenized mixture was centrifuged twice at 800 g for 5 min to remove the nuclear fraction. The supernatant was collected and

centrifuged twice at 10,000 g for 10 min for collection of mitochondria. All procedures were performed on ice.

The sub-mitochondrial fractions were obtained from isolated mitochondria after suspension in 10 mM KH_2PO_4 (pH 7.4) for 15 min, followed by addition of an equal volume of mitochondrial fractionation buffer [32% (w/v) sucrose, 30%(w/v) glycerol, 10 mM $MgCl_2$, 10 mM KH_2PO_4 (pH7.4)]. Following another 15 min incubation, the suspension was sonicated twice for 15 s with a 1 min interval between sonications. The suspension was centrifuged at 12,000 g for 10 min at 4°C to obtain a supernatant and a pellet. The pellet was resuspended in homogenization buffer (250 mM sucrose, 10 mM MOPS-pH 7.4, 1 mM EGTA, 2 mM $MgCl_2$, 0.1% bovine serum albumin). The pellet and supernatant fractions then were loaded on separate discontinuous sucrose gradients composed of 2 ml each of 25, 37.5, and 50% (w/v) sucrose in 10 mM KH_2PO_4 (pH 7.4) and centrifuged at 210,000 g for 3 h at 4°C. The outer mitochondrial membrane (OMM) fraction was collected at the 25/37.5% interface of the gradient loaded with the supernatant. The mitoplast fraction (inner mitochondrial membrane plus matrix) was collected as the pellet from the gradient loaded with the original pellet suspension. The OMM and mitoplast fractions were washed with homogenization buffer and centrifuged at 160,000 g for 1 h or at 12,000 g for 10 min, respectively. All steps were performed on ice or at 4°C.

2.2. ETFDH cloning and expression constructs for fusion proteins

For cloning of mouse ETFDH, total cDNA from the liver tissue of a 10-week old C57BL/6J mouse was used to amplify specific ETFDH cDNA using PCR. The amplified cDNA was inserted into a vector, pCMV6-AC-HA. The sense primer was 5′ CTCTCCTTTGTATCTGGTCAAGTCTCCGGGTCAC −3′ and antisense primer 5′-CCTTCATTCACTGGCACACCTGACAGCCAGTTTC −3′. The sequencing results revealed that the cloned mouse ETFDH cDNA exactly matched NM_025794.2. Mouse VWA8a cDNA and VWA8b cDNA were cloned as described [9].

A cDNA fragment coding for enhanced green fluorescent protein (GFP), ATG-TCCGGA-eGFP, was inserted into a plasmid, pCMV6-AC-HA (Origene #PS100004), to generate the pCMV-SG-eGFP expression construct. Within the cDNA fragment were the following: ATG (start codon); TCCGGA, which encodes two amino acids SG, is a restriction endonuclease BspE I site for subcloning use; and cDNA coding for 238 amino acids comprising eGFP. cDNA for the matrix targeting sequence of VWA8 (vMTS, encoding the first N-terminus 35 amino acids of VWA8) and the matrix targeting sequence of ETFDH (eMTS cDNA, encoding the first N-terminus 33 amino acids of ETFDH) were inserted into the expression vector pCMV-SG-eGFP to generate pCMV-vMTS-eGFP and pCMV-eMTS-eGFP, respectively.

A cDNA fragment ATG-C10-eGFP was inserted into a plasmid pCMV6-AC-HA (Origene #PS100004) to generate pCMV-C10-eGFP. Within this cDNA fragment were the following: ATG (start codon); C10, encoding ten amino acids SGAGSGGSAG, is a link for subcloning fusion proteins; and cDNA coding for 238 amino acids comprising eGFP. VWA8a, VWA8b, VWA8b with deletion of vMTS, and ETFDH cDNA were inserted into this vector to

generate pCMV-VWA8b-eGFP, pCMV-VWA8b(ΔvMTS)-eGFP, and pCMV-ETFDH-eGFP, respectively.

All constructs were verified by DNA sequencing. Please see Figure 1A for a summary of the use of these vectors.

