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MBOAT7 is anchored to endomembranes by six transmembrane domains

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ABSTRACT

Membrane bound O-acyltransferase domain- containing 7 (MBOAT7, also known as LPIAT1) is a protein involved in the acyl chain remodeling of phospholipids via the Lands' cycle. The *MBOAT7* is a susceptibility risk genetic locus for non-alcoholic fatty liver disease (NAFLD) and mental retardation. Although it has been shown that MBOAT7 is associated to membranes, the MBOAT7 topology remains unknown. To solve the topological organization of MBOAT7, we performed: A) solubilization of the total membrane fraction of cells overexpressing the recombinant MBOAT7-V5, which revealed MBOAT7 is an integral protein strongly attached to endomembranes; B) *in silico* analysis by using 22 computational methods, which predicted the number and localization of transmembrane domains of MBOAT7 with a range between 5 and 12; C) *in vitro* analysis of living cells transfected with GFP-tagged MBOAT7 full length and truncated forms, using a combination of Western Blotting, co-immunofluorescence and Fluorescence Protease Protection (FPP) assay; D) *in vitro* analysis of living cells transfected with FLAG-tagged MBOAT7 full length forms, using a combination of Western Blotting, selective membrane permeabilization followed by indirect immunofluorescence. All together, these data revealed that MBOAT7 is a multispanning transmembrane protein with six transmembrane domains. Based on our model, the predicted catalytic dyad of the protein, composed of the conserved asparagine in position 321 (Asn-321) and the preserved histidine in position 356 (His-356), has a lumenal localization. These data are compatible with the role of MBOAT7 in remodeling the acyl chain composition of endomembranes.

1. Introduction

Membrane bound O-acyltransferase domain-containing 7 (MBOAT7), also named as lysophosphatidylinositol acyltransferase (LPIAT1), is a member of the membrane-bound O-acyltransferase (MBOAT) superfamily (Hofmann, 2000), which is composed by enzymes involved in lipid metabolism (Riekhof et al., 2007; Shindou and Shimizu, 2009). MBOAT7 is a 472 amino acids-long protein present in endomembranes (endoplasmic reticulum, mitochondria-associated membranes and lipid droplets) (Mancina et al., 2016). MBOAT7 has a putative catalytic dyad composed of an asparagine and a histidine residue at position 321 and 356 of the protein (Lee et al., 2008), respectively. However, it is not known whether the catalytic dyad faces the lumen of the organelle or the cytosol.

MBOAT7 is involved in the Lands' cycle, a metabolic pathway carried out in the endoplasmic reticulum compartment (Moessinger et al., 2014). This pathway influences the composition of membranes by remodeling the acyl chain composition of phospholipids (Lands, 1958, 2000).

MBOAT7 contributes to maintaining brain and liver homeostasis in humans (Johansen et al., 2016b). Indeed, individuals homozygous for loss of function mutations in *MBOAT7* have mental retardation (Johansen et al., 2016a; Lee et al., 2012), while a common genetic variant (rs641738) have been associated with the entire spectrum of alcoholic (Buch et al., 2015) and nonalcoholic fatty liver disease (NAFLD) (Mancina et al., 2016). NAFLD is characterized by increased hepatic triglyceride content and progression to liver inflammation, fibrosis and hepatocarcinoma (Day, 2006; Kleiner et al., 2005). Despite the fact that NAFLD will be soon the most common problem in internal medicine (Guerrero et al., 2009), the biology behind the disease progression is not clear.

The role of MBOAT7 in the pathogenesis of NAFLD is poorly

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understood. The rs641738 variant is associated with a reduction in MBOAT7 intracellular expression levels that *bona fide* affect the acyl chain composition and remodeling of phospholipids in membranes. Although MBOAT superfamily members are multispanning transmembrane enzymes with a variable number of transmembrane domains (Chang and Magee, 2009), it is not known whether this applies to MBOAT7. Moreover, the topology of MBOAT7, which refers to the exact orientation and organization of the protein within the membrane bilayer, remains unknown.

The topology of any protein can be inferred by examining the specific composition and distribution of the amino acid protein sequence. Indeed, transmembrane domains are predominantly composed of hydrophobic amino acid residues consisting of alpha-helices and spanning the phospholipid-bilayer from side to side (Almen et al., 2009).

In this study, we solved for the first time MBOAT7 protein orientation and organization in membranes of living cells, combining *in silico* and *in vitro* analyses.

2. Material and methods

2.1. MBOAT7 transiently transfected in HEK293T/17 cells

MBOAT7 cDNA was synthesized and cloned into pcDNA3.1 vector having a V5 epitope tag at the C-terminus, by GeneArt Gene Synthesis (Thermo Fisher Scientific, Rockford, IL, USA). Human embryonic kidney cells 293 stably expressing the SV40 large T antigen (HEK293T/17) were purchased from American Type Culture Collection (Manassas, VA, USA). HEK293T/17 cells were cultured in high glucose (4500 mg/L) Dulbecco's Modified Eagle's Medium (DMEM) containing 10% Fetal Bovine Serum (FBS) and 5% penicillin-streptomycin (GE Healthcare Life Sciences, Pittsburgh, PA, USA). HEK293T/17 cells were transiently transfected with the plasmid containing the *MBOAT7* cDNA using a cationic lipid-mediated method (Lipofectamine3000 transfection reagent, Thermo Fisher Scientific, Rockford, IL, USA) according to the manufacturer's instructions.

2.2. Cell total membrane fractionation and solubilization

Cells were collected after 48 h of transfection. As previously described (He et al., 2010), cells were lysed using a 27-gauge needle in buffer A (50 mM Tris-HCl pH 7.0, 250 mM sucrose (Sigma-Aldrich, St. Louis, MO, USA), 1 mM ethylenediaminetetraacetic acid (EDTA) (Thermo Fisher Scientific, Rockford, IL, USA), 10% Mammalian Protein Extraction Reagent (MPER) (Thermo Fisher Scientific, Rockford, IL, USA) containing protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA)). Lysate was centrifuged at 1,000 g for 1 min at 4 °C. Supernatant was saved and centrifuged at 100,000 g for 45 min at 4 °C. The pellet (total membranes) was resuspended in buffer A and centrifuged at 100,000 g for 1 h at 4 °C. The pellet was resuspended in 10 mM Tris HCl pH 7.4 and divided into equal parts. The membrane fractions were incubated for 1 h on ice with: A) a salt solution (1 M NaCl, Sigma-Aldrich, St. Louis, MO, USA), B) an alkaline buffer pH 11.3 (0.2 M Na_2CO_3, Sigma-Aldrich, St. Louis, MO, USA), C) a steroidal saponin (2% digitonin, Millipore Corporation, Billerica, MA, USA), D) a mild non-ionic detergent (2% Triton X-100 in phosphate buffer solution (PBS)), E) a Zwitter-ionic detergent (2% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), Sigma-Aldrich, St. Louis, MO, USA), or F) a harsh ionic detergent (2% sodium dodecyl sulfate (SDS) in PBS, VWR International, Leuven, Belgium), used as control. Solubilized membranes were centrifuged at 100,000 g for 1 h at 4 °C, then supernatants were saved and pellets resuspended in 50 µl of buffer A.

2.3. SDS-PAGE and immunoblot analysis

An equivalent volume of each fraction (supernatants and resuspended pellets) were mixed with Laemmli buffer, and boiled for 10 min at 95 °C. Proteins were size-separated by SDS PAGE (12% acrylamide gel, 120 V, 2 h) and transferred onto a nitrocellulose membrane (0.4 A, 1 h). Membranes were incubated in tris-buffered saline containing 0.2% Tween (0.2% TBS-T buffer) with 5% nonfat dried milk (PanReac AppliChem, Darmstadt, Germany) for 1 h, and incubated overnight with primary antibodies, washed 3 times for 10 min with 0.2% TBS-T buffer, incubated 1 h with HRP-conjugated secondary antibodies and washed 3 times for 10 min with 0.2% TBS-T buffer. Membranes were incubated for 5 min with chemiluminescent HRP substrate (Millipore Corporation, Billerica, MA, USA) and visualized by Chemidoc XRS System (BioRad, Hercules, CA, USA). The following antibodies were used: mouse anti-V5 (Thermo Fisher Scientific P/N46-0705, Rockford, IL, USA) diluted 1:5000, rabbit anti-calnexin (Sigma-Aldrich, St. Louis, MO, USA) diluted 1:2000.

