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MGARP is ultrastructurally located in the inner faces of mitochondrial membranes☆

Shuping Zhang

School of Life Sciences, Tsinghua University, Beijing, 100084, China

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ABSTRACT

Mitochondria, the centers of energy production, are highly organized with inner membranes, cristae and outer membranes. The mitochondrial architecture determines their functions in all cellular processes. Changes in the mitochondrial ultrastructure are tightly related to a wide variety of diseases. MGARP, a mitochondria-localized protein, was predicted by bioinformatics and confirmed by cellular and biochemical methods to be located in mitochondria, but there is no direct and clear evidence for its precise location. This report demonstrates the precise ultrastructural location of MGARP within mitochondria by the ascorbate peroxidase 2 (APEX2) system in combination with electron microscopy (EM). EM revealed that more MGARP is located in the inner/cristae membranes, with its C-terminus at the inner faces of the intramembrane spaces, than in the outer membranes. MGARP overexpression caused both mitochondrial remodeling and cristae shaping, leading to the collapse of the mitochondrial network. The mitochondrial morphologies in MGARP-overexpressing cells were diverse; the cells became round or short, and their cristae were deformed and became discontinuous or circular. An engineered MGARP mutant deficient in its transmembrane domain no longer localized to the mitochondria and lost its effects on mitochondrial structure, confirming that the localization of MGARP in the mitochondria depends on its structural integrity. Collectively, our findings define the location of MGARP within the mitochondria, which is associated with its functional implications for the architecture and organization of mitochondria.

1. Introduction

Mitochondria play important roles in various physiological processes, including energy production, metabolism and autophagy [1]. Mitochondrial dynamics and quality control are tightly associated with sickness and health. Defects in mitochondria caused by mutations in mitochondrial and/or nuclear DNA lead to diverse and complex human diseases [2]. Within cells, mitochondria form a dynamic, interconnected network that is intimately integrated with other cellular compartments [3]. A mitochondrion consists of an outer membrane, an intermembrane space, an inner membrane, cristae and a matrix. Different regions of mitochondria carry out specialized functions. At the outer membrane, proteins, nucleotides, ions, fatty acids and metabolites can be transported between the cytosol and the intermembrane space [4,5]. At the inner mitochondrial membrane (IMM)/cristae, energy conversion and ATP production occur, and proteins are shuttled into the matrix [6–8]. Mitochondrial cristae shape determines respiratory chain supercomplex assembly and respiratory efficiency [9]. Mitochondrial morphology and inner structure critically depend on mitochondria-shaping proteins [10].

Many proteins are located in the mitochondrial membranes. These mitochondrial proteins, many of which have important functions, usually contain a mitochondrial targeting signal [7,11,12]. Among mitochondria-localized proteins, the majority are encoded by nuclear DNA [13]. MGARP, a mitochondria-localized, glutamic acid-rich protein, is also encoded by a nuclear chromosome and was first discovered from a large-scale screen of the ovary [14]. MGARP was later demonstrated to be highly expressed in steroid tissues (ovary, testes and adrenal gland) and visual system [15–17]. MGARP is involved in steroidogenesis and negatively mediates neocortical development by regulating mitochondrial distribution and motility in neocortical neurons [15,18]. Its expression regulated by the hypothalamic–pituitary–gonadal axis (also called the HPG

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E-mail address: bczhang@tsinghua.edu.cn.

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axis) and fluctuates with the estrogen level [17]. MGARP upregulation in U17 snoRNA-deficient cells promoted the formation of ER-mitochondrial contacts to regulate cholesterol flux to mitochondria [19].

However, there is no direct and clear evidence for the precise location of MGARP. A previous analysis of MGARP topology predicted that the C-terminus of MGARP is located inside the membrane, suggesting that the transmembrane domain (TMD) is in the outer mitochondrial membrane (OMM) with the C-terminus in the intermembrane space (IMS) [20]. Experiments performed by Li et al. demonstrated that osmotic shock alone and without trypsin did not alter MGARP protein abundance [21]. Combined treatment with osmotic shock and trypsin degraded MGARP, which strongly suggests that the C-terminus of MGARP is in the IMS, but MGARP was not released from the IMS following treatment with osmotic shock [21]. The precise localization of MGARP within mitochondria will help to clarify and understand these observations.

