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# Role of $NH_2$-terminal hydrophobic motif in the subcellular localization of ATP-binding cassette protein subfamily D: Common features in eukaryotic organisms

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ABSTRACT

In mammals, four ATP-binding cassette (ABC) proteins belonging to subfamily D have been identified. ABCD1–3 possesses the $NH_2$-terminal hydrophobic region and are targeted to peroxisomes, while ABCD4 lacking the region is targeted to the endoplasmic reticulum (ER). Based on hydropathy plot analysis, we found that several eukaryotes have ABCD protein homologs lacking the $NH_2$-terminal hydrophobic segment (H0 motif). To investigate whether the role of the $NH_2$-terminal H0 motif in subcellular localization is conserved across species, we expressed ABCD proteins from several species (metazoan, plant and fungi) in fusion with GFP in CHO cells and examined their subcellular localization. ABCD proteins possessing the $NH_2$-terminal H0 motif were localized to peroxisomes, while ABCD proteins lacking this region lost this capacity. In addition, the deletion of the $NH_2$-terminal H0 motif of ABCD protein resulted in their localization to the ER. These results suggest that the role of the $NH_2$-terminal H0 motif in organelle targeting is widely conserved in living organisms.

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## 1. Introduction

ATP-binding cassette (ABC) proteins comprise a superfamily of membrane-bound proteins found in almost all organisms [1]. The structure of ABC proteins is highly conserved, and they catalyze the ATP-dependent transmembrane transport of a wide variety of substrates [2]. ABC proteins in eukaryotic cells are localized to the plasma membrane as well as organelles such as the endoplasmic reticulum (ER) and peroxisomes. A number of the ABC proteins consist of a two transmembrane domain (TMD), which forms a tunnel, and a nucleotide-binding domain (NDB) for hydrolyzing ATP. On the other hand, the organelle associated ABC transporters in the peroxisomes or ER contain just one TMD and one NBD. They are half-sized and are thought to require another unit to form the homo or heterodimer required for functionality. In humans, 48 ABC proteins have been identified and are classified into seven subfamilies, A–G, based on their evolutionary similarity [2].

To date, four ABC proteins have been included in subfamily D (ABCD proteins) in mammals: adrenoleukodystrophy protein (ALDP/ABCD1), ALDP-related protein (ALDRP/ABCD2), the 70-kDa peroxisomal membrane protein (PMP70/ABCD3) and the PMP70-related protein (P70R/ABCD4) [3]. ABCD1–3 are known as peroxisomal proteins, and are suggested to be involved in the transport of long and very long chain fatty acids (VLCFA) or their CoA-derivatives into peroxisomes [4,5]. Notably, the dysfunction of ABCD1 is the cause of the human genetic disorder X-linked adrenoleukodystrophy (X-ALD). ABCD4 was identified in homology search for ABCD1 and ABCD3 related sequences in a database of expressed sequence tags (ESTs). We have shown that ABCD4 is localized in ER, not in peroxisomes [6]. Very recently, it was reported that ABCD4 is localized in lysosomes and involved in the export of cobalamin from lysosomes in human fibroblasts. The dysfunction of ABCD4 is the cause of vitamin $B_{12}$ deficiency type cblJ [7]. ABCD4 is thought to be co-translated on and inserted into the ER membrane, and a portion of ABCD4 is transported to lysosomes.

During translation, the peroxisomal ABCD proteins, ABCD1–3 form a complex with Pex19p, a peroxisomal biogenesis factor,

*Abbreviations:* ABC, ATP-binding cassette; ER, endoplasmic reticulum; EGFP, enhanced green fluorescent protein; H0 motif, hydrophobic segment; NBD, nucleotide binding domain; TMD, transmembrane domain.

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through the interaction of the $NH_2$-terminal hydrophobic segment (H0 motif) in the ABCD proteins in the cytosol. Then the complex binds to Pex3p on the peroxisomal membranes, and the ABCD proteins are inserted [3,8]. In contrast, ABCD4 hardly interacts with Pex19p because of its lack of the $NH_2$-terminal H0 motif, and as a result, transmembrane domain 1 (TMD1) of ABCD4 is recognized by certain signal recognition particles (SRP) and integrated into the ER membrane [6,9]. Interestingly, the $NH_2$-terminal region of ABCD3 itself is targeted to the mitochondrial outer membrane, and then the $NH_2$-terminal H0 motif, together with TMD1 and 2, brings about the targeting to the peroxisomes [9]. We suggest that the $NH_2$-terminal H0 motif is essential for suppressing ER-targeting function and allows the ABCD proteins to localize to the peroxisomes by cooperating with TMD1–2.