2.4. In silico analysis of MBOAT7 membrane topology

To predict human MBOAT7 number and localization of TM domains, a set of 22 different transmembrane prediction algorithms were selected based on their performance in several benchmarks (Kernytsky and Rost, 2003; Rath et al., 2013; Reeb et al., 2015; Tsirigos et al., 2012). Final consensus model was obtained after removing incorrect predictions, using MATLAB Bioinformatics toolbox built-in function *seqconsensus*, with a custom scoring matrix (1 for match and 0 for mismatch between TM and non-TM segments).

2.5. Evaluation of algorithm prediction performance

Sensitivity and specificity were calculated for each algorithm separately on a per-residue basis, as:

$$Sensitivity = TP/(TP + FN)$$

$$Specificity = TN/(TN + FP)$$

where TP = true positive, FN = false negative, TN = true negative, and FP = false positive.

The number of false positives and false negatives were calculated comparing the predicted topology of each algorithm with final consensus model.

2.6. Fluorescence Protease Protection (FPP) assay set up

HEK293T/17 cells were seeded and, when they reached 60% confluence, were transfected using Lipofectamine 3000 transfection reagent, according to the manufacturer's instructions. To set up the FPP assay (Lorenz et al., 2006; White et al., 2015), HEK293T/17 were transiently transfected with: A) pmaxFP-Green-N vector (Addgene, Cambridge, MA, USA), encoding the green fluorescent protein maxFP-Green, which freely diffuses in the cytoplasm; B) caveolin-1-GFP (Addgene, Cambridge, MA), an integral membrane protein with both its C- and N-terminal facing the cytosol (Liu et al., 2002; Smart et al., 1999); or C) pDsRed2-Mito Vector (Takara Bio Europe, Saint-Germain-en-Laye, France), encoding a variant of *Discosoma sp.* red fluorescent protein (Living Colors, 2001; Matz et al., 1999), which localizes in mitochondria (Nixon et al., 2013). After transfection, HEK293T/17 cells were incubated at 37 °C for 36–40 h. Medium was removed and cells were washed twice with KHM buffer (110 mM CH_3CO_2K, 3 mM $MgCl_2$, 20 mM HEPES NaOH pH 7.2) for 60 s at room temperature. All the reagents used for preparing KHM buffer were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cells were placed under Axio Vert.A1 inverted fluorescence microscope (Carl Zeiss AB, Oberkochen, Germany). All the images were taken by Axiocam 503 mono camera using a 63x non-oil objective. Due to the experimental limitations, it was not possible to take oil-immersion pictures. Images were analysed using Zeiss software ZEN 2.3. Cells were then perfused for 360 s at room

temperature using KHM buffer containing 50 μM high purity digitonin (Millipore Corporation, Billerica, MA, USA). Cell images were taken every 120 s during the digitonin treatment, for a total of four images, to capture the pre-permeabilization stage and the membrane permeabilization state (Supplementary Fig. 1A). Then, cells were washed twice with KHM buffer for 60 s at room temperature to remove the excess of digitonin. Cells were perfused for 360 s at room temperature using KHM buffer containing 50 μM proteinase K (Sigma-Aldrich, St. Louis, MO, USA). Cell images were taken every 120 s during the proteinase K incubation, for a total of four images, to record the decay (Supplementary Fig. 1B) or persistence (Supplementary Fig. 1C) of the fluorescent signal.

2.7. Fluorescent image quantification

To examine the persistence or decay of fluorescence, we performed an image segmentation using k-means clustering. The image with highest fluorescence intensity level (i.e. corresponding to zero-time point) for each GFP-tagged fusion protein was considered as reference image. The k-means clustering using Euclidean distance was applied to group the pixels within the reference image into two clusters of background and cell objects. The minimum intensity of pixels belonging to the cell objects was then used as a reference threshold for images from other time points. Next, to quantify the fluorescence decay over time, the sum of pixel intensities above the reference threshold was calculated for each time point, and normalized to the maximum calculated value across all images belonging to a specific GFP-tagged fusion protein. Hence, fluorescence decay values for all set of GFP-tagged fusion proteins form a matrix with rows corresponding to values for a particular GFP-tagged fusion protein and columns corresponding to different GFP-tagged fusion proteins (Supplementary Fig. 2). Finally, the resulting fluorescence was hierarchically clustered using Euclidean distance, to identify whether the GFP-tag faces the cytosolic or the lumenal side of the membrane. All the analyses were performed using in-house developed MATLAB scripts (Mathworks, Natick, MA, USA). The schematic overview of the approach is depicted in Supplementary Fig. 2.

2.8. GFP-tagged MBOAT7 full length and truncated forms construction

The lysophospholipid acyltransferase 7 isoform 1 [Homo sapiens] (NCBI Reference Sequence: NP_077274.3) (Supplementary Fig. 3A) was used for the synthesis of all the recombinant MBOAT7 forms. The full length MBOAT7 cDNA was synthesized and subcloned into pcDNA-DEST53_A322 vector having a GFP-epitope tag at the N-terminus (Supplementary Fig. 3B), or into pcDNA-DEST47_A095 vector having a GFP-epitope tag at the C-terminus (Supplementary Fig. 3C) by GeneArt Gene Synthesis (Thermo Fisher Scientific, Rockford, IL, USA). Eleven truncated forms of GFP-tagged *MBOAT7* cDNA were synthesized by GeneArt Gene Synthesis (Thermo Fisher Scientific, Rockford, IL, USA). GFP-tag was added to the N-terminal of five MBOAT7 truncated forms, or at the C-terminal of six MBOAT7 truncated forms, for a total of thirteen GFP-tagged MBOAT7 forms (Supplementary Fig. 3D). Moreover, neither the reported *MBOAT7* sequence, nor the synthesized GFP-tagged *MBOAT7* forms, had a signal peptide.

2.9. FLAG-tagged MBOAT7 forms construction

Full length *MBOAT7* cDNAs with a FLAG epitope tag (nucleotide sequence: GACTACAAAGACGATGACGACAAG; amino acid sequence: DYKDDDDK) inserted in amino acid positions 65, 135, 220, 310, 350 or 423 of the MBOAT7 protein, and with a V5 epitope tag at the C-terminal of the MBOAT7 protein, were synthesized and subcloned into pcDNA3.1 vector by GeneArt Gene Synthesis (Thermo Fisher Scientific, Rockford, IL, USA) (Supplementary Fig. 4A-B).

2.10. GFP-tagged MBOAT7 forms overexpressed in HEK293T/17 cells

HEK293T/17 cells were transiently transfected with the plasmids containing the GFP-tagged *MBOAT7* forms cDNA as described above. The efficiency of the transfection was confirmed using the Axio Vert.A1 inverted fluorescence microscope (Carl Zeiss AB, Oberkochen, Germany) after 48 h.

2.11. Total membrane fractionation and solubilization of cells overexpressing GFP-tagged MBOAT7 forms

HEK293T/17 cells were collected after 48 h of transfection. Cell total membrane fractionation was performed as described in materials and methods. The membrane fractions were incubated for 1 h on ice with a salt solution (1 M NaCl, Sigma-Aldrich, St. Louis, MO, USA). Solubilized membranes were centrifuged at 100,000 g for 1 h at 4 °C, then supernatants were saved and pellets resuspended in 50 μl of buffer A. Resuspended pellets and supernatants were size-separated by SDS PAGE and immunoblotted as described above. The following antibodies were used: rabbit anti-GFP (Cell Signaling Technology, Danvers, MA, USA) diluted 1:1000, or rabbit anti-calnexin (Sigma-Aldrich, St. Louis, MO, USA) diluted 1:2000.