In this study, we established an enhanced ascorbate peroxidase 2 (APEX2) system [22] and combined this system with electron microscopy (EM) to monitor the precise location of MGARP within mitochondria. We found that MGARP proteins were predominantly located in the inner/cristae membranes, with less MGARP in the outer membranes, and that the C-terminus of MGARP was located in the inner faces of intermembrane spaces. In addition, MGARP overexpression (OE) caused mitochondria remodeling and cristae shaping. The transmembrane domain and the rest of the *N*-terminal regions of MGARP were essential for MGARP localization and function. Together, our studies show for the first time the precise location of MGARP and identify MGARP as a cristae-shaping protein.

2. Materials and methods

2.1. Antibodies and reagents

Restriction and modifying enzymes were obtained from Takara Biotechnology (Dalian, China). *Anti*-myc and *anti*-GAPDH were purchased from ABclonal (Woburn, MA). *Anti*-V5 was purchased from HuaXingBio (Beijing, China). The fluorescent secondary antibodies TRITC-conjugated goat anti-rabbit and FITC-conjugated goat anti-mouse IgG and anti-mouse IgG were purchased from ZSGB-BIO (Beijing, China). The MGARP antibody was generated as previously described [17]. Hoechst 33342 was purchased from Sigma (St. Louis, MO). MitoTracker was purchased from Invitrogen (Waltham, MA).

2.2. Plasmids, cell culture, transfection and stable cell line selection

The pcDNA3.1(−)-V5-APEX2-NES vector was obtained from Prof. Li Yu (School of Life Sciences, Tsinghua University, China) and Prof. Peng Zou (School of Life Sciences, Peking University, China). To generate MGARP + APEX2 fusion plasmids, cDNA for the MGARP coding region and MGARP containing a transmembrane deletion (Del-MGARP) was ligated to the pcDNA3.1(−)-V5-APEX2-NES (APEX2) vector by *Eco*RI and *Xho*1 to form the pcDNA3.1(−)-V5-APEX2-NES (MGARP + APEX2) and pcDNA3.1(−)-V5-APEX2-NES (Del-MGARP + APEX2) plasmids, respectively.

Cell culture was carried out as reported [23]. HeLa cells were grown in DMEM supplemented with 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin at 37 °C. A total of 2×10^5/ml HeLa cells were cultured for 12 h, and the above plasmids were transfected into the HeLa cells. Next, 800 μg/ml G418 was added to the cell culture medium to select and maintain stable cell lines. HeLa cells were authenticated by Beijing Microread Gene Technology Co., Ltd. using short tandem repeat profiling. No cross-contamination from other human cell lines was found, and the most similar cell line in the ATCC was the HeLa cell line.

2.3. Western blotting analysis

Western blotting was carried out as previously reported [23]. The cells were lysed, and the proteins in the sample were separated by SDS-PAGE with a 5% stacking gel and a 12% separating gel. The proteins were transferred onto a nitrocellulose membrane using a wet transfer system for 2 h at 100 V. The transferred membrane was blocked with 5% milk (5 g of skimmed milk powder dissolved in 100 ml TBST) and incubated with *anti*-V5 or *anti*-MGARP. The transferred membrane was blotted with the corresponding secondary antibodies conjugated to HRP for 2 h at room temperature, followed by washing with TBST (1000 ml TBS with 1 ml Tween-20) and development using a DAB Horseradish Peroxidase Color Development Kit (Amresco, OH).

2.4. Validation of the APEX2 system

The APEX2 signal was detected as described [22,24]. HeLa cells stably expressing APEX2, MGARP + APEX2 or Del-MGARP + APEX2 were cultured for 48 h before staining. The cells were stained with 3,3′-diaminobenzidine (DAB) and hydrogen peroxide (H_2O_2) for 10 min on ice. The positive cells were stained brown in a white light field. A fresh solution of DAB containing 1 × DAB (0.5 mg/ml) and 10 mM H_2O_2 in cold (0–4 °C) 1 × sodium cacodylate was prepared for development.