The ABCD proteins are present in almost all of the eukaryotic taxa and are comprised from one to five members in each. Interestingly, several species possesses 2 types of ABCD proteins that differ in terms of having or not having an $NH_2$-terminal H0 motif, as seen in mammals. In this study, in order to determine whether "the N-terminal hydrophobic rule" is conserved across eukaryotic organisms, we transiently expressed *Caenorhabditis elegans*, *Arabidopsis thaliana* or *Mucor circinelloides* ABCD proteins in fusion with GFP in CHO cells and investigated their subcellular localization using immunofluorescence microscopy.

## 2. Materials and methods

### 2.1. Materials

*M. circinelloides* was obtained from The National Institute of Agrobiological Sciences (NIAS) (Tsukuba, Japan). The plasmids pRAFL07-92-H13 and pRAFL09-20-L02 that contain the *A. thaliana* cDNA sequence encoding AtABCD1 and AtABCD2, respectively were obtained from RIKEN BioResource Center (Tsukuba, Japan). cDNAs encoding the *C. elegans* ABCD proteins (pmp-1–5) were obtained from the National Institute of Genetics (Mishima, Japan). pEGFPN-1 was purchased from Clontech Laboratories (Mountain View, CA). The KOD-plus mutagenesis kit was from TOYOBO (Osaka, Japan). Rabbit anti-catalase antibody, mouse anti-KDEL antibody and rabbit lysosomal-associated membrane protein 1 (LAMP1) antibody were purchased from Rockland (Gilbertsville, PA), Calbiochem (Darmstadt, Germany) and Sigma (St. Louis, MO), respectively. Cy3™-labeled goat anti-rabbit and anti-mouse IgG were from GE healthcare (Little Chalfont, UK). Mitotracker Red CMXRos was purchased from Life Technologies Corporation (Carlsbad, CA).

### 2.2. Plasmid constructions

All primers used in this study were listed in Supplementary Table 1. To construct the *C. elegans* pmp-1–5-GFP, cDNAs of pmp-1–5 were amplified using pME18S-FL/pmp-1, pmp-2, pmp-4, pmp-5 and pBlueScript/pmp-3 as the respective templates. PCR was performed using two sets of oligonucleotide primers having a *Kpn*I site and a *Bam*HI site. The amplified products were cloned into the same site of the pEGFPN-1 vector to yield pEGFP/pmp-1–5. To construct the $NH_2$-terminal truncated mutants of pmp-1-GFP, pmp-2-GFP and pmp-4-GFP, which had the hydrophobic motif (H0 motif) before TMD1 deleted, inverse PCR was performed using pEGFP/pmp-1, -2, and -4 as templates and the cDNAs were designated as pEGFP/ΔH0-pmp-1, -2 and -4, respectively. The sets of the oligonucleotide primers were designed on the basis of their sequences (pmp-1, -2 and -4 without H0 as shown in Supplementary Table 1). All of the inverse PCRs were performed using the KOD-plus mutagenesis kit. In addition, to construct pmp-2(AA.1–64)-pmp-5(AA.1–598)-GFP, in which the $NH_2$-terminal 64 amino acids of pmp-2 were inserted just upstream of pmp-5-GFP, an overlapped extension PCR was performed. The cDNAs of the $NH_2$-terminal region of pmp-2 and pmp-5 were generated by PCR using oligonucleotide primer sets Fw-pmp-5(+H0–1)/Rw-pmp-5(+H0–1) and Fw-pmp-5(+H0–2)/Rv-pmp-5-Bam, and pEGFP/pmp-2 and pEGFP/pmp-5 as templates, respectively. The cDNA fragments with each overlap sequence were fused by PCR using a oligonucleotide primer set Fw-pmp-5(+H0–1)/Rv-pmp-5-Bam. The amplified fragments of pmp-2(AA.1–64)-pmp-5(AA.1–598) were cloned into the same site of the pEGFPN-1 vector.