2.12. Co-immunofluorescence microscopy

Glass coverslips were coated with poly-L-lysine hydrobromide (Sigma-Aldrich, St. Louis, MO, USA) for 1 h at 37 °C. After incubation, coated coverslips were washed twice with sterile water. HEK293T/17 cells were seeded onto coverslips in 24-well plates and cultured for 48 h. HEK293T/17 cells were cultured for an additional 48 h or transiently transfected with: A) pmaxFP-Green-N vector (Addgene, Cambridge, MA, USA), encoding the green fluorescent protein maxFP-Green; B) pDsRed2-Mito Vector (Takara Bio Europe, Saint-Germain-en-Laye, France), encoding a variant of *Discosoma sp.* red fluorescent protein (Living Colors, 2001; Matz et al., 1999); or C) GFP-tagged MBOAT7 forms, as described above. After 48 h, all cells were fixed using 4% formaldehyde for 10 min at room temperature and then washed 3 times with PBS. All cells, except cells transfected with pmaxFP-Green-N vector and pDsRed2-Mito Vector, were permeabilized using 0.3% Triton-X100 in PBS for 10 min at room temperature. Permeabilized cells were washed twice with PBS and incubated with 4% BSA in PBS for 1 h at room temperature. Permeabilized cells were incubated with primary antibody for 1 h at room temperature, washed twice with PBS and incubated with red or green fluorophore conjugated secondary antibody for 1 h at room temperature in the dark. All cells were stained with DAPI (4′,6-diamidino-2-phenylindole dihydrochloride) (Sigma-Aldrich, St. Louis, MO, USA) diluted 1:8000 in PBS for 5 min in the dark. Glass coverslips were washed twice with PBS and mounted on microscope slides (VWR International, Leuven, Belgium) using Dako fluorescence Mounting Medium (Dako North America, Carpinteria, CA, USA). Glass coverslips were placed under Apotome fluorescence microscope (Carl Zeiss AB, Oberkochen, Germany). All the images were taken by Axiocam MRm Rev2 camera (Carl Zeiss AB, Oberkochen, Germany) using a 63x oil-immersion objective. Images were analysed using Zeiss AxioVision Microscope software. The following primary antibodies were used: rabbit anti-calnexin (Sigma-Aldrich, St. Louis, MO, USA) diluted 1:2000, or mouse anti-alpha 1 Na,K-ATPase (Abcam, Cambridge, UK) diluted 1:2000. The following secondary antibodies were used: anti-rabbit Alexa Fluor 594, or anti-mouse Alexa Fluor 488 (Thermo Fisher Scientific, Rockford, IL, USA).

2.13. FLAG-tagged MBOAT7 forms overexpressed in HEK293T/17 cells

HEK293T/17 cells were transiently transfected with the plasmids containing the FLAG-tagged *MBOAT7* forms cDNA as described above. Transfected cells were harvested after 36 h of incubation at 37 °C. The

efficiency of the transfection was confirmed by Western Blot analysis (data not shown).

2.14. Total membrane fractionation and solubilization of cells overexpressing FLAG-tagged MBOAT7 forms

Cells were harvested after 36 h of transfection. Cell total membrane fractionation was performed as described above. The membrane fractions were incubated for 1 h on ice with a salt solution (1 M NaCl, Sigma-Aldrich, St. Louis, MO, USA). Solubilized membranes were centrifuged at 100,000 g for 1 h at 4 °C, then supernatants were saved and pellets were resuspended in 50 µl of buffer A. Resuspended pellets and supernatants were size-separated by SDS PAGE and immunoblotted as described above. The following antibodies were used: mouse anti-V5 (Thermo Fisher Scientific P/N46-0705, Rockford, IL, USA) diluted 1:5000, or rabbit anti-calnexin (Sigma-Aldrich, St. Louis, MO, USA) diluted 1:2000.

2.15. Selective membrane permeabilization followed by co-immunofluorescence microscopy

Glass coverslips were coated using poly-L-lysine hydrobromide (Sigma-Aldrich, St. Louis, MO, USA) for 1 h at 37 °C. After incubation, coated coverslips were washed twice with sterile water. HEK293T/17 cells were split onto coverslips in 24-well plates and transiently transfected with FLAG-tagged MBOAT7 forms. Then, 36 h after transfection, cells were fixed with 4% formaldehyde for 10 min at room temperature, washed 3 times with PBS, and selectively permeabilized as previously described (Matevossian and Resh, 2015) with some modifications. Briefly, to selectively permeabilize the plasma membrane, cells were incubated with KHM buffer containing 50 µM high purity digitonin (Millipore Corporation, Billerica, MA, USA) for 10 min at room temperature and washed 3 times with PBS. To permeabilize all cell membranes, cells were incubated with 0.3% Triton-X100 in PBS for 10 min at room temperature. Permeabilized cells were washed twice with PBS and incubated with 4% BSA in PBS for 1 h at room temperature. Cells were incubated with primary antibody for 1 h at room temperature. Next, cells were washed twice with PBS and incubated with red or green fluorophore conjugated secondary antibody for 1 h at room temperature in the dark. Cells were washed twice with PBS and stained with DAPI (4′,6-diamidino-2-phenylindole dihydrochloride) (Sigma-Aldrich, St. Louis, MO, USA) diluted 1:8000 in PBS for 5 min in the dark. Glass coverslips were washed twice with PBS for 5 min and mounted on microscope slides (VWR International, Leuven, Belgium) using Dako fluorescence Mounting Medium (Dako North America, Carpinteria, CA, USA). Coverslips were placed under Apotome fluorescence microscope (Carl Zeiss AB, Oberkochen, Germany). All the images were taken by Axiocam MRm Rev2 camera (Carl Zeiss AB, Oberkochen, Germany) using a 63x oil-immersion objective. Images were analysed using Zeiss AxioVision Microscope software. The following primary antibodies were used: mouse anti-FLAG (Sigma-Aldrich, St. Louis, MO, USA) diluted 1:2000, or mouse anti-V5 (Thermo Fisher Scientific P/N46-0705, Rockford, IL, USA) diluted 1:400, and rabbit anti-protein disulfide-isomerase (PDI) (Cell Signaling Technology, Danvers, MA, USA) diluted 1:50. The following secondary antibodies were used: anti-rabbit Alexa Fluor 594, or anti-mouse Alexa Fluor 488 (Thermo Fisher Scientific, Rockford, IL, USA) diluted 1:2000.

3. Results

3.1. Cell total membrane fractionation and solubilization revealed MBOAT7 to be an integral polytopic membrane protein

To test whether MBOAT7 is a peripheral, integral or transmembrane protein, we performed total membrane fractionation followed by solubilization trials of the recombinant MBOAT7-V5 overexpressed in human HEK293T/17 cells. Total membrane fractions were purified and incubated with: 1 M NaCl; 0.2 M Na_2CO_3 pH 11.3; 2% digitonin; 2% Triton X-100; 2% CHAPS; or 2% SDS. MBOAT7-V5 was detected: A) only in the pellet (membrane fraction) of the samples incubated with 1 M NaCl, 0.2 M Na_2CO_3 pH 11.3, 2% digitonin, or 2% CHAPS; B) in both pellet and supernatant (solubilized protein) fractions, to a larger extent in the pellet (membrane fraction), after incubation with 2% Triton X-100; C) only in the supernatant fraction after incubation with 2% SDS (Fig. 1A). These results suggested that MBOAT7 is a transmembrane protein tightly attached to membranes.