2.3. Cell culture and transfection

C2C12 myoblasts were purchased from ATCC (CRL-1772) and cultured in medium MEM alpha (Gibco 12571–063) supplemented with 10% fetal bovine serum (16000–044), 0.1% penicillin and streptomycin (Gibco 15140–122). Transfection of vectors was conducted using Lipofectamine 3000 (Invitrogen#L3000015) and performed as described previously [11].

2.4. Confocal microscopy and Immunofluorescence staining

C2C12 cells were cultured on glass cover slips in 6-well dishes. Cells were transfected by the constructs with eGFP for 48 h. Cells were fixed with 3% paraformaldehyde in PBS for 20 min and cover slips were mounted on glass slides using Vectashield before confocal microscopy. For immunofluorescenc staining, fixed cells also were permeabilized with 0.5% triton X-100 in PBS for 5 min, and then quenched with 100 mM glycine in PBS for 20 min. The cells were blocked with 1% BSA in PBS for 1 h, exposed to primary antibodies for 2 h at room temperature and then treated for 1 h with Alexa Fluor 594 secondary antibodies (Invitrogen #A21207) diluted 1:1000. Images were obtained using a Zeiss 63x oil immersion object in an inverted Zeiss LSM 880 microscope. eGFP and MitoTracker Red were detected by Airyscan detector using Argon 488nm laser and DPSS 561–10 laser, respectively.

2.6. Western blotting

Cell lysates or proteins were separated by SDS-PAGE, transferred onto nitrocellulose membranes, and detected by Western blotting with primary antibody followed by detection using horseradish peroxidase-conjugated secondary antibodies.

2.7. Bioinformatics analyses

The amino acid sequences of representative proteins in different compartments of mitochondria were analyzed for the presence of a matrix targeting signals using MitoProt [12].

3. Results

3.1. VWA8 contains an N-terminal Matrix-Targeting Signal.

Representative proteins that are known to reside in one of four mitochondrial sub-compartments (Uniprot, https://www.uniprot.org) were selected and their amino acid sequences were analyzed by MitoProt II software in order to compare their sequences with that of VWA8. Table 1 shows that proteins in the outer mitochondrial membrane (OMM) and intermembrane space (IMS) either completely lack a matrix targeting sequence (MTS; VDAC, CPT-1, AK, Cyto-c) or have a truncated MTS (CK, 15 amino acids). Proteins can associate with the inner mitochondrial (IMM) from either theIMS or matrix side. Proteins

from the IMS entering the IMM (*e.g.*, adenine nucleotide translocase or ANT) do not have a MTS, but proteins that associate with the matrix surface of the IMM do (*e.g.*, CPT-2 and OXA-1L). VWA8 has a full 34-amino acid MTS, which suggests it would localize to the mitochondrial matrix based on theoretical considerations.

3.2 vMTS is essential for VWA8 to enter mitochondria

To test the bioinformatics-based theoretical predictions, several constructs were designed to track protein localization using eGFP (Figure 1A). C2C12 myoblasts, chosen because of their ease of tranfection, were transfected with these constructs and the transfected cells also were stained with 50 nM of MitoTracker Red (Invitrogen #7512) for 30 min to visualize mitochondria. The fixed cells then were visualized using confocal microscopy. Figure 1B shows that eGFP without a matrix targeting sequence (vector containing only eGFP) is primarily targeted to nuclei while eGFP with matrix targeting sequences from either VWA8 (VWA8 MTS) or ETFDH (ETFDH MTS) localized to mitochondria. Figure 1C shows that native sequences of VWA8a, VWA8b and ETFDH all are targeted to mitochondria. Figure 1D shows that a fusion protein of VWA8b and eGFP localizes to mitochondria, but a construct of VWA8b and eGFP with deletion of vMTS [VWA8b(ΔMTS)] is retained in the cytosol, indicating that vMTS is necessary for mitochondrial targeting of VWA8.