Full-length cDNAs of *Arabidopsis ABCD*, RAFL07-92-H13 and RAFL09-20-L02, were provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan [10,11]. The GFP-tagged *A. thaliana* ABCD1 (AtABCD1) expressing vector was constructed as follow. The 4.0-kb fragment was amplified using a oligonucleotide primer set Fw-AtABCD1-Kpn/Rv-AtABCD1-Bam and RAFL07-92-H13 as the template. Then the amplified products were digested with *Kpn*I and *Bam*HI, and introduced into the same site of pEGFP-N1 to yield pEGFP/AtABCD1. Next, N-half AtABCD1 expressing vector was constructed as follow. The 6.7-kb fragment was amplified by inverse PCR using a oligonucleotide primer set Fw-inv-AtABCD1-N/Rv-inv-AtABCD1-N and pEGFP/AtABCD1 as the template, and this fragment was self-ligated using T4 Polynucleotide Kinase (Toyobo) and Ligation high (Toyobo) to form pEGFP/AtABCD1(N-half). The AtABCD2 expressing vector pEGFP/AtABCD2 was constructed from RAFL09-20-L02 according to the same procedure described above.

cDNA of *M. circinelloides* was synthesized from the mycelial total RNA extracted using Direct-zol RNA MiniPrep Kit (Zymo Reseatch, CA). *M. circinelloides ABCD* encoding regions were amplified using oligonucleotide primer sets listed in Supplementary Table 1 and the cDNA as the template. The amplified products were inserted into pEGFP-N1 to prepare pEGFP/MC28553, pEGFP/MC72946 and pEGFP/MC91590 as described above.

### 2.3. Cell culture and transient transfection

Cell culture of CHO-K1 using a Lab-Tek™ Chamber Slide™ System (Nalge Nunc, Rochester, NY) and transfections using Effectene Transfection Reagent (Qiagen, Valencia, CA) were performed as described in [6]. At 16 h post-transfection, the medium was replaced with new medium and incubated for a further period of 24 h or 48 h. After incubation, the cells were fixed for immunofluorescence analysis. In certain cases with the expression of truncated mutant ABCD proteins, MG132 (final concentration 20 μM) was added to the culture medium for 12 h before fixation to inhibit the degradation of the genetically modified ABCD proteins.

### 2.4. Immunofluorescence

Immunofluorescence analysis was performed as described in [12]. The primary antibodies used in this study were rabbit antibodies against catalase and LAMP1, and a mouse antibody against KDEL. Mitochondria were labeled with the mitochondria-specific dye Mitotracker Red CMXRos before fixation [6]. The cells were mounted in Slow Fade Gold (Invitrogen) to allow examination using TCS SP5 confocal microscopy (Leica, Wetzlar, Germany).

## 3. Results and discussion

### 3.1. Hydropathy plot analysis of ABCD proteins in eukaryotes

We have previously reported that the subcellular targeting of human ABCD proteins depend on the $NH_2$-terminal H0 motif that is recognized by Pex19p [13]. Mammalian ABCD1–3 possess the

$NH_2$-terminal H0 motif that results in the targeting to peroxisomes. In *Saccharomyces cerevisiae*, 2 ABCD protein homologs (Pxa1p and Pxa2p) are known to be functional in peroxisomes, both of them have the H0 motif [14]. It is thus important to determine whether the role of the $NH_2$-terminal H0 motif in organelle targeting is conserved across eukaryotic organisms. In the GTOP database (http://spock.genes.nig.ac.jp), 193 organisms have ABCD proteins with homology to the mammalian, yeast or plant ABCD proteins in eukaryotes. We performed Kyte–Doolittle hydropathy plot analysis on the known ABCD proteins and found that certain organisms, including *C. elegans*, *Helobdella robusta*, *A. thaliana*, *Schizophyllum commune* and *M. circinelloides* have an ABCD protein that lacks the typical $NH_2$-terminal H0 motif, as in the case of ABCD4 in *Homo sapiens* (Fig. 1). From the results of the hydropathy plot analysis of 143 organisms, at least 60 organisms appeared to have ABCD proteins lacking the typical $NH_2$-terminal H0 motif (Table 1).

In the metazoa, 66 organisms have ABCD protein homologs and most of the metazoa have 3 or more ABCD protein homologs. Among the 57 organisms we analyzed, 49 have ABCD proteins lacking the $NH_2$-terminal H0 motif. In particular, almost all mammals have ABCD4 protein homologs lacking the $NH_2$-terminal H0 motif. However, the arthropoda, including *Drosophila melanogaster*, *Anopheles gambiae*, *Aedes aegypti* and *Apis mellifera*, appear to have only 2 ABCD protein homologs that have the $NH_2$-terminal H0 motif. In plants including Viridiplantae, Haptophyceae and Stramenopiles, there are 25 organisms that have ABCD protein homologs. *A. thaliana* is well known to have a full-sized peroxisomal ABCD protein, AtABCD1 (also known as CTS/Ped3/Pxa1), and a half-sized ABCD protein, AtABCD2 [15,16]. Among the 19 organisms we analyzed, 5 were found to have AtABCD1 protein homologs lacking the $NH_2$-terminal H0 motif.