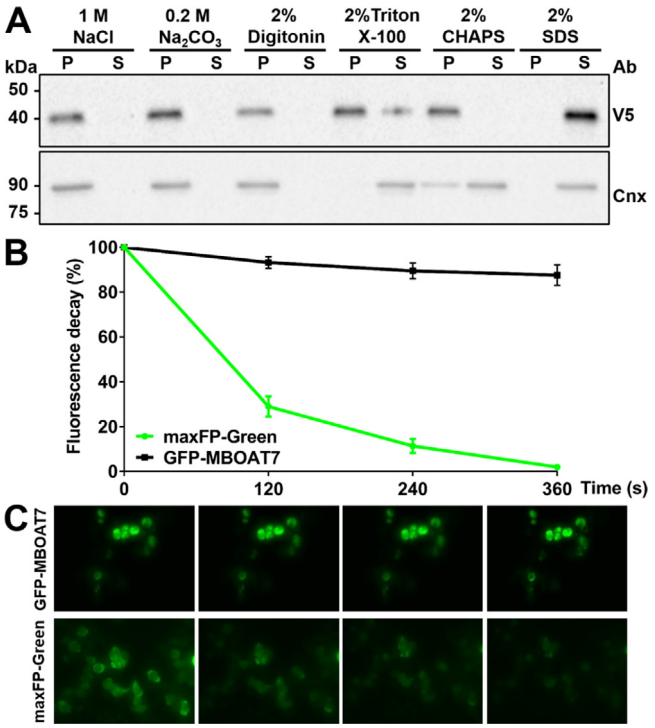

Fig. 1. MBOAT7 is an integral membrane protein tightly anchored to endomembranes. A) HEK293T/17 cells were transiently transfected with MBOAT7-V5 and total membrane fractions were purified as described in material and methods. Equal amount of purified membrane fractions was incubated with 1 M NaCl; 0.2 M Na_2CO_3 pH 11,3,; 2% digitonin; 2% Triton X-100; 2% CHAPS; or 2% SDS. Supernatant (S) and pellet (P) fractions were size-separated by 12% SDS-PAGE analysis and immunoblotted with anti-V5 antibody or anti-calnexin antibody. B) HEK293T/17 cells were transiently transfected with GFP-MBOAT7 and incubated with 50 µM of digitonin. There was no fluorescent decay for GFP-MBOAT7 signal suggesting GFP-MBOAT7 is an integral membrane protein. maxFP-Green protein was used as control for the plasma membrane permeabilization. Time is expressed in seconds. Error bars represent standard error mean (SEM) of N ≥ 4. C) Under each of the four time points a representative figure of intracellular fluorescence is shown. Abbreviations: NaCl, sodium chloride; Na_2CO_3, sodium carbonate; SDS, sodium dodecyl sulfate; Cnx, calnexin.

Calnexin, an endoplasmic reticulum transmembrane protein with one transmembrane (TM) domain (Ho et al., 1999), was used as a control and, as expected, it was completely solubilized only after incubation with 2% Triton X-100 or 2% SDS (Fig. 1A).

3.2. Fluorescence Protease Protection (FPP) assay revealed MBOAT7 to be an endomembrane-associated protein

To confirm our data, we overexpressed the full length GFP-MBOAT7 or, as positive control, the unbound cytosolic protein maxFP-Green, in HEK293T/17 cells. We measured GFP-fluorescence signal at time 0, 120, 240, and 360 s after incubation with digitonin (Fig. 1B, C). We observed more than 90% persistence of the GFP-MBOAT7 fluorescence

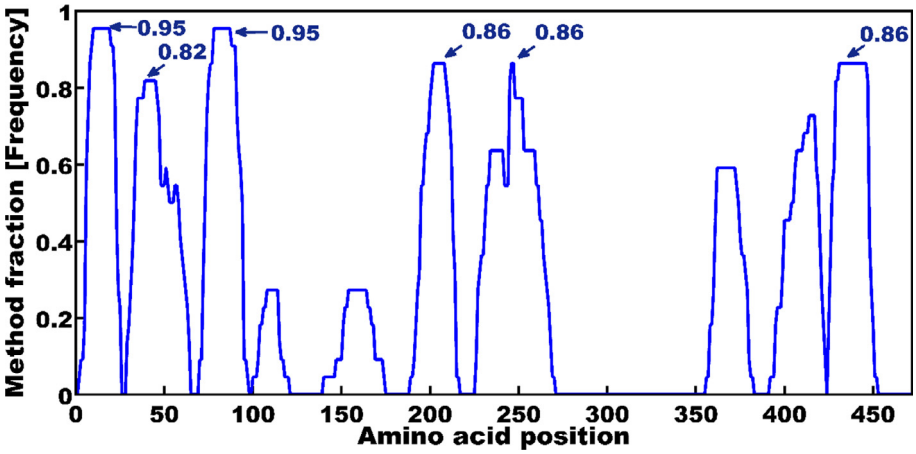

Fig. 2. Consistency of the software prediction. Method fraction denotes the frequency of software algorithms predicting an amino acid as part of a transmembrane segment.

up to 360 s. These data suggest that MBOAT7 is not freely diffused in the cytosol, but it is an endomembrane-associated protein. This result is consistent with the cell total membrane fractionation and solubilization data.

3.3. *In silico MBOAT7 analysis predicted 5 to 12 transmembrane (TM) domains*

To gain insight into human MBOAT7 topology, we performed *in silico* analysis by using 22 computational methods predicting the number and localization of its TM domains. The number of predicted TM domains ranged from 5 to 12 (Supplementary Fig. 5).

Overall prediction consistency for each amino acid was plotted as the fraction of algorithms predicting that amino acid within a TM domain (Fig. 2).

3.4. *GFP-tagged MBOAT7 forms localize to the endoplasmic reticulum*

To verify that the addition of the GFP moiety and the truncation of MBOAT7 did not alter the localization of the protein to the endoplasmic reticulum, we used a combination of: A) total cell membrane fractionation and solubilization followed by Western Blot analysis (Schlegel and Lisanti, 2000); and B) co-immunofluorescence microscopy (Hirata et al., 2013). Firstly, we performed total membrane fractionation of HEK293T/17 cells overexpressing GFP-tagged MBOAT7 forms. Secondly, we incubated the cell total membrane fractions with a high concentration of salt (1 M NaCl). After centrifugation, GFP-tagged MBOAT7 forms were detected only in the pellet (membrane fraction) of the samples (Fig. 3A). Calnexin, an integral membrane protein, was used as a control. These results suggest that all the GFP-tagged MBOAT7 forms are transmembrane proteins and the GFP moiety or the truncation of the protein do not affect the membrane localization of the recombinant proteins.

Furthermore, to show that all the GFP-tagged MBOAT7 forms localize to the endoplasmic reticulum, we performed co-immunoblotting coupled with fluorescence microscopy on HEK293T/17 cells overexpressing GFP-tagged MBOAT7 forms. We observed that all GFP-tagged MBOAT7 forms co-localized with calnexin, an endoplasmic reticulum membrane protein (Fig. 3B), indicating that the addition of the GFP moiety and the truncation of the proteins did not affect the endoplasmic reticulum localization of the MBOAT7 forms. Moreover, to further validate that GFP-tagged MBOAT7 forms are endoplasmic reticulum membrane proteins, we showed the different distribution patterns of A) calnexin, an ER membrane protein; B) DsRed2, a mitochondrial protein; C) Na,K-ATPase, a plasma membrane protein; or D) maxFP-Green, a protein free in the cytosol (Supplementary Fig. 6). Our analysis suggested that GFP-tagged MBOAT7 forms had a typical ER fluorescence pattern.

3.5. *MBOAT7 has the N- and C-terminal facing the cytosol*

To understand the folding of MBOAT7 within the membrane bilayer, we overexpressed the full length MBOAT7 forms with a GFP-tag either at the N- (GFP-MBOAT7) or at the C-terminal (MBOAT7-GFP) of the protein, in HEK293T/17 cells. After incubation with digitonin, cells were incubated with proteinase K, and GFP-fluorescence was measured at time 0, 120, 240, and 360 s (Fig. 4). We observed more than 95% GFP-fluorescence decay after 360 s in cells overexpressing GFP-MBOAT7 (Fig. 4A, B) or MBOAT7-GFP (Fig. 4C, D). These results suggest that both N- and C-terminal of MBOAT7 face the cytosol (Matevossian and Resh, 2015).