2.5. Electron microscopy (EM)

Transmission electron microscopy to detect the APEX2 signal was performed as described [22]. HeLa cells stably expressing APEX2, MGARP + APEX2 or Del-MGARP + APEX2 were cultured in 35 mm Petri dishes for 3 days, and the same volume of 2.5% glutaraldehyde in 100 mM sodium cacodylate buffer (pH 7.4) containing 2 mM $CaCl_2$ warmed to 37 °C was added for 5 min at room temperature, followed by replacement with fresh 2.5% glutaraldehyde for 5 min. The cells were placed on ice and incubated for 60 min, after which the cells were rinsed five times for 2 min each in ~1.5–2 ml of cold (0–4 °C) 1 × sodium cacodylate. The following steps were similar to those described [22]. For staining, the cells were incubated in a freshly diluted solution of 0.5 mg/ml (1.4 mM) DAB tetrahydrochloride (Sigma, D5637) combined with 0.03% H_2O_2 (Sigma, 88597) in cooled buffer for 5 min. The DAB solution was then removed, and the cells were washed and postfixed with 2% osmium tetroxide for 5 min, dehydrated, embedded and sliced into ultrathin sections. The ultrathin sections were stained with uranyl acetate and lead citrate. DAB-stained areas of the embedded cells were examined by transmitted light, and the areas of interest were cut out using a razor blade and mounted on resin blocks with cyanoacrylic adhesive. Ultrathin sections (70 nm) were cut, and images were captured by TEM (Hitachi, H7650) at the Tsinghua University Branch of the China National Center for Protein Sciences (Beijing).

2.6. Immunofluorescence staining

Immunofluorescence staining was carried out with a modified previously described method [25]. Cells grown on glass cover slips in 6-well plates were washed twice with ice-cold PBS, fixed with 4% paraformaldehyde in PBS for 20 min at room temperature, and permeabilized with 0.2% Triton X-100 in PBS for 10 min at room temperature. The cells were blocked with 1% BSA in PBS for 1 h at 37 °C. The membrane was incubated with *anti*-MGARP or *anti*-V5

primary antibodies diluted in 0.2% BSA in PBS for 1 h at 37 °C, followed by incubation with FITC-, TRITC-, or AMCA-conjugated goat anti-rabbit or anti-mouse IgG. The nuclei were counterstained with or without Hoechst 33342. Cover slips were mounted with a glycerol-based anti-fade mounting medium and analyzed with a Zeiss confocal laser scanning microscope (LSM710) at the Center of Biomedical Analysis, Tsinghua University.

2.7. Statistics

All data were analyzed with the Excel and GraphPad programs and are expressed as the mean ± standard error of at least three independent experiments. Significant differences were determined with Student's *t*-test. Significance is indicated when $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

3. Results and discussion

Validation of the activity and localization of the APEX2 and MGARP + APEX2 fusion constructs by western blotting, immunostaining and light microscopy.

MGARP has a single transmembrane domain [14–16,21], but there is no direct and clear evidence of its precise location within mitochondria. Thus, we established an enhanced ascorbate peroxidase 2 (APEX2) system in HeLa cells that can emit a robust signal through APEX2 activity that can be readily captured by electron microscopy (EM) [22,24]. This system was verified by western blotting, DAB staining, cellular immunostaining and light microscopy, as detailed below. First, western blotting verified that the construction and expression of this system were successful (Fig. 1A). Furthermore, DAB staining showed that APEX2 alone had no background staining signal, while the MGARP + APEX2 fusion protein was markedly expressed, as shown by the brown staining of the cytoplasm in a punctuate pattern (Fig. 1B). Significantly, laser scanning confocal microscopy also showed that APEX2 alone had no fluorescent signal, while the MGARP + APEX2 fusion protein was clearly observed in the cytoplasm and overlapped with the staining pattern of MitoTracker, a fluorescent marker for mitochondria (Fig. 1C). MGARP overexpression caused significant mitochondrial remodeling, as demonstrated by the pronounced morphological changes in the overall shape of the mitochondria, which was in agreement with previous reports [15,16,18,21].

EM reveals that MGARP is located in the inner faces of the mitochondrial membranes.