In fungi, 94 organisms have ABCD protein homologs and most of them have only 2 ABCD protein homologs, except for *Spizellomyces punctatus* (3), *M. circinelloides* (4), *Phycomyces blakesleeanus* (4), *Capsaspora owczaraki* (3) and *Rhizopus oryzae* (5). Among the 60 fungi we analyzed, *S. commune*, *Tremella mesenterica*, *S. punctatus* and *M. circinelloides* have ABCD protein homologs without the

**Table 1**
Number of organisms that have mammalian ABCD protein homologs.

| Kingdom | Organism No. | Analyzed | Lack of H0 motif |
|---|---|---|---|
| Metazoa | 66 | 57 | 49 |
| Plant | 25 | 19 | 5[b] |
| Fungi | 94 | 60 | 4 |
| Protozoa | 8 | 7 | 2 |
| Total | 193[a] | 143 | 60 |

[a] In the GTOP database, 193 organisms with mammalian ABCD protein homologs are listed. Among the 143 organisms on which we performed hydropathy plot analysis, 60 organisms were found to possess ABCD protein homologs lacking the $NH_2$-terminal hydrophobic segment (H0 motif).

[b] In plants (including Viridiplantae, Haptophyceae and Stramenopiles), 15 organisms have ABCD protein homologs with a similar hydropathy plot profile to AtABCD1. Among them, 5 organisms have ABCD proteins that lack the H0 motif. In contrast, 14 organisms have ABCD proteins with homology to AtABCD2 (some with both types of ABCD proteins). AtABCD2 possesses a different type of $NH_2$-terminal sequence (see Fig. 1) and its subcellular distribution pattern has not yet been determined.