3.6. *MBOAT7 has six transmembrane domains*

To understand the number and localization of TM domains of the protein, we generated a total of eleven MBOAT7 truncated forms with a GFP-tag located at either N- (n = 5) or C- (n = 6) terminal of the protein (Supplementary Fig. 3D).

We overexpressed GFP-tagged MBOAT7 truncated forms in HEK293T/17 cells. After incubation with digitonin, cells were incubated with proteinase K, and GFP-fluorescence was measured at time 0, 120, 240, and 360 s (Fig. 5). We observed a complete GFP-fluorescence decay only in cells overexpressing GFP-MBOAT7-Δ65 or MBOAT7-Δ220-GFP, while we detected more than 65% mean preservation of the GFP-fluorescence in cells overexpressing other GFP-tagged MBOAT7 truncated forms, as compared to the DsRed2. This indicates that only the GFP-tags attached to the amino acid in position 65 or 220 have a cytosolic localization, whilst the GFP-tags attached to the N- or C-terminal of other GFP-tagged MBOAT7 truncated forms have a lumenal localization.

To examine the clusters obtained in the FPP assay experiments, we used hierarchical clustering by applying the Euclidean distance metric (Supplementary Fig. 7). We found two different clusters including: A) DsRed2 and all the proteins with the GFP-tag facing the lumen of organelles (GFP-MBOAT7-Δ29, GFP-MBOAT7-Δ100, GFP-MBOAT7-Δ135, GFP-MBOAT7-Δ180, MBOAT7-Δ275-GFP, MBOAT7-Δ310-GFP, MBOAT7-Δ350-GFP, MBOAT7-Δ385-GFP and MBOAT7-Δ423-GFP); B) Caveolin-1-GFP and all the proteins with the GFP-tag facing the cytosol (GFP-MBOAT7, GFP-MBOAT7-Δ65, MBOAT7-Δ220-GFP and MBOAT7-GFP).

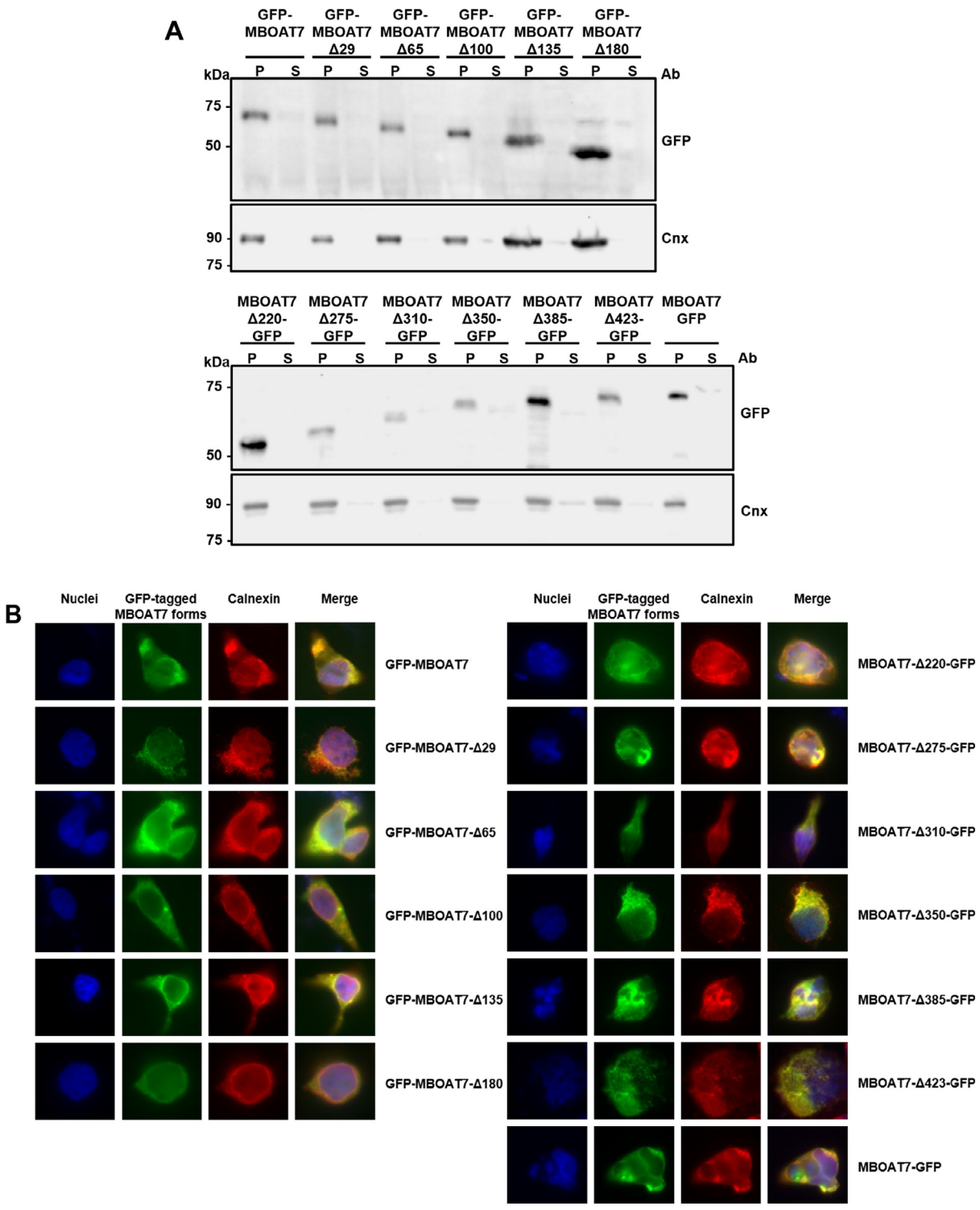

Fig. 3. GFP-tagged MBOAT7 forms are endoplasmic reticulum proteins. A) HEK293T/17 cells were transiently transfected with GFP-tagged MBOAT7 forms, and total membrane fractions were then purified as described in materials and methods. Purified membrane fractions were incubated with 1 M NaCl. Supernatant (S) and pellet (P) fractions were size-separated by 12% SDS-PAGE analyses and immunoblotted with anti-GFP antibody or anti-calnexin antibody. B) HEK293T/17 cells were transiently transfected with GFP-tagged MBOAT7 forms and subjected to immunoblotting and fluorescence microscopy as described in materials and methods. The rabbit anti-calnexin antibody recognized the endogenous calnexin used as ER marker (Red). Cell nuclei were stained with DAPI (Blue). Right panels show the merged images. Abbreviations: Cnx, calnexin.

3.7. FLAG-tagged MBOAT7 forms have a membrane localization

To verify that the addition of an internal FLAG-tag and of a C-terminal V5-tag did not affect membrane localization of the recombinant FLAG-tagged MBOAT7 forms, we performed total membrane fractionation and solubilization with 1 M NaCl coupled with protein analyses by Western Blot, showing that all the FLAG-tagged MBOAT7 forms are membrane proteins (Fig. 6A).