The cellular localization of a protein is generally associated with its functions. A recent report indicated that the C-terminus of MGARP may protrude into the mitochondrial intermembrane space and that much of the C-terminus of MGARP does not seem to interact with Miros, a group of proteins anchored in the outer mitochondrial membrane [21]. MGARP is considered to be an outer mitochondrial membrane adaptor protein that modulates ER-mitochondria contacts [19]. Despite various reports on the structure and functions of MGARP, its exact location within the mitochondria remains undetermined. Using advanced electron

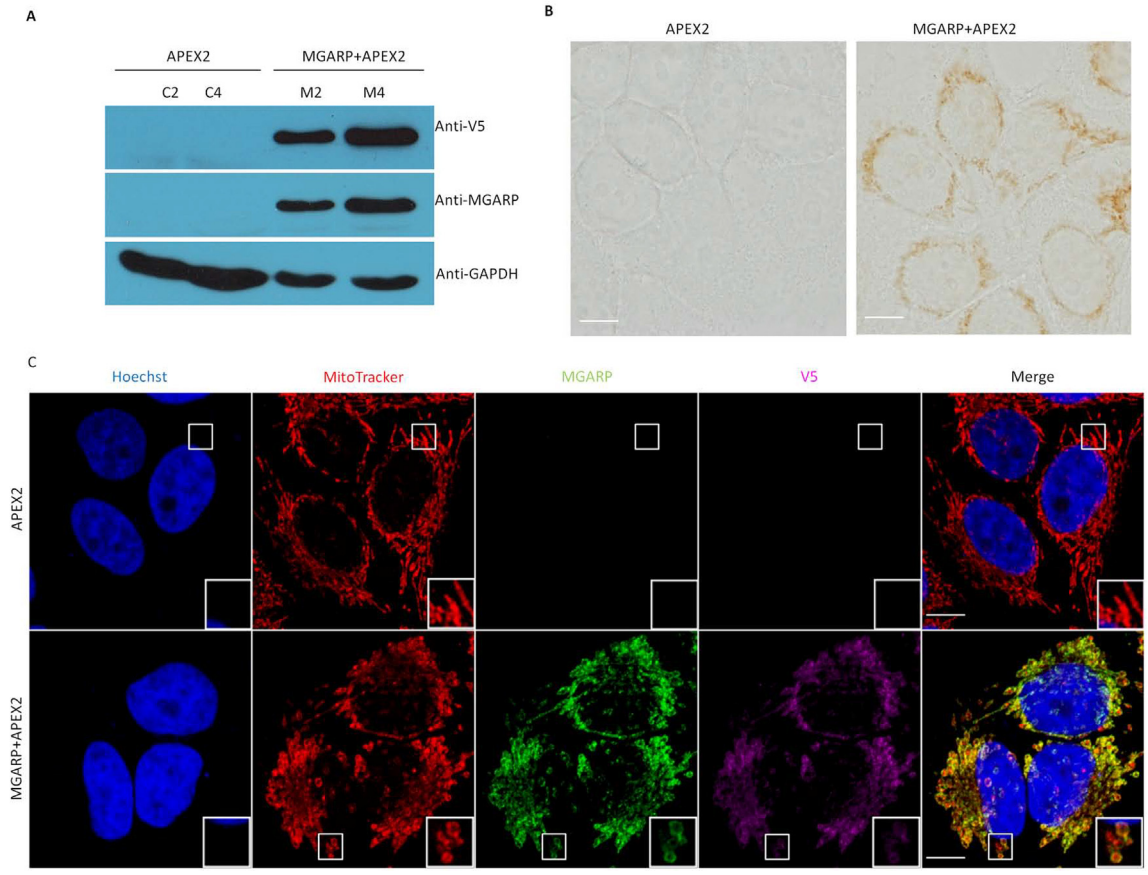

Fig. 1. Validation of the activity and localization of APEX2+MGARP fusion constructs. HeLa cells were transfected with pcDNA3.1(−)-APEX2 or pcDNA3.1(−)-MGARP + APEX2 and then screened with G418 (800 μg/ml) for 3–4 weeks to obtain stable cell lines. The brown color indicates APEX activity. MGARP expression in stable cell lines was detected by western blotting (A), immunostaining (B), and immunofluorescence (C). (B) APEX activity was achieved by DAB staining and observed by bright field imaging. Scale bars, 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