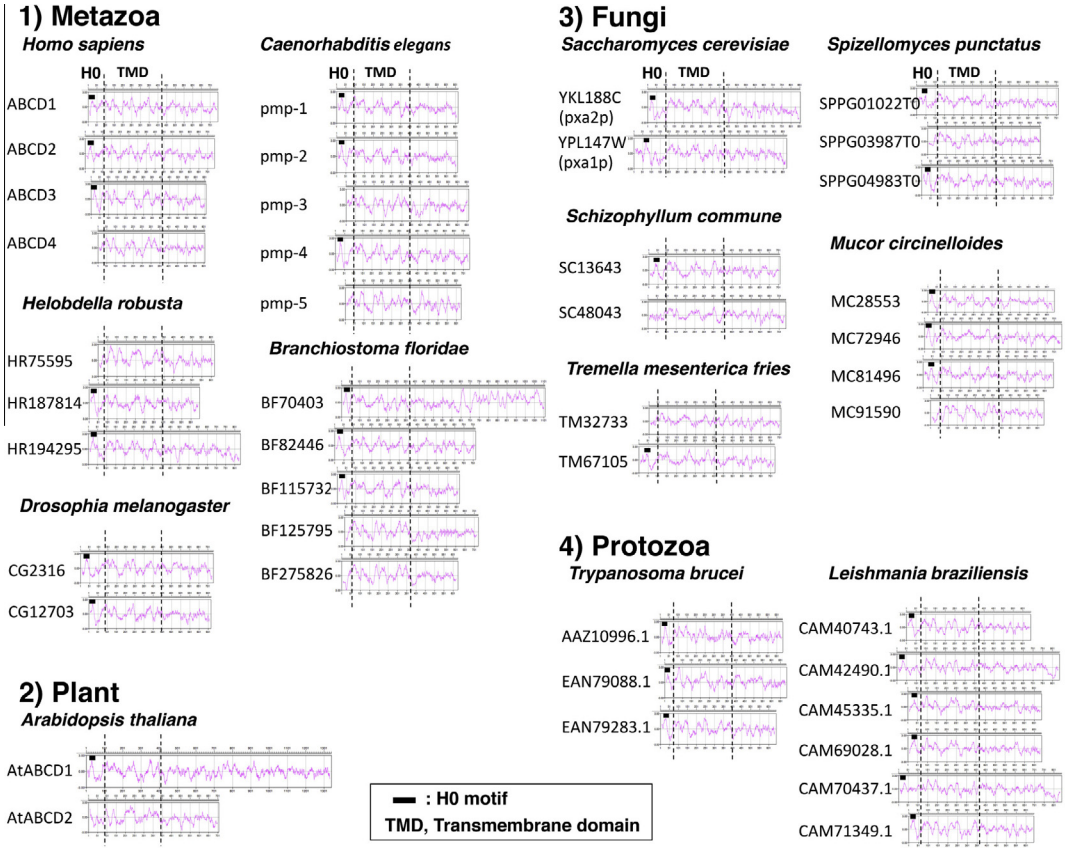


**Fig. 1.** Hydropathy plots of the ABCD proteins. ABCD protein homologs were identified with the GTOP database using human ABCD1, Pxa1p and AtABCD1 as the input sequences. The hydropathy plot of the ABCD proteins was calculated according to the method of Kite and Doolittle (with the window set at 15 residues). The data indicates the representative organisms in metazoan, plants, fungi and protozoa that have ABCD protein homologs. The solid lines on the plots show the typical $NH_2$-terminal hydrophobic segment (H0 motif).

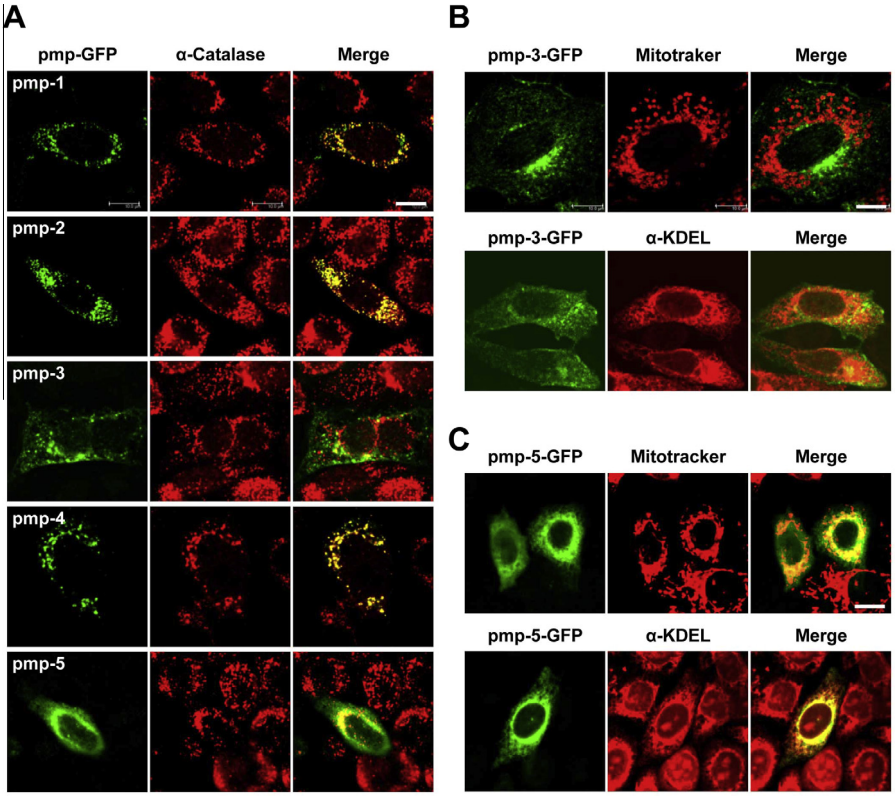


**Fig. 2.** Subcellular localization of *C. elegans* ABCD proteins (pmp-1–5) transiently expressed in CHO cells. CHO cells were transfected with pEGFP/pmp-1–5 and analyzed for the subcellular localization of the protein by immunofluorescence. The distribution of pmp-GFPs was compared with that of catalase, a peroxisomal marker enzyme, detected by immunofluorostaining with rabbit anti-catalase antibody followed with Cy3-conjugated goat anti-rabbit IgG (A). The GFP fluorescence is shown on the left, peroxisomal staining pattern in the center, and a superimposition of both stains on the right. The subcellular distribution of pmp-3-GFP (B) or pmp-5-GFP (C) was compared with those of ER and mitochondria. The distribution of ER is shown by the immunofluorostaining using anti-KDEL antibody. Mitochondria were labeled by the organelle specific dyes, Mitotracker. The ER or mitochondrial staining pattern is shown in the center. Scale bar, 10 μm.

typical $NH_2$-terminal H0 motif. In the protozoa (Alveolata, Amoebozoa, Choanoflagelia and Euglenozoa), *Trypanosoma brucei* and *Leishmania braziliensis* have more than 3 ABCD protein homologs and all of these ABCD protein homologs we analyzed have an $NH_2$-terminal H0 motif.

