Co-operative function and mutual stabilization of the half ATP-binding cassette transporters HAF-4 and HAF-9 in Caenorhabditis elegans

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Caenorhabditis elegans HAF-4 and HAF-9 are half ABC (ATP-binding-cassette) transporters that are highly homologous to the human lysosomal peptide transporter TAPL [TAP (transporter associated with antigen processing)-like; ABCB9]. We reported previously that both HAF-4 and HAF-9 localize to the membrane of a subset of intestinal organelles, and are required for the formation of these organelles and other physiological aspects. In the present paper, we report the genetic and physical interactions between HAF-4 and HAF-9. Overexpression of HAF-4 and HAF-9 did not rescue the intestinal organelle defect of the haf-9 and haf-4 deletion mutants respectively, indicating that they cannot substitute for each other. Double haf-4 and haf-9 mutants do not exhibit more severe phenotypes than the single mutants, suggesting their co-operative function. Immunoprecipitation experiments demonstrated their physical interaction. The results of the present study suggest that HAF-4 and HAF-9 form a heterodimer. Furthermore, Western blot analysis of the deletion mutants and RNAi (RNA interference) knockdown experiments in GFP (green fluorescent protein)-tagged HAF-4 or HAF-9 transgenic worms suggest that HAF-4–HAF-9 heterodimer formation is required for their stabilization. The findings provide a clue as to how ABC transporters adopt a stable functional form.

Key words: ATP-binding-cassette (ABC) transporter, Caenorhabditis elegans, heterodimer, intestine, organelle biogenesis.

INTRODUCTION

ABC (ATP-binding cassette) transporters form the largest superfamily of transporters [1,2]. Their transport substrates, tissue distributions, subcellular localizations and physiological functions are diverse; however, many have not yet been characterized.

Eukaryotic ABC transporters are categorized into two types: full transporters and half transporters [3]. A full transporter contains two sets of transmembrane domains and two nucleotide-binding domains, and functions as a monomer. In contrast, a half transporter contains one set of transmembrane domains and one nucleotide-binding domain, thus it must form a heterodimer and/or homodimer to produce a functional transporter. To elucidate the physiological function and transport substrates of a half transporter, its functional unit must be identified. The mitochondrial ABC transporters ABCB6, ABCB7, ABCB8 and ABCB10 are homodimeric half transporters [4], whereas the heterodimeric transporter ABCG5/8 is involved in cholesterol transport in the liver and intestine [5]. In contrast, multiple combinations of heterodimers and homodimers have been reported for ABCD transporters (peroxisomal half transporters) [6,7]. However, the correlation between each functional unit and the physiological function of ABCD transporters is still largely unknown [8].

Among the half ABC transporters, the functional units of the TAP (transporter associated with antigen processing) family are the best characterized. The TAP transporter localizes to the endoplasmic reticulum, and transports antigenic peptides from the cytosol to the endoplasmic reticulum [9]. Generally, TAP consists of TAP1 (ABCB2) and TAP2 (ABCB3), i.e. TAP1 and TAP2 form a heterodimer [10]. Several reports suggested the TAP function as a homodimer [11–13]; however, no biochemical evidence for its transport activity has been presented [14,15]. Another TAP family transporter, TAPL (TAP-like; ABCB9), which is highly homologous to TAP1 and TAP2 [16–18], forms a homodimer [19,20] and transports peptides of various sizes in vitro [19,21]. Unlike TAP, TAPL is localized to the lysosome, at least in TAPL-overexpressing mammalian cultured cells and Sertoli cells [18,22]. Moreover, TAPL overexpression does not restore MHC class I surface expression in TAP-deficient cells [23]. Those reports, together with the difference in tissue-specific expression between mammalian TAPL and TAP1/TAP2, suggest that TAPL has a role which differs from that of the MHC class I-restricted antigen transporter TAP [24,25].

Genetic and biochemical approaches are thought to be valuable methods to determine the possible combinations of half transporter dimers and elucidate which unit of the half transporter dimer accounts for each physiological function. To investigate a half transporter using both genetic and biochemical approaches, we utilized a model organism, the nematode Caenorhabditis elegans, for which genetic and molecular biological methods are well established.

Although no TAP homologues were found in the C. elegans genome, we found genes homologous to TAPL using a phylogenetic analysis [26]. Of the ten half ABCB transporters in C. elegans (HAF-1–9 and ABTM-1), HAF-2, HAF-4, HAF-7, HAF-8 and HAF-9 constitute a single clade with TAPL. In particular, HAF-4 and HAF-9 are highly homologous with TAPL.
and are very similar to each other, with a 47% identity in the full-length protein and a 68% identity in the ABC domain.