3.3 VWA8 is targeted to the matrix side of inner mitochondrial membrane

The above results show that VWA8 is localized to mitochondria, that the matrix targeting sequence of either VWA8 or ETFDH confers mitochondrial localization to eGFP, and that the VWA8 matrix targeting sequence is necessary for targeting of VWA8 to mitochondria. To determine biochemically if these putative matrix targeting sequences actually target VWA8 to the matrix, rat livers were used to isolate mitochondria and sub-fractionate them into mitochondrial sub-compartments. Protein localization was tracked using immunoblotting. The scheme for these experiments is detailed in Figure 2. Sprague Dawley rats were sacrificed and mitochondria were prepared from the excised livers. Mitochondrial sub-compartments were isolated by differential fractionation techniques (Figure 2A, B). Several protein markers were selected to validate the sub-fractionation technique. These markers and their known locations included Adenine Nucleotide Translocase (ANT1/2, inner mitochondrial membrane), Superoxide Dismutase (SOD2, matrix), Voltage Dependent Anion Channel (VDAC, outer mitochondrial membrane), Electron Transfer Flavoprotein-ubiquinone Oxidoreductase (ETFDH, matrix surface of inner mitochondrial membrane), and Glyceraldehyde Phosphate Dehydrogenase (GAPDH, cytosol). Figure 2C biochemically confirms the imaging data showing that VWA8a localizes to mitochondria. Note that the band around the molecular weight of VWA8a in the cytosol fraction migrates slightly lower than VWA8a and is non-specific (Figure 2C). Because of the lack of an antibody, we could not visualize VWA8b.

To further define the subcompartment containing VWA8, mitochondria were differentially subfractionation into mitoplasts and then proteins were release from mitoplasts (IMM plus matrix) using either sonication or Brij58 detergent. A graphical depiction of fractionation by these two methods and an immunoblot showing the results from the four fractions that result from this method are given in Figure 2D. After gentle sonication, the OMM was disrupted

and separated from mitoplasts. VWA8a protein was retained in mitoplasts and was absent from the OMM. A higher intensity sonication was used to disrupt IMM and release contents of the mitochondrial matrix. In this case VWA8a, like ETFDH, mostly was retained in IMM. When IMM preparations were incubated in a strong detergent, Brij58, VWA8a and ETFDH both were released from IMM, suggesting that VWA8a, like ETFDH, is localized to the matrix side of IMM.

3.4. VWA8 colocalizes with ETFDH

Since the submitochondrial location of VWA8 localization is similar to ETFDH, it would be predicted that VWA8 would co-localize with ETFDH in imaging experiments. To answer this question, C2C12 myoblasts were transfected with pCMV-VWA8a-eGFP or pCMV-VWA8b-eGFP so both isoforms could be tested. The transfected cells were subjected to immunofluorescence staining with anti-ETFDH antibody followed by an Alexa Fluor 594 secondary antibody. The cells, grown on cover slips were mounted on glass slides, and then imaged using confocal microscopy. The overlay of these images in Figure 3A shows that VWA8a colocalizes with ETFDH and Figure 3B shows that VWA8b is co-localized with ETFDH in mitochondria.

4. Discussion

About 99% of mitochondrial proteins are synthesized in the cytoplasm and imported into the mitochondrion [13]. The outer mitochondrial membrane and intermembrane space each contain about 10% of the total mitochondrial proteins, while the inner mitochondrial membrane and matrix each contain about 40% of the total [13]. The machinery for mitochondrial protein import and sorting involves integral membrane complexes in both the outer and inner mitochondrial membranes. There are two major protein complexes, TOM40 (Translocase of Outer Membrane 40) and TIM23 (Translocase of Inner Membrane 23), in the outer and inner membranes, respectively. The TOM40 complex spans the outer mitochondrial membrane while TIM23 spans the inner membrane [14–16]. After import through TOM40, proteins pass into the intermembrane space. From there, imported proteins may remain in the intermembrane space, traffic to the outer membrane (*e.g.*, VDAC), be inserted into the inner membrane (*e.g.*, ANT) or pass through the inner membrane via TIM23 and enter the mitochondrial matrix (*e.g.*, CPS-1). To enter the matrix, proteins usually contain specific sequences called Matrix-Targeting Signals/Sequences (MTS) [14,16]. Usually the MTS is located in the N-terminus of the protein (up to the first 70 amino acids). Upon entry into the matrix, the MTS usually is cleaved by a peptidase.