During these analyses, we found that *C. elegans*, a nematoda, has 5 ABCD protein homologs (pmp-1–5), and among them, pmp-1, pmp-2 and pmp-4 possess the $NH_2$-terminal H0 motif, while pmp-3 and pmp-5 do not. *M. circinelloides*, a fungus, also has 4 ABCD protein homologs in which one of them dose not have the H0 motif. To unravel the importance of the $NH_2$-terminal H0 motif, we chose *C. elegans*, *A. thaliana* and *M. circinelloides* ABCD proteins as the model in the following experiments.

### 3.2. Subcellular localization of *C. elegans*, *A. thaliana* and *M. circinelloides* ABCD proteins expressed in CHO cells

Concerning the subcellular localization of the peroxisomal proteins, both the targeting signals and translocation machinery are well conserved across species and a heterologous expression system for the peroxisomal proteins, including the ABCD proteins, is readily available. For example, when human ABCD proteins were expressed in tobacco epidermal cells, ABCD1–3 were correctly targeted to the peroxisomes and ABCD4 was targeted to ER [17]. In the present study, using CHO cells as the host, we transiently expressed *C. elegans*, *A. thaliana* and *M. circinelloides* ABCD proteins and examined the subcellular localization by immunofluorescence microscopy. These organisms have several ABCD protein homologs with or without $NH_2$-terminal H0 motif (Fig. 1).

*C. elegans* has 5 ABCD protein homologs (pmp-1–5), and 2 of them (pmp-3 and 5) lack the H0 motif. First, we transiently expressed pmp-1–5 in fusion with GFP (pmp-1–5-GFP) in CHO cells and examined the subcellular localization by immunofluorescence microscopy. As shown in Fig. 2A, pmp-1-GFP, pmp-2-GFP and pmp-4-GFP exhibited a punctate staining pattern that was superimposable on the distribution of peroxisomes stained by catalase. On the other hand, under the same conditions, the distribution of pmp-3-GFP and pmp-5-GFP did not overlap with that of catalase in the same cells (Fig. 2A). These data suggest that pmp-3 and pmp-5 are present in organelles other than peroxisomes. Next, we compared the subcellular localization of pmp-3-GFP and pmp-5-GFP with those of ER and mitochondria (Fig. 2B and C). The distribution of pmp-5-GFP was completely superimposable on that of ER-resident proteins possessing the COOH-terminal KDEL sequence, but not on that of mitochondria stained by Mitotracker. On the other hand, the distribution of pmp-3-GFP was not superimposable on ER, mitochondria (Fig. 2B) or lysosomes (data not shown).

In *A. thaliana*, AtABCD1, a full-size ABC protein, has the $NH_2$-terminal H0 motif and reside in peroxisome in plant [18]. When AtABCD1-GFP was expressed in CHO cells, the expression was weak and peroxisomal localization was not observed. It is conceivable that mammalian cells do not have a capacity to take full-size ABC protein to peroxisomes. Therefore, we constructed an expression vector harboring the $NH_2$-terminal half of the *AtABCD1* cDNA