Our previous study showed that both HAF-4 and HAF-9 specifically localize to the membrane of a subset of intestinal granular organelles that are not acidic, but are positive for the LAMP (lysosome-associated membrane protein) homologue LMP-1 using transgenic worms expressing GFP (green fluorescent protein)-tagged proteins [26]. We also showed that haf-4 and haf-9 deletion mutants have similar phenotypes, e.g. defective formation of organelles, slow growth, reduced brood size and prolonged defecation cycle. Those results imply the presence of co-operative function of HAF-4 and HAF-9, and that they might form a heterodimer. Furthermore, an inactive form of HAF-4 with a mutation in the ATP-binding Walker A motif did not rescue the organelle defect, suggesting that its transport activity is required for organelle formation. However, whether HAF-4 and HAF-9 form heterodimers or homodimers, and which mode of dimerization contributes to the physiological phenotype were yet to be investigated.

In the present paper, we report genetic and biochemical analyses of the interaction between HAF-4 and HAF-9. Genetic analyses revealed that they cannot substitute for each other, and that the double mutants do not have a more severe phenotype than the single mutants. Biochemical analyses demonstrated the physical interaction between HAF-4 and HAF-9, which require each other for stabilization.

MATERIALS AND METHODS

General methods and mutant strains

Maintenance, husbandry and genetic crosses of C. elegans were performed according to the standard protocols described by Brenner [27]. Strains were cultured at 20°C. The Bristol strain N2 was used as the standard wild-type strain, and the following mutant strains were obtained from Caenorhabditis Genetics Center (University of Minnesota, MN, U.S.A.): haf-2(gk13), haf-4(ak1042), haf-4(gk240), haf-9(gk23) and lmp-1(n2045).

Transgenic strains

To generate the haf-9::mCherry construct, the DsRed\textsuperscript{\textregistered}\textsuperscript{\textcopyright} gene in the pST-RFP2 vector (provided by Dr Toshihiko Oka, Kyushu University) was replaced with the DsRed\textsuperscript{\textcherry} gene [28], and then an Sphl-BamHI haf-9 genome clone containing the promoter region (a fragment that includes 2086 bp upstream of the initiation codon) was inserted into the vector.

Microinjection of DNA into the C. elegans germline was performed as described by Mello et al. [29] using pRF4 (rol-6(su1006)) as a selection marker. Integrant formation was performed using UV irradiation [30]. Obtained transgenic lines were outcrossed with the Bristol N2 strain six times. For rescue experiments, mutant worms were crossed with the integrant strains Is[haf-4::GFP], Is[haf-9::GFP] and/or Is[haf-9::mCherry].

Optical microscopic observation

RNAi (RNA interference) images were obtained using fluorescence stereomicroscopy (SZX16; Olympus) combined with a mercury vapour lamp and the SZX2-FGFPHQ or SZX2-FRFP1 filter unit (Olympus) fluorescence intensities of RNAi-treated transgenic worms were measured using the NIH ImageJ software. Confocal fluorescence images were obtained using a confocal microscope (FV1000; Olympus) with 473 nm or 559 nm laser excitation. Autofluorescence was visualized by pre-exposure to blue light. Spectral scanning and unmixing were performed with a spectral deconvolution program in the FV1000 software FV10-ASW. All pictures were obtained using hermaphrodites on adult day 1. All confocal pictures presented are oriented with the anterior to the left-hand side. For granular counts, DIC (differential interference contrast) images were taken using microscopy (BX51; Olympus) with DIC optics, and then the number of granules with a diameter size of \(\geq 1\mu m\) was manually counted within a 300 \(\mu m^2\) area in the image of each worm. Student’s \(t\) test or Tukey’s test for multiple comparisons was used for the statistical analyses.

Growth rate measurement and other assays

Growth rate measurements, brood size measurements and defecation assays were performed as described previously [26]. Student’s \(t\) test or Tukey’s test for multiple comparisons was used for the statistical analyses.

RNAi

RNAi feeding on agar plates was performed according to the WormMethod [31]. The RNAi clones for haf-9, lmp-1 and haf-2 were prepared from the C. elegans RNAi v1.1 Feeding Library (Open Biosystems). The mock pL4440-dest-RNAi feeding vector was constructed from the lmp-1 pL4440-dest-RNAi feeding vector by removing the insert by EcoRV digestion, followed by self-ligation. The haf-4 RNAi vector was constructed by digesting the lmp-1 RNAi pL4440-dest-RNAi feeding vector with KpnI and EcoRV and ligating it with the RT (reverse transcription)–PCR-amplified cDNA of the KpnI-haf-4 open reading frame (except the first adenosine and the last 31 nucleotides)–EcoRV fragment.