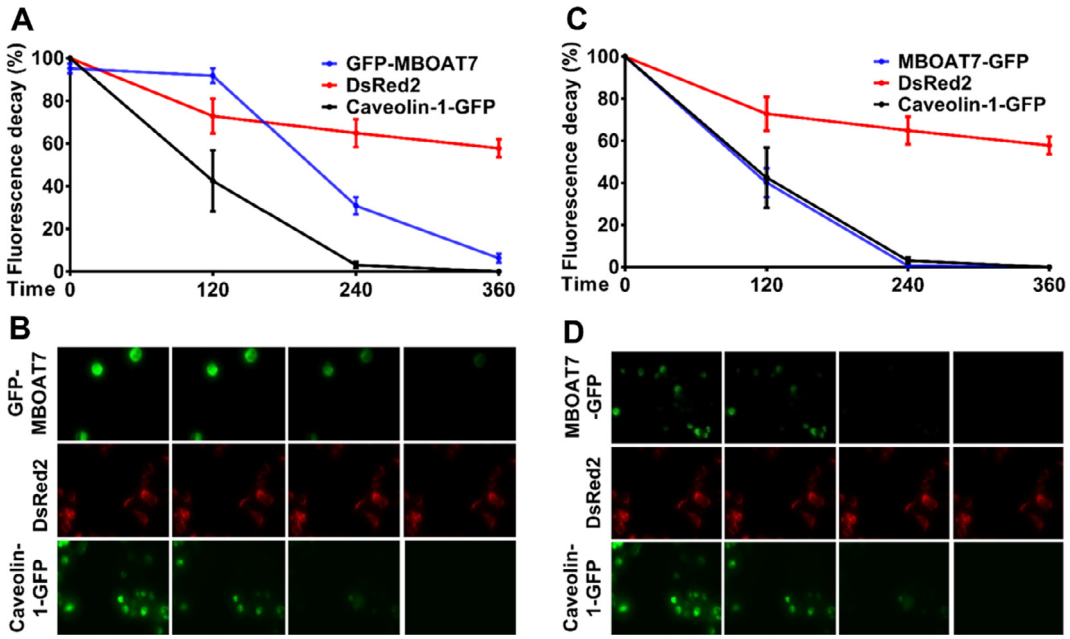

Fig. 4. The N- and C-terminal of MBOAT7 face the cytosolic side of the membrane. HEK293T/17 cells were transiently transfected with A-B) GFP-MBOAT7, or C-D) MBOAT7-GFP. After incubation with 50 μM of digitonin, cells were incubated with 50 μM of proteinase K. Fluorescence decay of the signal suggests that the GFP-tag attached to both GFP-tagged MBOAT7 full length forms have a cytosolic localization. Caveolin-1-GFP and DsRed2 were used as controls. Time is expressed in seconds. Under each of the four time points a representative figure of intracellular fluorescence is shown (B-D). Error bars represent standard error mean (SEM) of $N \geq 4$.

3.8. Differential permeabilization of cell membranes followed by immunofluorescence microscopy confirms FPP assay data

We confirmed collected data performing the FPP assay by differential permeabilization of cell membranes coupled with immunofluorescence microscopy of cells transfected with the recombinant FLAG-tagged MBOAT7 forms (Matevossian and Resh, 2015). The orientation of each FLAG-tag with respect to the endomembranes was tested by selectively permeabilizing cell compartments using digitonin, which permeabilized only the plasma membrane, or Triton X-100, which permeabilized all the cell endomembranes. Cells were co-immunoblotted for the exogenous FLAG-tag and for the endogenous PDI (protein disulfide isomerase), which was used as control for the efficiency of permeabilization of endomembranes (Matevossian and Resh, 2015). The incubation with digitonin was sufficient to detect the FLAG-tag attached to the amino acids in positions 65 and 220 of MBOAT7, confirming that both amino acids had a cytosolic localization. The incubation with Triton X-100 was necessary to detect the FLAG-tag attached to the amino acids in positions 135, 310, 350 and 423, confirming that these amino acids had a lumenal localization (Fig. 6B).

By selective membrane permeabilization coupled with indirect immunoblotting for the C-terminal V5-tag, we observed that the V5-tag attached to the C-terminal of each recombinant FLAG-tagged MBOAT7 form faces the cytosol. These data confirmed that the addition of the internal FLAG-tag did not affect the orientation of TM domains of the proteins (Supplementary Fig. 8).

Taken together, all these findings suggest that MBOAT7 has six TM domains (Fig. 7).

3.9. Algorithm prediction performance

To evaluate the performance of the *in silico* predictions, we calculated sensitivity and specificity for each of the 22 algorithms separately (Supplementary Table 1). The number of false positives (FP) and false negatives (FN) were calculated comparing each algorithm prediction with the final consensus model. We found that the average sensitivity and specificity was 78% and 89% respectively. The most sensitive algorithm was SVMTM with 88% sensitivity and 88% specificity, while the most specific algorithms were Phobius and PolyPhobius, both with 96% specificity and 85 or 77% sensitivity respectively.

4. Discussion

In this study, by combining *in silico* and *in vitro* analyses, we described for the first time the topology of MBOAT7 (Lysophospholipid acyltransferase 7 isoform 1, NCBI Reference Sequence: NP_077274.3). We observed that MBOAT7 is anchored to endomembranes by six transmembrane (TM) domains with its putative catalytic dyad facing the lumen of cellular organelles.

We started by examining the solubilization of the recombinant MBOAT7-V5 after total membrane fractionation and incubation with several compounds changing ionic charge, polarity and stability of the membrane and protein structure. The recombinant MBOAT7-V5 did not solubilize in the supernatant after incubation with high concentration of salt (1 M NaCl), high pH (0.2 M Na_2CO_3 pH 11.3), a steroidal saponin (2% digitonin) or a Zwitter-ionic detergent (2% CHAPS). In addition, the treatment with high concentration of a mild non-ionic detergent (2% Triton X-100) was not sufficient to completely separate MBOAT7-V5 from the membrane fraction, suggesting that MBOAT7 is not a peripheral membrane protein. Peripheral membrane proteins are easily dislodged from the membrane by using high concentration of salt, high pH or mild non-ionic detergents. To detach MBOAT7-V5 from the membrane, we incubated the membrane fraction with a harsh ionic detergent with denaturing effects (2% SDS) suggesting that MBOAT7 is strongly attached to endomembranes. To further confirm that MBOAT7 is attached to endomembranes, we incubated HEK293T/17 cells transiently transfected with the fluorescent GFP-MBOAT7 with digitonin for 360 s, and we observed a persistence of the fluorescent signal. These data are consistent with our findings from the cell total membrane fractionation and solubilization experiments. Taken together, we showed that MBOAT7 is an integral membrane protein tightly anchored to endomembranes.

Next, we predicted the number of TM domains by *in silico* analyses using 22 state-of-art transmembrane prediction algorithms. We found a