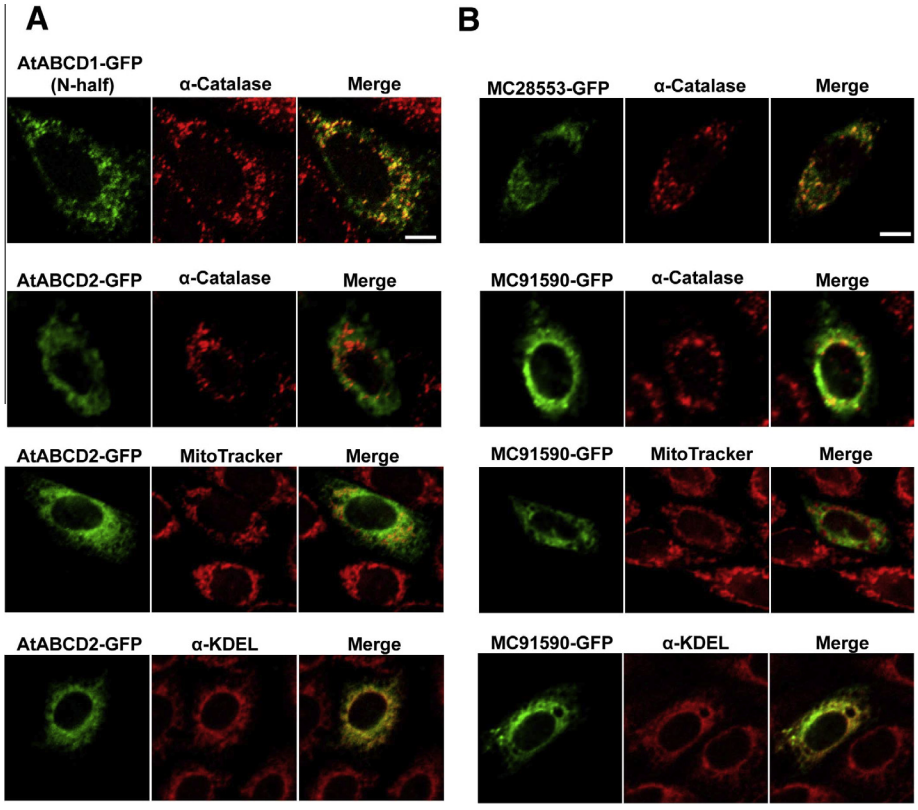


**Fig. 3.** Subcellular localization of *A. thaliana* and *M. circinelloides* ABCD proteins transiently expressed in CHO cells. CHO cells were transfected with pEGFP/AtABCD1(N-half), pEGFP/AtABCD2, pEGFP/MC28553 or pEGFP/MC91590 and the distribution of the GFP-tagged *A. thaliana* ABCD protein (A) and the GFP-tagged *M. circinelloides* ABCD protein (B) was analyzed as in Fig. 2. The GFP fluorescence is shown on the left, peroxisomal, ER and mitochondrial staining pattern in the center, and a superimposition of both stains on the right.

and expressed AtABCD1(N-half)-GFP in CHO cells. As shown in Fig. 3A, the GFP fluorescence dots were superimposed to catalase dots, suggesting that AtABCD1(N-half)-GFP was correctly localized in peroxisomes. This result is consistent with recent finding that AtABCD1(N-half) targeted to yeast peroxisomes [19]. In contrast to AtABCD1, AtABCD2 is a half-size ABC protein and dose not have the H0 motif [20]. As expected, AtABCD2-GFP was not superimposed to catalase dots but exhibited reticulum-like distribution pattern. The distribution of AtABCD2-GFP coincided with that of ER but not mitochondria.

Among fungi, *M. circinelloides* have 4 ABCD protein homologs with (MC28553, MC72946 and MC81496) or without (MC91590) H0 motif (Fig. 1). As expected, MC28553, but not MC91590, was localized in peroxisomes (Fig. 3B). MC91590 appeared to be localized in ER but not in mitochondria. Expression of MC72946 showed cytotoxicity and MC81496 cDNA was not cloned because of the incompleteness of the sequence. Therefore, we did not express these 2 ABCD proteins in this experiment.

Taken together, these results indicate that the subcellular localization of ABCD protein largely depends on the $NH_2$-terminal H0 motif in all eukaryotic organisms. It is thus conceivable that the importance of the $NH_2$-terminal H0 motif for the targeting of ABCD proteins to peroxisomes is highly conserved across species.

### 3.3. Subcellular localization of truncated and chimeric ABCD proteins

To further elucidate the role of the $NH_2$-terminal H0 motif, we deleted the H0 motif and examined the subcellular localization of the truncated proteins. In the following experiments, we used *C. elegans* ABCD proteins as model proteins because there are 3 ABCD proteins with H0 motif and 2 ABCD proteins without H0 motif. As shown Fig. 4A, the truncated pmp-2-GFP lost its peroxisomal localization and instead was distributed to ER. A similar distribution pattern was observed in the truncated pmp-1-GFP and pmp-4-GFP. We next attached 64 $NH_2$-terminal amino acids of pmp-2, which consist of the $NH_2$-terminal cytosolic domain adjacent to the TMD1 of pmp-2, to the $NH_2$-terminal of pmp-5, and examined the subcellular localization of the chimeric protein. As shown in Fig. 4B, pmp-2(AA.1–64)-pmp-5(AA.1–598)-GFP exhibited a reticulum-like distribution pattern, and the distribution was not consistent with that of ER, but rather, with mitochondria. In other words, the chimeric protein escaped from the ER-targeting pathway and was localized to mitochondria. The subcellular distribution pattern of the chimeric protein was similar to that of chimeric ABCD4 that had the 61 $NH_2$-terminal amino acids of ABCD3 [6,21]. These data also suggest that the $NH_2$-terminal H0 motif is required for escape for the ER targeting, but the region is not sufficient for the peroxisomal targeting. Hydrophobic property around TMD1–2 is important for the targeting for peroxisomes but not mitochondria.