Briefly, a bacterial culture was seeded on to NG (nematode growth) agar containing 25 \(\mu g/ml\) carbencillin, 5 \(\mu g/ml\) nystatin and 0.5 mM isoprropyl \(\beta\)-D-thiogalactopyranoside in 12-well plates. At 1 day later, approximately 20 synchronized L (larva) 1 stage worms were transferred on to each plate. They were incubated for 5 days at 20°C until use in stereomicroscopic analyses.

Antisera and antibody preparation

An antiserum against a HAF-4 peptide fragment from Lys\textsuperscript{744} to Asn\textsuperscript{764} (KRPVARSQPPAQASIN) was obtained from an immunized rabbit (Medical & Biological Laboratories). An antiserum against a HAF-9 peptide fragment from Gly\textsuperscript{781} to Ser\textsuperscript{796} (GGSGRSTAGARRIRS) was obtained from an immunized rabbit (Thermo Fisher Scientific).

Monoclonal anti-LMP-1 and anti-DLG-1 (EMBL accession CCD65680.1) antibodies from LMP1 and DLG1 hybridomas respectively (Developmental Studies Hybridoma Bank at the University of Iowa, IA, U.S.A.) were prepared according to [32]. The conditioned medium (10-fold dilutions) was applied to the immunoreaction.

Immunoprecipitation

Worms were harvested from nine NG agar plates (9 cm in diameter) as described previously [26]. The frozen worms were suspended in 400 \(\mu l\) of MTKS (magnesium-, Tris-, potassium- and sucrose-containing buffer) buffer [50 mM Tris/HCl (pH 7.5), containing 250 mM sucrose, 25 mM potassium acetate, 5 mM magnesium acetate and a protease inhibitor cocktail (Complete\textsuperscript{TM}, EDTA-free; Roche Diagnostics)], sonicated eight times for 20 s, each with 1 min rest in ice-cold water using an ultrasonic...
homogenizer (output level 6, Ultra S Homogenizer VP-5S; Taisotec), and then separated into fractions by centrifugation at 1500 \( g \) for 15 min, followed by ultracentrifugation at 33,000 rev./min for 1 h at 4°C (TLA-100.4 rotor, Optima TL Ultracentrifuge; Beckman Coulter). The precipitates were resuspended in 400 \( \mu l \) of binding buffer [20], containing 1% (w/v) n-dodecyl-\( \beta \)-d-maltoside, 10% (v/v) glycerol and protease inhibitor cocktail, incubated at 4°C for 16 h, and then subjected to ultracentrifugation. The resulting supernatants were collected as membrane fractions.

Membrane fractions (200 \( \mu g/500 \mu l \)) were pre-cleared for 30 min with 40 \( \mu l \) of 50% (v/v) slurry of Protein G–Sepharose 4 Fast Flow (GE Healthcare). The resulting supernatants were incubated with 1 \( \mu g \) of anti-GFP antibody (Roche Diagnostics), anti-RFP (red fluorescent protein) antibodies (5F8, ChromoTek; and 3G5, Medical & Biological Laboratories), normal rabbit IgG (Santa Cruz Biotechnology) or anti-HAF-4 serum (16G–5G5, Medical & Biological Laboratories) or anti-RFP (red fluorescent protein) antibodies (5F8, ChromoTek; and 3G5, Medical & Biological Laboratories), normal rabbit IgG (Santa Cruz Biotechnology) or anti-HAF-9 serum (1:1000 dilution), anti-LMP-1 antibody, anti-DLG-1 antibody, anti-GFP antibody (catalogue number 11814460001; Roche Diagnostics) (1:1000 dilution), followed by reaction with a DirecT-conjugated anti-RFP antibody (Medical & Biological Laboratories), and the supernatants were collected after centrifugation. Input (5 \( \mu g \) of protein per lane), unbound fractions (13 \( \mu l \) per lane) and immunoprecipitates (20 \( \mu l \) per lane) were separated by SDS/PAGE (7.5% gel) and electroblotted (ATTO Model-AE6677) on to an Immobilon \( \text{®} \) transfer membrane (Millipore) that was subsequently incubated in blocking solution (5% (v/v) anti-goat serum (BioWest) in PBS-T (PBS containing 0.1% (v/v) Tween 20) for 1 h, and then incubated in blocking solution (5% (w/v) non-fat dried skim milk powder (Hokkaido Milk Products) in PBS-T for the others). The blot was probed with anti-HAF-4 serum (1:5000 dilution in blocking solution), anti-HAF-9 serum (1:10000 dilution), anti-LMP-1 antibody, HRP (horseradish peroxidase)-conjugated anti-GFP antibody (Nacalai Tesque) (1:10000 dilution) and HRP-DirectT-conjugated anti-RFP antibody (Medical & Biological Laboratories) (1:2000 dilution), followed by reaction with a HRP-conjugated goat anti-(rabbit IgG) antibody (Thermo Fisher Scientific) (1:4000 dilution) and 5% (w/v) blocked non-fat dried skimmed milk powder in PBS-T or a HRP-conjugated goat anti-(mouse IgG) antibody (Promega) (1:2500 dilution) as necessary. Target protein detection was performed using SuperSignal \( \text{®} \) West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) and the LAS-3000 image analyser (Fujifilm).