We used a bioinformatics approach to analyze the sequences of VWA8 and other mitochondrial proteins in order to provide a theoretical basis for our experiments. Such analyses can aid in identification of putative mitochondrial proteins and distinguish them from proteins in other subcellular compartments [12]. This bioinformatics analysis showed that all proteins we examined that are known to pass through the inner mitochondrial membrane and enter the matrix have a MTS consisting of at least 16 amino acids. However, for inner mitochondrial membrane proteins in general the situation is more complex, as described above. The protein most similar to VWA8 with regard to submitochondrial

location that we analyzed is ETFDH (Electron Transferring Flavoprotein Dehydrogenase or Electron Transfer Flavoprotein-Ubiquinone Oxidoreductase). ETFDH is a dehydrogenase that accepts electrons from electron-transfer flavoprotein and reduces ubiquinone in the inner membrane. ETFDH, like CPT-2, contains an N-terminal MTS and enters the matrix. But unlike CPT-2, which is integrated into the inner membrane, ETFDH attaches more loosely to the matrix side of the inner membrane [17,18]. ETFDH, like VWA8, does not contain any predicted transmembrane domains. It associates with the inner membrane by interactions via a parallel hydrophobic α-helix and β-sheet.

We previously showed that VWA8 is targeted to mitochondria, but further bioinformatics analysis predicted that VWA8 should be targeted to the matrix through its 34 amino acid matrix targeting sequence. To determine whether the VWA8 matrix targeting sequence (vMTS) is sufficient to target proteins to mitochondria, we used fusion proteins consisting of eGFP together with the vMTS or eMTS (matrix-targeting sequences of VWA8 or ETFDH, respectively). eGFP without targeting sequences traffics primarily to nuclei, but when vMTS or eMTS are added to the N-terminus of eGFP, the fusion proteins are targeted to mitochondria. All fusion proteins we tested that include a vMTS or eMTS (VWA8a-eGFP, VWA8b-eGFP and ETFDH-eGFP) were targeted to mitochondria. In addition, when vMTS is deleted in mitochondrial proteins, as in the construct VWA8b(ΔvMTS), the protein no longer localizes to mitochondria, but remains in the cytosol. These data strongly suggest that vMTS is necessary and sufficient for VWA8 to enter mitochondria.

To gain further detail on the submitochondrial compartment to which VWA8 is targeted, we used a biochemical mitochondrial subfractionation approach. Isolation of mitochondria from rat liver is straightforward, but subfractionation of these mitochondria is more complex. After mitochondria are isolated, as a first step the outer mitochondrial membrane must be disrupted. There are two generally used methods for doing this, solubilization with digitonin or sonication. However, neither completely removes the outer membrane. Because the sub-fractionation efficiency of digitonin is very sensitive to concentration and incubation time, the use of sonication has become more common, as it is more reproducible. Whether to use digitonin or sonication also depends empirically on the protein of interest. In the experiments conducted here, sonication resulted in more consistent identification of the sub-fraction containing VWA8. We found that in our subfractionation scheme, VWA8 protein tracked that of EFTDH. When mitoplasts (inner membrane and matrix) were treated with moderate sonication, ETFDH and VWA8 both tracked with the inner membrane. However, when mitoplasts were incubated with the detergent Brij58, the interaction between either ETFDH or VWA8 and the inner membrane was disrupted. That is likely due to the fact that neither ETFDH nor VWA8 is an integral to the inner membrane (like ANT1/2, for example, which remains in the inner membrane after Brij58 treatment), but instead only attaches more loosely to the inner membrane, mainly by hydrophobic interactions [17,18]. Integral inner membrane proteins, such as ANT1/2, as predicted, remain in the inner membrane fraction even after Brij58 incubation. Based on these results, we hypothesize that VWA8, like ETFDH, interacts with the inner membrane more loosely, probably by hydrophobic interactions. VWA8 shares several other features with ETFDH. In N-terminus, ETFDH has a 32-amino acid MTS and VWA8 has a 34-amino acids MTS. ETFDH has a predicted matrix targeting probability to the matrix 0.95 and VWA8 has a predicted 0.89 probability of matrix

targeting. Taken together, these data suggest that VWA8 interacts loosely with the matrix side of the inner mitochondrial membrane.