Our results clearly indicate that the $NH_2$-terminal H0 motif is important for peroxisomal targeting even in the nematode. In a previous study, we reported that the NBD phylogenetic tree showed that pmp-1 and pmp-2 cluster with human ABCD3, and pmp-4 clusters with human ABCD1 and ABCD2. Interestingly, pmp-3 and pmp-5 cluster with human ABCD4 [3].

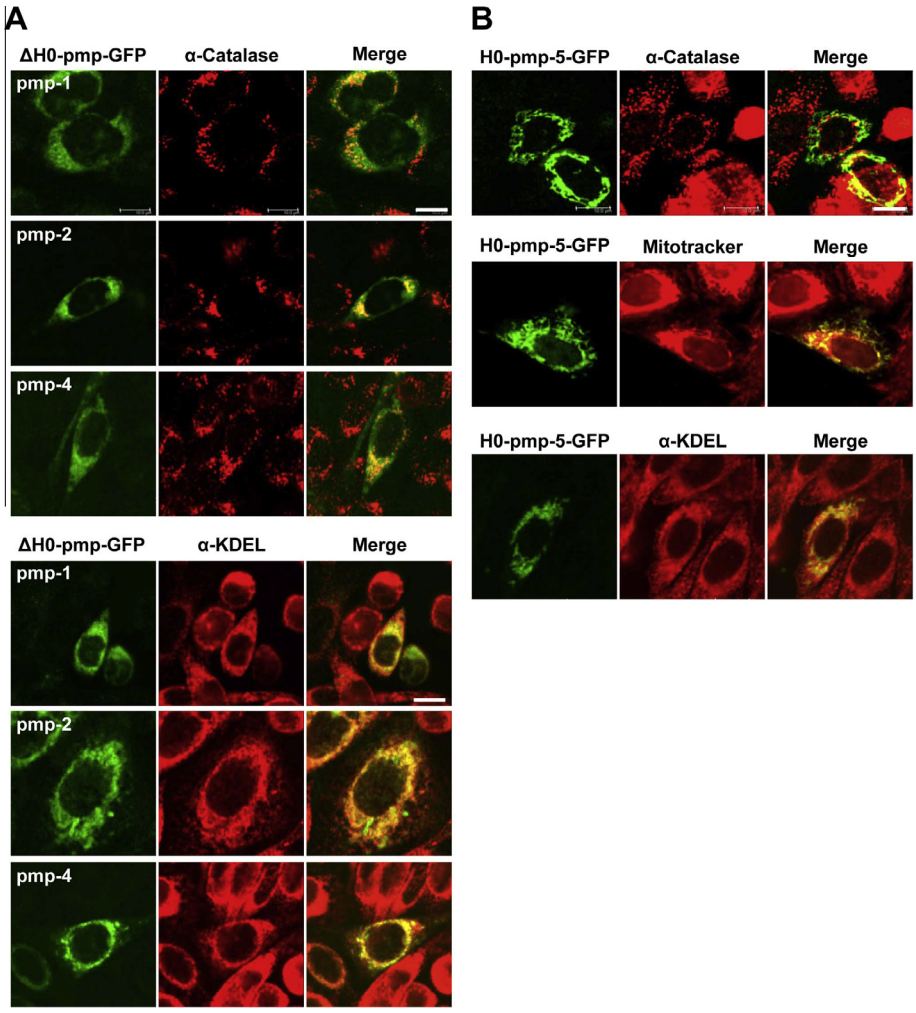


**Fig. 4.** Expression of truncated or chimeric *C. elegans* ABCD proteins in CHO cells. (A) pmp-1-, pmp-2- and pmp-4-GFP with deletion of the $NH_2$-terminal hydrophobic region were expressed in CHO cells. The subcellular distribution pattern of the truncated protein was compared with the pattern in peroxisomes and the ER as in Fig. 2, (B) pmp-5-GFP fusion with the $NH_2$-terminal hydrophobic motif of pmp-2 (pmp-2 (AA.1–64)-pmp-5(AA.1–606)-GFP was expressed in CHO cells and analyzed as in A).

Finally, we suggest that the "$NH_2$-terminal hydrophobic rule" is conserved across eukaryotic organisms and the organelle targeting system of the peroxisome seems to have been highly conserved through evolutionary processes.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.09.133.

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