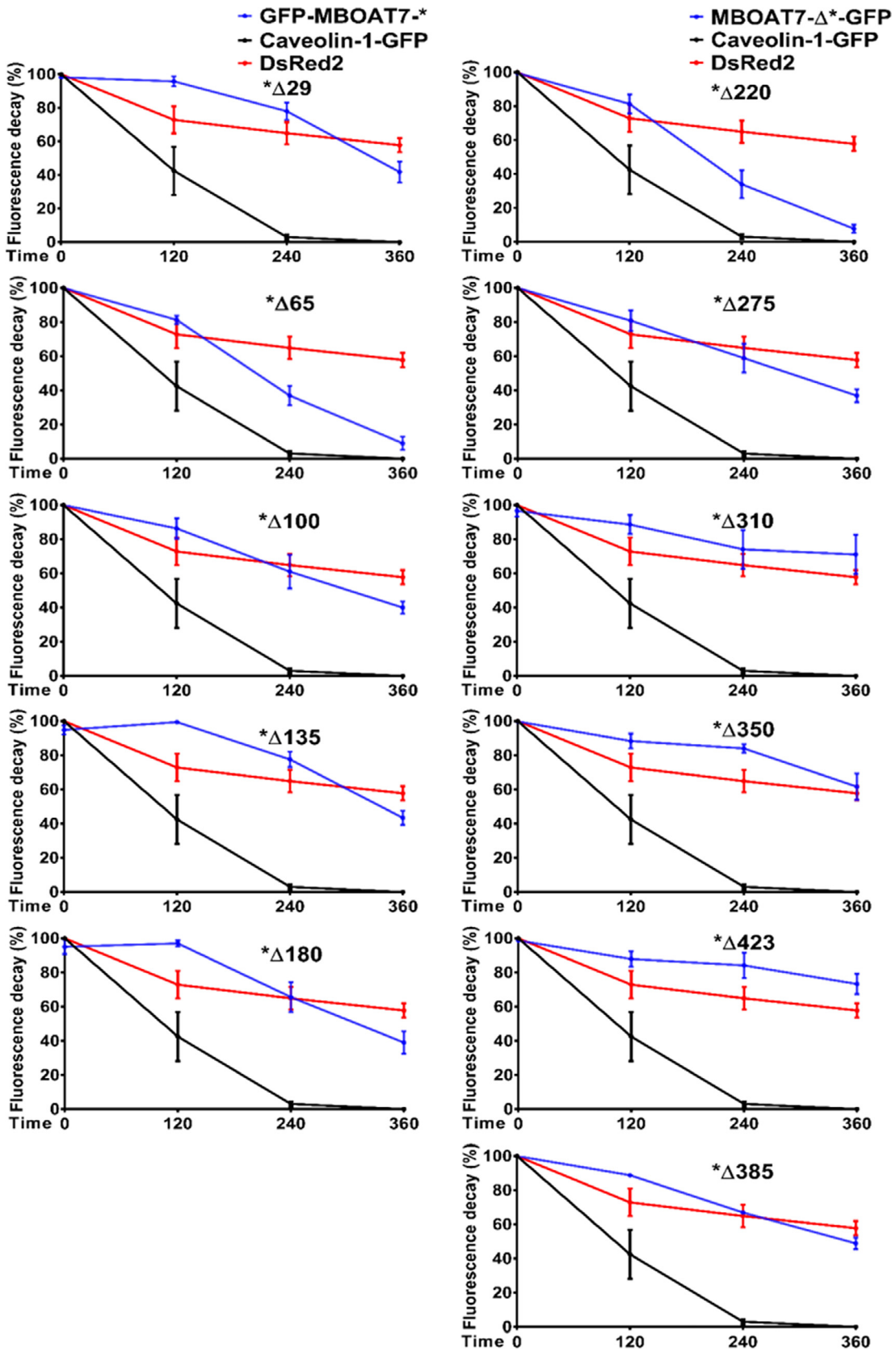

Fig. 5. The GFP-tag attached to the amino acid in position 65 or 220 face the cytosolic side of the membrane. HEK293T/17 cells were transiently transfected with GFP-tagged MBOAT7 truncated forms. After incubation with 50 μM of digitonin, cells were incubated with 50 μM of proteinase K. The graphs show the decay of the GFP fluorescence in cells transfected with GFP-tagged MBOAT7 truncated forms. Caveolin-1-GFP and DsRed2 were used as controls. Time is expressed in seconds. Error bars represent standard error mean (SEM) of N ≥ 4.

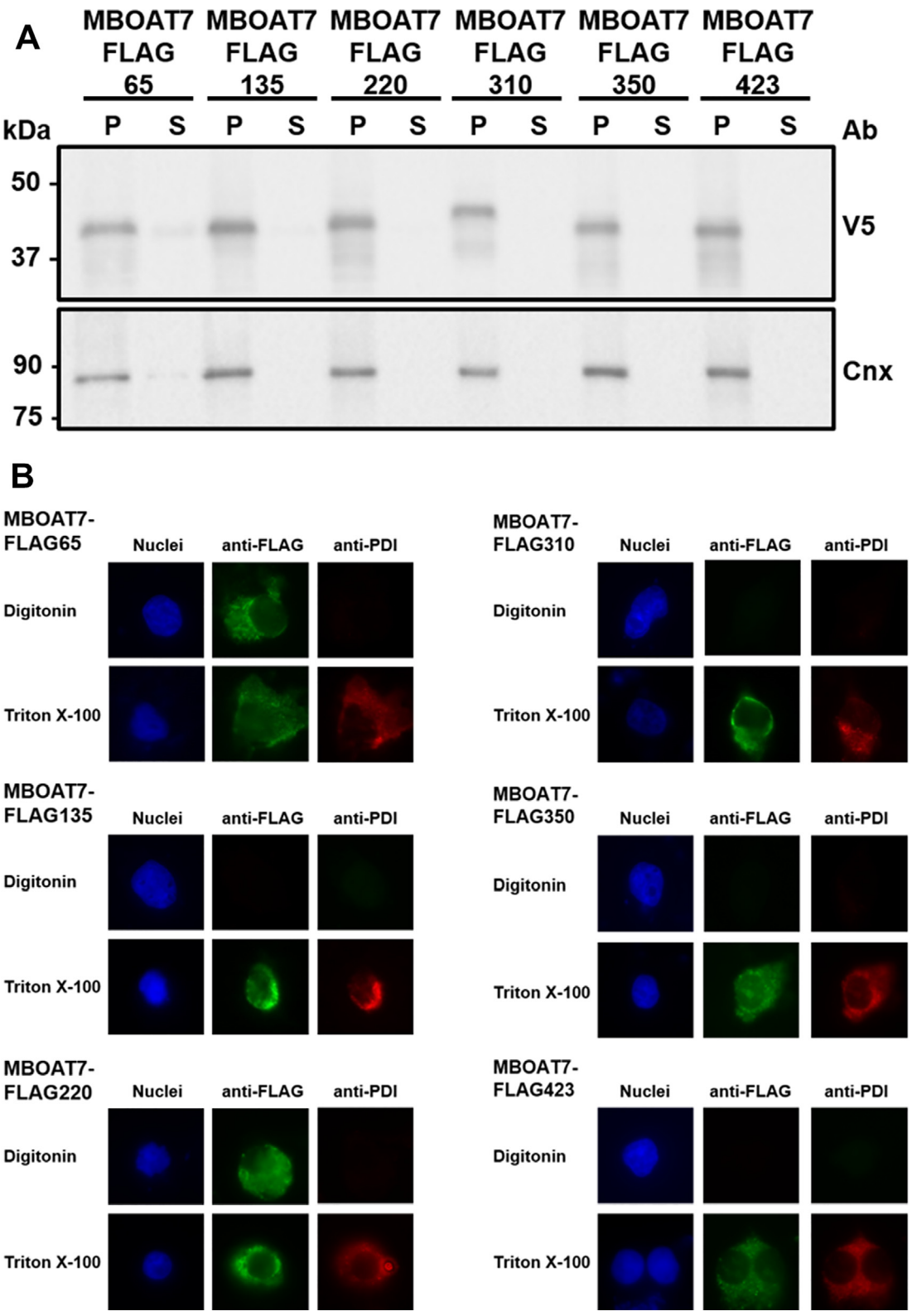

Fig. 6. MBOAT7 topology mapping using differential membrane permeabilization followed by indirect immunofluorescence. A) HEK293T/17 cells were transiently transfected with the recombinant FLAG-tagged MBOAT7 forms, total membrane fractions were purified and incubated with 1 M NaCl. Pellet (P) and supernatant (S) fractions were size-separated by 12% SDS PAGE analysis, and immunoblotted with anti-V5 antibody or anti-calnexin antibody. B) HEK293T/17 transiently transfected with FLAG-tagged MBOAT7 forms underwent differential membrane permeabilization followed by indirect immunofluorescence. The mouse anti-FLAG antibody recognized the exogenous FLAG-tagged MBOAT7 forms, the rabbit anti-PDI antibody recognized the endogenous PDI. Cell nuclei were stained with DAPI (blue). Abbreviations: Cnx, calnexin; PDI, protein disulfide-isomerase.