Determining the submitochondrial location of VWA8 localization could help predict the function of VWA8. VWA8 isoforms are large proteins, the long isoform in the mouse (VWA8a) consisting of 1905 amino acids and the shorter isoform (VWA8b) having 1038 amino acids. VWA8a has a AAA+ ATPase domain, Walker A and B motifs, and a more C-terminal VWA domain. VWA8b lacks the VWA domain but retains the ATPase domain and ATP binding site. We have reported that VWA8 has ATPase activity *in vitro* [9]. AAA+ ATPase domain-containing proteins often function as chaperones or motor proteins. Considering the location of VWA8 at the matrix side of the inner mitochondrial membrane, we conjecture that VWA8 might have chaperone and/or protease activities and be involved in processing of proteins that are targeted to the matrix or inner membrane. We cannot rule out the possibility that VWA8 might, like ETFDH, be involved in electron transport chain activity because of its proximity to the inner membrane. However, it lacks the features of such a protein. Additional studies are needed to further define the function of VWA8 proteins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Abbreviations:

MTS	Matrix-Targeting Signals/Sequences
OMM	Outer Mitochondrial Membrane
IMS	Intermembrane Space
IMM	Inner Mitochondrial Membrane
ETFDH or ETFQO	Electron Transferring Flavoprotein Dehydrogenase/ Electron Transfer Flavoprotein-Ubiquinone Oxidoreductase

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Highlights

- VWA8 (Von Willebrand A Domain-containing Protein 8) is a AAA+ ATPase that has a predicted N-terminal mitochondrial matrix targeting sequence.
- Immunofluorescence experiments show that the predicted matrix targeting sequence is necessary for trafficking of VWA8 (and other fusion proteins) to the mitochondrion.
- Differential biochemical sub-fractionation techniques using sonication or detergent treatment of isolated mitochondria reveal that VWA8 likely is associated with the matrix face of the inner mitochondrial membrane.