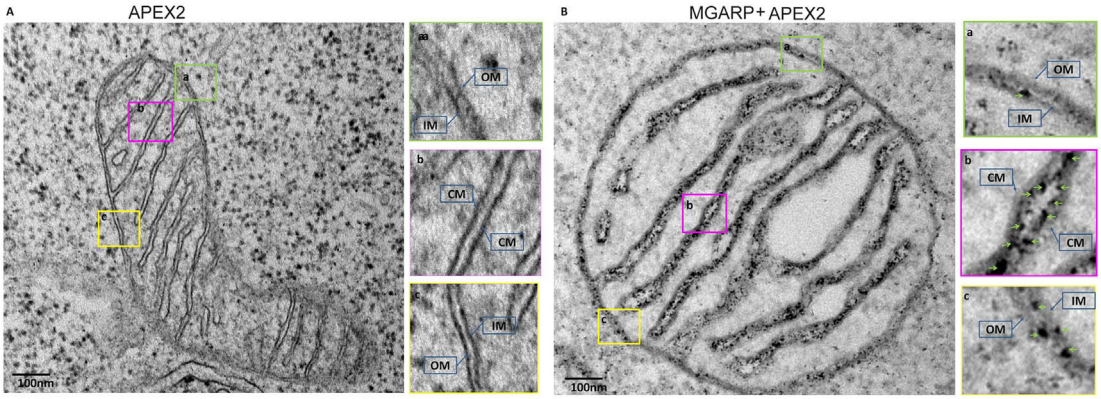

Fig. 2. EM images showing that MGARP is located in the inner faces of the mitochondrial membranes. The mitochondria were from HeLa cells that overexpressed APEX2 (A) or APEX2+MGARP (B). All images were obtained with EM. The insets show areas at higher magnifications. Positive dots of APEX2 staining are indicated with green arrows. OM: outer membrane; IM: inner membrane; CM: cristae membrane. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

microscopy (EM) in our core facility, we initiated an investigation to define the location of MGARP in mitochondria. As shown in the EM images, there were many dotted signals for MGARP + APEX2 distributed in the inner faces of the inner/cristae and outer membranes, with especially intense signals in the cristae membranes (Fig. 2), suggesting that MGARP functions in the inner/cristae membranes, the outer membranes and the IMS. This is the first ultrastructural localization of MGARP in the inner faces of the mitochondrial membranes, particularly in the cristae membranes, and this location supports the notion that the transmembrane domain (TMD) is in the inner mitochondrial membrane (IMM) with the C-terminus of MGARP in the IMS [21]. The cristae membranes are enriched in proteins involved in iron-sulfur biogenesis, protein translocation and synthesis, and mitochondrial nucleoid maintenance and house assembled respiratory complexes and supercomplexes [9,26–28]. It has been documented that a reduction in MGARP levels results in a decrease in the cellular content of mitochondrial DNA [15]. Thus, these findings further extend the knowledge that MGARP is mainly located in the cristae, implying that the cristae membranes are the major platforms for MGARP functioning.

MGARP overexpression causes mitochondrial remodeling both the outside and inside of mitochondria.

The cristae membranes have remarkable structural variability and diversity [29]. From the EM images, we also observed overall ultrastructural changes in the mitochondrial morphology and cristae shape in MGARP-overexpressing cells (Fig. 3). Quantification analysis showed that the mitochondria in MGARP-overexpressing cells became rounder and shorter than those in control cells (Fig. 3A–C). The mitochondrial size, length, aspect ratio, and cristae number were significantly reduced, while the intermembrane space (IMS) was significantly enlarged (Fig. 3C). Moreover, some