Membrane fractionation and Western blot analysis
Approximately 6000 synchronized young adult worms were sonicated six times for 20 s each in 300 \( \mu l \) of MTKS buffer. After ultracentrifugation, the resulting supernatants were collected as soluble fractions. The precipitates were suspended in 200 \( \mu l \) of 17 mM Tris/HCl (pH 7.0), containing 2% (w/v) SDS, 10% (v/v) glycerol and 100 mM dithiothreitol, and incubated at 75°C for 10 min. The supernatants were then collected as membrane fractions after centrifugation at 1500 \( g \) for 10 min.

Fractions of samples (containing 5 \( \mu g \) of protein) were incubated at 75°C for 10 min and then separated by SDS/PAGE (10% gel) and electroblotted on to an Immobilon \( \text{®} \) transfer membrane, then subsequently incubated in blocking solution. The blot was probed with anti-HAF-4 serum, anti-HAF-9 serum, anti-LMP-1 antibody, anti-DLG-1 antibody, anti-GFP antibody (catalogue number 11814460001; Roche Diagnostics) (1:1000 dilution) and HRP-DirectT-conjugated anti-RFP antibody. The immunoreaction with the secondary antibodies, and target protein detection were performed as described above.

RESULTS
HAF-4 and HAF-9 colocalize to intestinal organelles in \textit{C. elegans}

We reported previously that both HAF-4 and HAF-9 localize specifically to non-acidic, but LMP-1-positive, intestinal organelles from the larval stage to the adult stage [26]. However, whether they localize to identical organelles was yet unknown. To examine the colocalization of HAF-4 and HAF-9, we established \textit{Is[haf-4::GFP]};\textit{Is[haf-9::mCherry]} transgenic worms. The overlap of HAF-4::GFP and HAF-9::mCherry fluorescence was detected in a subset of granules (Figure 1), indicating that HAF-4 and HAF-9 colocalized to the organelles.

\textit{haf-4} and \textit{haf-9} cannot functionally substitute for each other

To clarify the functional correlation between HAF-4 and HAF-9, we performed several genetic interaction analyses. We established previously the \textit{Is[haf-4::GFP]} and \textit{Is[haf-9::GFP]} allelics and showed that they can rescue the intestinal granular defect phenotypes of the \textit{haf-4} and \textit{haf-9} deletion mutants respectively (Supplementary Figure S1 at http://www.biochemj.org/bj/452/bj4520467add.htm) [26]. We also performed the reciprocal analyses: we examined whether introduction of the \textit{haf-4::GFP} and \textit{haf-9::mCherry} genes can rescue the intestinal granular defect phenotype in the \textit{haf-9} and \textit{haf-4} deletion mutants respectively. As shown in Figure 2, \textit{Is[haf-4::GFP]} rescued the defects in \textit{haf-4(ok1042)} (Figures 2C and 2D) and \textit{haf-4(gk240)} (Figures 2E and 2F), but not in \textit{haf-9(gk23)} (Figures 2G and 2H). In contrast, \textit{Is[haf-9::mCherry]} did not rescue the defects in the \textit{haf-4} deletion mutants (Figures 2K–2N), but did rescue the defects in the \textit{haf-9} deletion mutant (Figures 2O and 2P).