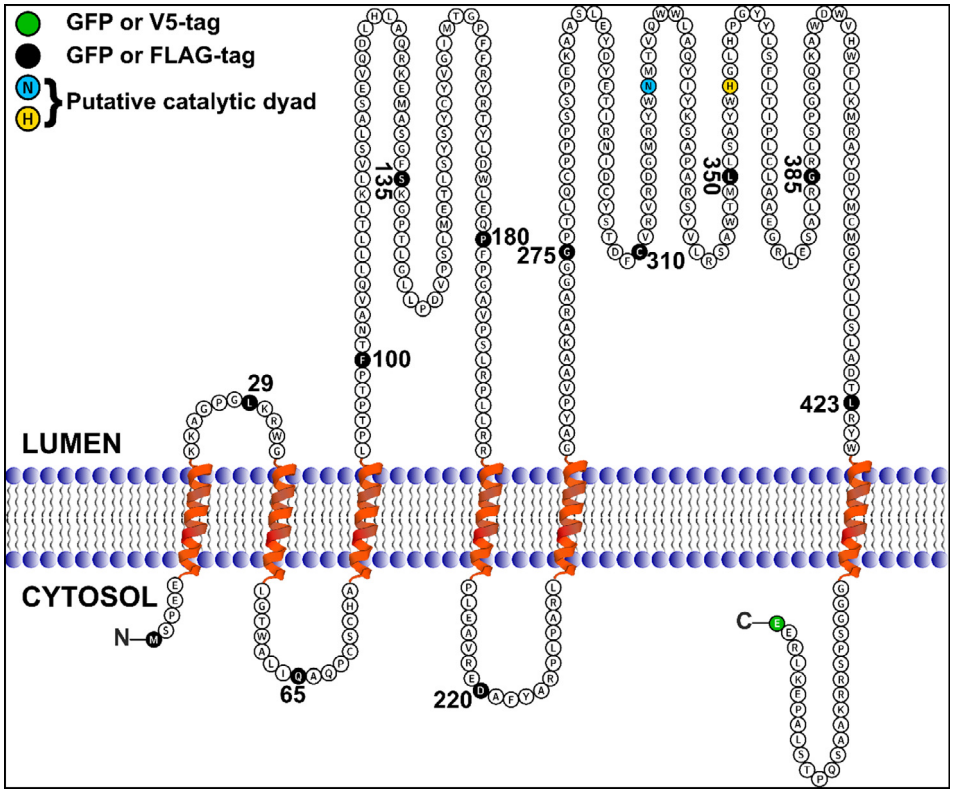

Fig. 7. MBOAT7 topology is a multispanning integral membrane protein with 6 transmembrane (TM) domains. The membrane is showed as a phospholipid-bilayer. TM domains are showed as red alpha-helices crossing the phospholipid-bilayer. Amino acids in black indicate the position of GFP or FLAG-tags installed in GFP or FLAG-tagged MBOAT7 forms. Amino acid in green indicates the position of GFP-tag or V5-tags installed in GFP or FLAG-tagged MBOAT7 forms. Based on the data obtained by *in silico* and *in vitro* analysis, MBOAT7 is a multispanning integral membrane protein with 6 TM domains, and the putative catalytic dyad is in the lumen.

large variability in the number of predicted TM domains, which ranged from 5 to 12. This high variability in TM domains number and position along the sequence can be attributed to approaches adopted by these methods that are mainly based on machine learning methods, such as Support Vector Machines (SVM), Neural Networks (NN), and Hidden Markov Models (HMM). The performance of these algorithms largely depends on the datasets of known protein structures, or sequence alignment methods used to train these algorithms (Venko et al., 2017). Moreover, it has been shown that the prediction performance has an inverse relationship with the number of TM domains, which again may partly explain the high variability among prediction methods (Reeb et al., 2015). However, we show a more accurate prediction by using all the software predictions simultaneously.

We synthesized and sublcloned two GFP-tagged full length MBOAT7 forms with the GFP-tag attached either to the N- or C-terminal of the protein. In addition, eleven GFP-tagged truncated MBOAT7 forms were synthesized, cleaving the MBOAT7 sequence every 35–55 amino acids, and adding the GFP-tag to the N- or C-terminal of the protein.

We showed, using a combination of cell total membrane fractionation and solubilization coupled with protein analyses by Western Blotting, that all GFP-tagged MBOAT7 forms are transmembrane proteins. Furthermore, we performed co-immunoblotting coupled with fluorescence microscopy (Rossi et al., 2016) showing that the addition of the GFP moiety and the truncation of the protein did not affect the endoplasmic reticulum localization of the recombinant proteins always co-localizing with the ER marker (calnexin).

We therefore took advantage of experimental Fluorescence Protease Protection (FPP) assay to assess the number and localization of TM domains of MBOAT7. The FPP assay is based on the proteinase K activity after plasma membrane permeabilization conferred by digitonin. In principle, a rapid decay of the fluorescence signal in our experiments indicates that the GFP-tag faces the cytosol, and conversely the persistence of the signal indicates a lumenal localization of the GFP-tag.

First, to understand where the extremities of MBOAT7 are located with respect to the phospholipid-bilayer, we performed the FPP assay in cells transfected with the GFP-tagged full length MBOAT7 forms. We observed a rapid decay of the fluorescence in cells transfected with each of the full length recombinant protein, indicating that both N- and C-terminal of the protein face the cytosol and that MBOAT7 has an even number of TM domains. The faster decay of the fluorescence detected in cell transfected with MBOAT7-GFP can be due to an easier access for the proteinase K to the GFP-tag at the C-terminal where there is a longer cytosolic segment of the protein as compared to the N-terminal.

We next performed the FPP assay in cells transfected with GFP-tagged truncated MBOAT7 forms. We observed a decay of the fluorescence only in cells transfected with the GFP-MBOAT7-Δ65 or MBOAT7-Δ220-GFP, suggesting that the GFP moiety attached to amino acid in position 65 and 220 face the cytosol. On the contrary, we observed a persistence of the fluorescence in cells transfected with all the other GFP-tagged MBOAT7 truncated forms, indicating that those GFP-tags face the lumenal side of the endomembranes.

To further confirm the data collected from the FPP assay, we employed another method based on the selective permeabilization of cell membranes coupled with immunofluorescence microscopy on cells transfected with FLAG-tagged MBOAT7 proteins (Matevossian and Resh, 2015). The differential permeabilization of cell membranes using digitonin or Triton X-100 followed by immunofluorescence microscopy (Fig. 6B) further confirmed the data obtained from FPP assay. All these data suggest that MBOAT7 has six TM domains (Fig. 7).

MBOAT family members have a cytosolic conserved asparagine and a preserved histidine which may represent a putative catalytic active site (Bosson et al., 2006; Matevossian and Resh, 2015; Taylor et al., 2013). Based on our findings, this putative active site, composed in MBOAT7 by Asn-321 and His-356 (Lee et al., 2008), faces the lumen (Pagac et al., 2011). The vast majority of algorithms predicted two TM domains extending approximately within residues 350 and 423 (Supplementary Table 3). In contrast, we showed that amino acid residues 350, 385 and 423 are lumenal. The only model compatible with these results is the presence of two reentrant loops within these amino acids. In this scenario, the His-356 would be located adjacent to the membrane, while the Asn-321 would stay in the lumen. The close proximity to the membrane would favor the access to the long chain

polyunsaturated fatty acid substrates for the phospholipid acyl chain remodeling (van Meer et al., 2008). Moreover, this model would be in agreement with other MBOAT superfamily protein members of known topology (Matevossian and Resh, 2015; Taylor et al., 2013).

Furthermore, the similarity with other members of the same protein family and the *in silico* analysis, which is based on machine learning methods and neuronal networks, support the *in vitro* data.

In conclusion, we propose that MBOAT7 topology consists of a multispanning integral membrane protein with six TMs domains and two possible reentrant loops. Based on this model, the putative catalytic domain of the protein faces the lumenal compartment.

Conflict of interest

Stefano Romeo has been consulting for AstraZeneca, Celgene, GSK and Pfizer for human and molecular genetics of NAFLD in the last 5 years, although not in relation to this specific project.

Author contributions

AC, PP and SR conceived and designed the experiments. AC performed the *in vitro* experiments. OJ performed the *in silico* experiments. AC, OJ, PP and RMM analyzed data and drafted the article. All authors analyzed the results and approved the final version of the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jsb.2019.04.006.

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