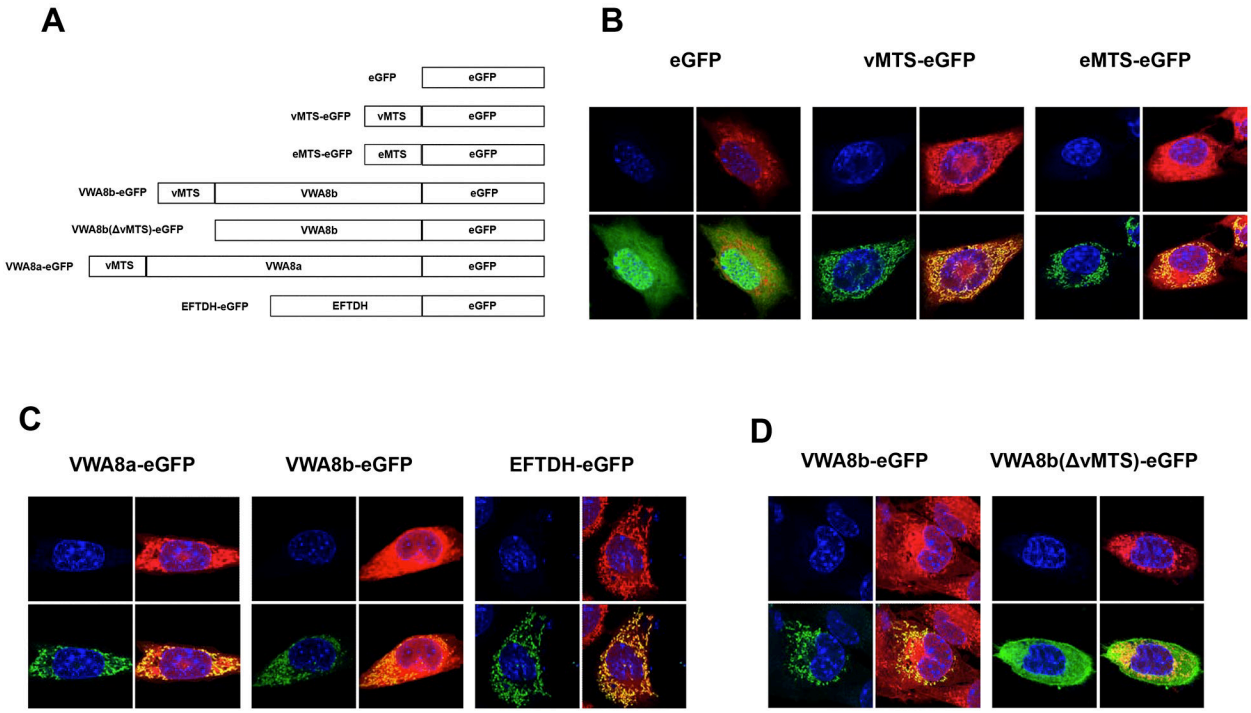

Figure 1. Matrix targeting sequences are necessary and sufficient for targeting proteins mitochondria.

A. Design of expression vector constructs. eGFP, enhanced Green Fluorescent Protein; vMTS, putative VWA8 matrix targeting sequence; eMTS, predicted ETFDH matrix targeting sequence; VWA8a, Von Willebrand A domain containing protein 8; VWA8b, Won Willebrand A domain containing protein 8b; EFTDH, Electron transfer flavoprotein-ubiquinone oxidoreductase. **B**. C2C12 myoblasts grown on coverslips were transfected with pCMV-SG-eGFP; pCMV-vMTS-eGFP or pCMV-eMTS-eGFP, and the transfected cells were stained with 50 nM MitoTracker Red for 30 min. The cells were fixed and confocal microscopy was used for visualization. Green, eGFP; Red, MitoTracker Red; Yellow, overlay. **C**. C2C12 myoblasts grown on coverslips were transfected with pCMV-VWA8a-eGFP, pCMV-VWA8b-eGFP or pCMV-ETFDH-eGFP, and the transfected cells were stained with 50 nM MitoTracker Red for 30 min. The cells were fixed and confocal microscopy was used for visualization. Green, eGFP; Red, MitoTracker Red; Yellow, overlay. **D**. C2C12 myoblasts were transfected with pCMV-VWA8b-eGFP and pCMV-VWA8b(ΔMTS-eGFP, and the transfected cells were stained with 50 nM of MitoTracker Red for 30 min. The cells were fixed and confocal microscopy was used for visualization. Green, eGFP; Red, MitoTracker Red; Yellow, overlay.