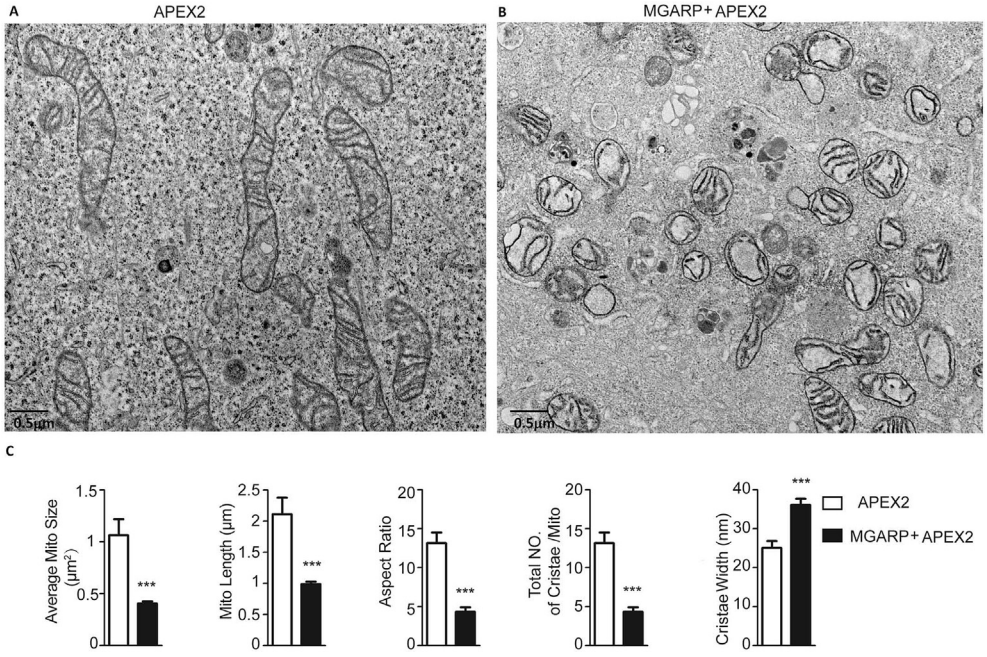

Fig. 3. The mitochondrial morphologies in MGARP-overexpressing cells were diverse, and the cristae were deformed. MGARP overexpression caused mitochondrial remodeling in both the outside and inside of the mitochondria. Mitochondrial ultrastructures in HeLa cells overexpressing APEX2 (A) or APEX2+MGARP (B). (C) Quantification of mitochondrial properties in different cell lines. Mito: mitochondria/mitochondrion. Data are shown as the mean ± SEM. ***P < 0.001.

cristae were deformed and became circular or discontinuous (Fig. 3B). These findings indicate that MGARP localization has profound impacts on mitochondrial remodeling and cristae shaping.

Mitochondrial cristae, as specialized compartments, function as dynamic biochemical reactors that limit the diffusion of molecules important for the oxidative phosphorylation (OXPHOS) system [8,9,30]. The shapes of the cristae change under different physiological conditions, and their membrane morphologies modulate the organization and function of the OXPHOS system and have a direct impact on cellular metabolism [8]. Cristae shaping not only affects mitochondrial function and determines the assembly and stability of supercomplexes [9], but also involves mitophagy, which is exemplified by Prohibitin 2, an inner mitochondrial membrane protein that functions as a mitophagy receptor [31]. Cristae shaping by widening cristae junctions and inverting cristae curvature occurs during apoptosis to allow the complete release of cytochrome *c* [32]. The width of the IMS was reported to respond to low ADP concentrations [33]. Thus, our data showing that high levels of MGARP cause cristae deformation and IMS enlargement suggest that MGARP functions in ATP production through OXPHOS, the maintenance of mitochondrial architecture, mitophagy, apoptosis and other respiration-related processes.

A MGARP mutant deficient in the transmembrane domain (Del-MGARP) no longer localizes to the mitochondria and lost the effects on mitochondrial architecture.

Since the APEX2 sequence in the vector has no start codon (ATG), control HeLa cells bearing APEX2 alone cannot change color after DAB staining (Fig. 1B). To further confirm that dots in the mitochondrial membranes were specifically produced by the MGARP + APEX2 fusion protein, we generated a new APEX2 fusion vector with a truncated MGARP mutant with the transmembrane domain and the rest of the *N*-terminal regions of MGARP deleted (Del-MGARP + APEX2). Its expression was verified by western blotting, DAB staining, cellular immunostaining and light microscopy (Fig. 4A and B). Fluorescence analysis showed that Del-MGARP + APEX2 no longer colocalized with MitoTracker (Fig. 4), in contrast to the above findings that the wild-type MGARP + APEX2 fusion protein exhibited an overlapping staining pattern with MitoTracker. Furthermore, EM imaging showed that there were no dots specific to Del-MGARP-APEX2 within the mitochondria (Fig. 4), which was also different from the observations of wild-type MGARP-APEX2. As mentioned above, much of the C-terminus of MGARP seems not to be involved in its interaction with a group of proteins anchored in the outer mitochondrial membrane [21]. Taken together, these results confirmed that the localization of MGARP in the mitochondria depends on its structural integrity and that the transmembrane and the *N*-terminal regions of MGARP are essential for its localization and function.