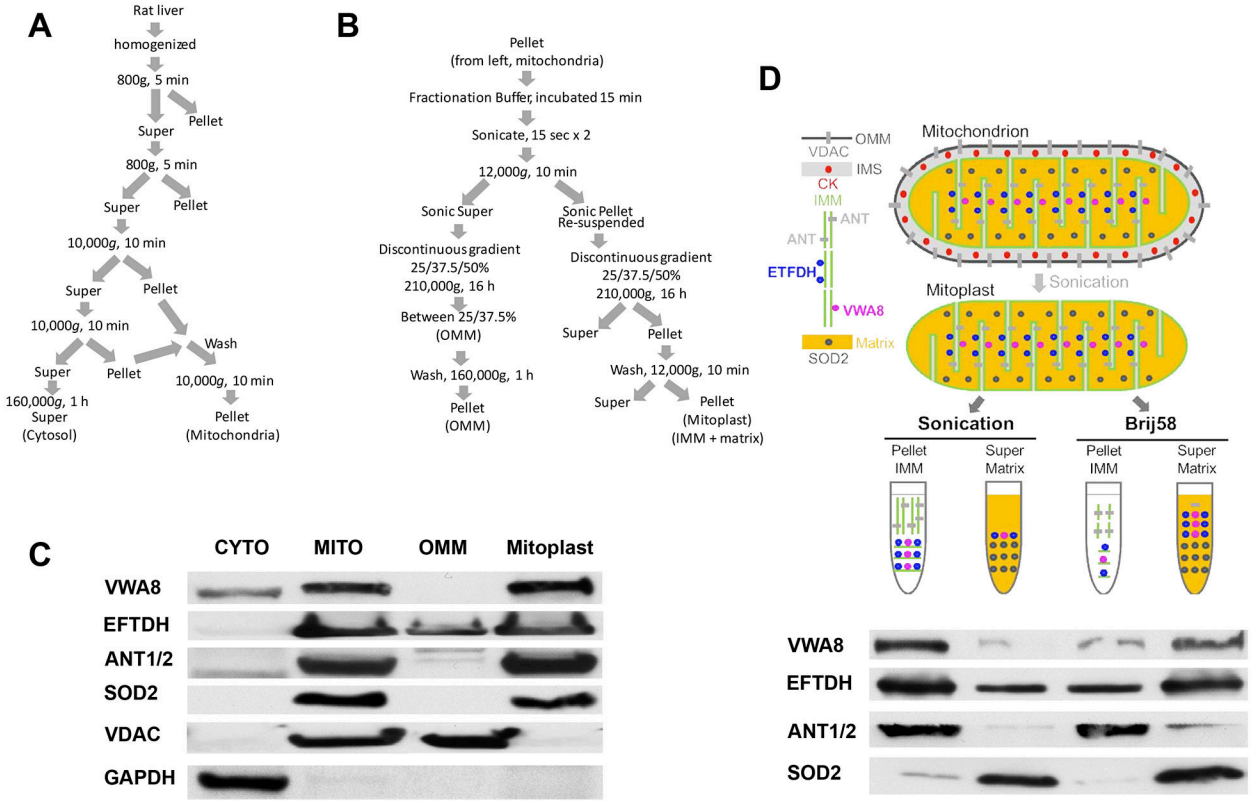

Figure 2. VWA8 and EFTDH localize to the matrix side of inner mitochondrial membrane.

A. Scheme for isolating mitochondria from rat liver. **B**. Scheme for subfractionating mitochondria isolated from rat liver. OMM, outer mitochondrial membrane; IMM, inner mitochondrial membrane; mitoplast, IMM plus matrix. **C**. Immunoblots of proteins present in mitochondrial compartments isolated by sub-fractionation, as described in B (n=3). CYTO, cytosol; MITO, unfractionated, whole mitochondria; OMM, outer mitochondrial membrane;, Mitoplast, outer mitochondrial membrane (IMM) plus matrix; ANT1/2, Adenine Nucleotide Translocase 1/2 isoforms (Inner Mitochondrial Membrane, IMM, found in mitoplast); SOD2, superoxide dismutase 2 (matrix, found in mitoplast); VDAC, Voltage dependent anion channel (outer mitochondrial membrane); GAPDH, Glyceraldehyde phosphate dehydrogenase (cytosol). **D**. Subfractionation of mitoplasts using sonication or detergent (Brij58). Mitoplasts were sonicated or incubated with Briji58 (n=3), as described in the Methods. All of samples were subjected to Western blotting.

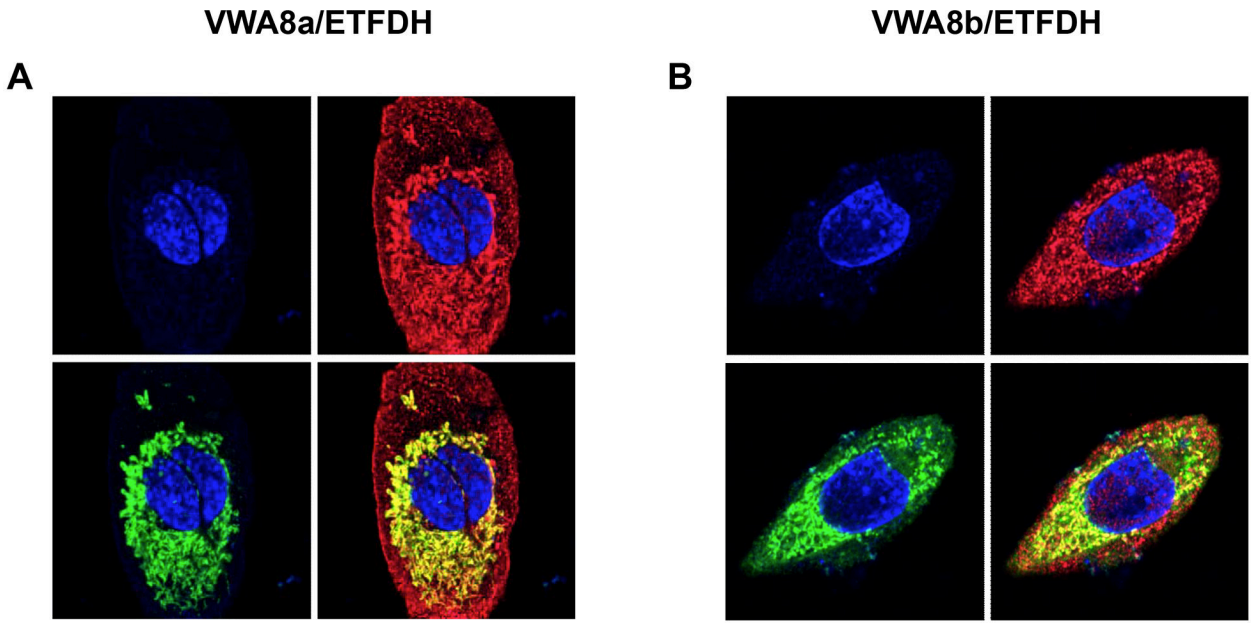

Figure 3. VWA8 and ETFDH co-localize in mitochondria.

C2C12 myoblasts grown on coverslips were transfected with pCMV-VWA8a-eGFP or pCMV-VWA8b-eGFP. The transfected cells were subjected to immunofluorescence staining with anti-ETFDH antibody followed by Alexa Fluor 594 secondary antibody (red). The cells on slips underwent confocal microscopy. **A**. VWA8a (green) co-localized with ETFDH (red) to mitochondria. Yellow, overlay. **B**. VWA8b (green) co-localized with ETFDH (red) in mitochondria. Yellow, overlay.

Table 1.

Bioinformatics analyses of representative proteins in sub-compartments of mitochondria.

Accession	Amino Acids	Compartment	P	Cleavage Site	Cleaved Sequence
VDAC-2 (NM_011695.2)	295	OMM	0.0459	NA	NA
CPT-1 (AF320000.1)	798	OMM	0.0261	NA	NA
Adenylate Kinase 2 MT (VM_001033966.4)	239	IMS	0.0359	NA	NA
Creatine Kinase (M74149.1)	381	IMS	0.0211	15	MPFSNSHNTQKLRF
Cytochrome C (X01756.1)	105	IMS	0.0720	NA	NA
ANT-1 (NM_007450.5)	298	IMM	0.2010	NA	NA
CPT-2 (AH000838.2)	658	IMM	0.7708	33	MMPRLLLRDWPRCPSLVLGAPSRPLSAVSGPA
OXA-1L (NM_026936.3)	433	IMM	0.8783	67	MARNLVCGRWQLLRLLRPQRSYHSVAVSLRPLAAELLAARRGNGRPPCALLAVFTPRCISTSATLF
ETFDH (NM_025794.2)	616	IMM-Matrix	0.9479	33	MLVRLTKLSCPAYHWFHALKIKKCLPLCAPRC
ATP Synthase F(0)C1 (BC003854.1)	136	IMM-Matrix	0.9877	62	MQTTKALLISPALIRSCTRGLIRPVSASLLSRPEAPSKQPSCSSSPLQVARREFQTSVISR
CPS-1 (NM_001080809)	1500	Matrix	0.9487	47	MTRILTACKVVKTLKSGFGFANVTTKRQWDFSRPGIRLLSVKAKTA
SOD2 (CT010273.1)	222	Matrix	0.9851	21	MLCRAACSTGRRLGPVAGAA
VWA8a (NM_027906.1)	1905	?	0.8904	35	MQSRLLLLGAPGGLGDVASRRVRLLLRQVLRGRP

MitoProt II prediction program was used for these predictions (see Methods). P, Probability of mitochondrial targeting; OMM, Outer mitochondrial membrane; IMM, Inner mitochondrial membrane; IMS, Inter membrane space.