In summary, this report defined the precise localization of MGARP within mitochondria and identified MGARP as a cristae-shaping factor. These findings provide novel insights into the

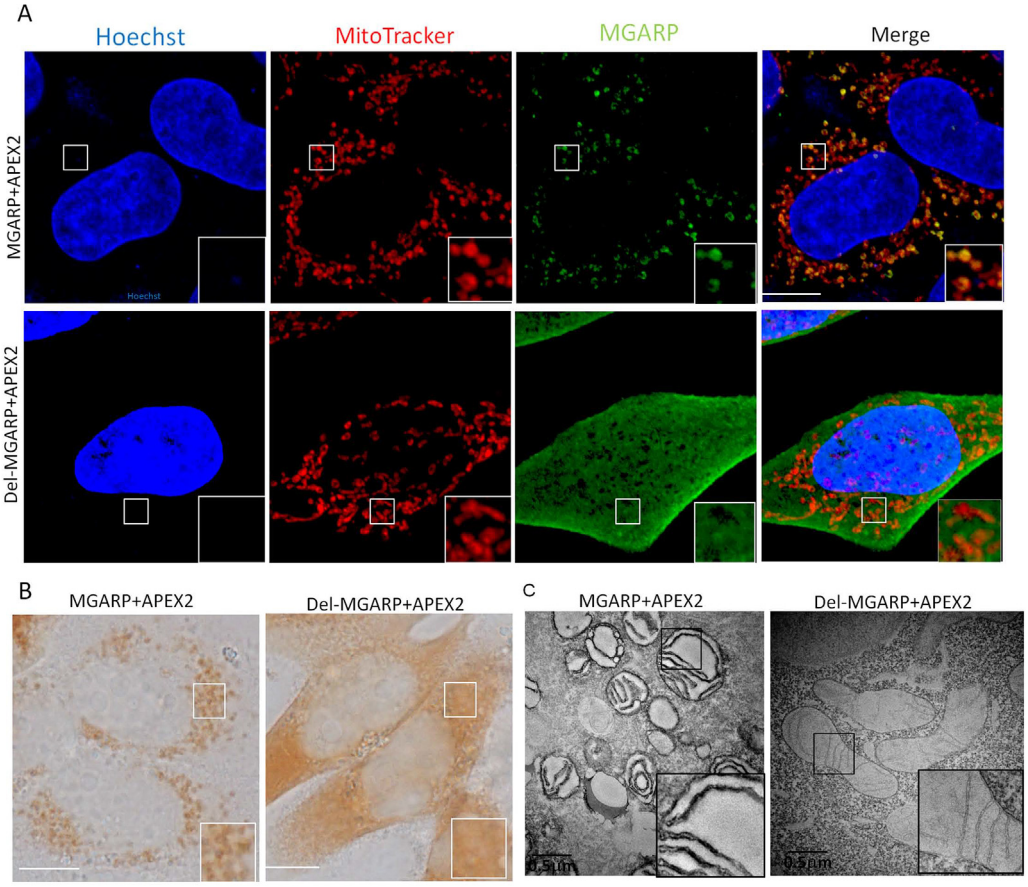

Fig. 4. The MGARP mutant (Del-MGARP) deficient in the transmembrane domain and *N*-terminus no longer localized to the mitochondria and had no effect on mitochondrial structure. HeLa cells were transfected with pcDNA3.1(−)-MGARP + APEX2 or pcDNA3.1(−)-Del-MGARP + APEX2 and then screened with G418 (800 μg/ml) for 3–4 weeks to obtain stable cell lines. (A) MGARP expression in stable cell lines was detected by immunofluorescence. (B) The APEX signal was achieved by DAB staining and observed by bright field imaging. The brown color indicates APEX activity. (C) EM images showing that Del-MGARP no longer localized to the mitochondria and lost its effects on mitochondrial structure. Scale bars, 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

physiological function of MGARP and its functional platforms. This study also provides a good guide for our ongoing and future work.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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