The \textit{C. elegans} intestine has another subset of lysosome-related organelles that are acidified and have autofluorescent contents [33–34]. Visualization of this autofluorescence in \textit{haf-9(gk23)};\textit{Is[haf-4::GFP]} and \textit{haf-4(ok1042)};\textit{Is[haf-9::GFP]}, in which the HAF-4 and HAF-9-positive intestinal granules are defective, showed that the GFP signals are not detected on the periphery of the autofluorescent signals (Supplementary Figure S2 at http://www.biochemj.org/bj/452/bj4520467add.htm). Therefore HAF-4 and HAF-9 do not mislocalize to the membrane of these autofluorescent granules even when the usual organelles are defective.

We also performed a genetic complementation test. \textit{haf-4(ok1042)} and \textit{haf-9(gk23)} are recessive alleles for the intestinal granular phenotype; the heterozygous mutants do not exhibit the intestinal granular defect, but the homozygous mutants do exhibit the defect. We observed \textit{haf-4(ok1042)} \textit{haf-9(+)} /
Figure 2  HAF-4::GFP and HAF-9::mCherry do not rescue the intestinal defect of the haf-9 and haf-4 deletion mutants respectively

DIC (A, C, E, I, K, M and O) and corresponding fluorescence images (B, D, F, H, J, L, N and P) are shown. (A and B) On a wild-type N2 background, HAF-4::GFP localized to the membrane of the intestinal granules. Introduction of the haf-4::GFP transgene rescued the intestinal granular defect in haf-4(ok1042) (C and D) and haf-4(gk240) (E and F). (G and H) In haf-9(gk23);Is[haf-4::GFP], HAF-4::GFP did not rescue the intestinal granular defect of haf-9(gk23). (I and J) On a wild-type N2 background, HAF-9::mCherry localized to the membrane of the intestinal granules. Introduction of the haf-9::mCherry transgene did not rescue the intestinal granular defect in haf-4(ok1042) (K and L) or haf-4(gk240) (M and N). (O and P) In haf-9(gk23);Is[haf-9::mCherry], HAF-9::mCherry rescued the intestinal granular defect of haf-9(gk23). The scale bar represents 10 μm. (Q) Quantification of intestinal granules. The number of granules ≥1 μm in diameter within a 300-μm² area in DIC images was manually counted. Five worms of each strain were analysed. Introduction of the haf-4::GFP and haf-9::mCherry transgenes significantly increased the number of granules in the haf-4 and haf-9 deletion mutants respectively (P < 0.001), but did not increase the number of granules in the haf-9 and haf-4 deletion mutants respectively (P > 0.15). The error bars represent the S.D.

haf-4(+) haf-9(gk23) adult worms using DIC microscopy and showed that they did not show the granular defect (Supplementary Figure S3 at http://www.biochemj.org/bj/452/bj4520467add.htm). The results of the present study indicate that haf-4 and haf-9 cannot functionally substitute for each other in the normal formation of the intestinal organelles.
**Interaction of half ABC transporters HAF-4 and HAF-9**

**Figure 3** The haf-4 and haf-9 double mutants do not exhibit more severe phenotypes than the single deletion mutants

(A) Intestinal granular phenotype. The numbers of granules \(\geq 1 \mu m\) in diameter within a 300-\(\mu m^2\) area in DIC images were manually counted. Five worms of each strain were analysed. Although each single mutant showed a significant reduction of the granule number \((P > 0.001)\), the double mutants did not show significant reductions compared with the corresponding single mutants \((P > 0.1)\). The representative DIC images are shown in Supplementary Figure S4 (at http://www.biochemj.org/bj/452/bj4520467add.htm).

(B) The time to reach adulthood. After eggs were laid on NG agar plates, the worms that reached adulthood were counted every 3 h \(\{n = 326 [N2], n = 253 [haf-4(ok1042)], n = 263 [haf-4(gk240)], n = 161 [haf-9(gk23)], n = 191 [haf-4(ok1042) haf-9(gk23)]\) and \(n = 279 [haf-4(gk240) haf-9(gk23)]\). Although each single mutant showed a significant slow growth \((P < 0.05)\), the double mutants did not show slower growth than haf-9(gk23).

(C) Brood size measurements. Ten worms of each strain were analysed. *\(P < 0.05\) and **\(P < 0.01\) against the wild-type N2. The double mutants did not show more reduced brood size than haf-9(gk23).

(D) Defecation assay. Ten worms of each strain were analysed. For each worm, ten measurements were averaged. Although each single mutant showed a significant prolonged defecation cycle \((P < 0.001)\), the double mutants showed only slight prolonged cycle compared with haf-9(gk23). The error bars represent the S.D.

**haf-4 and haf-9 double mutants do not exhibit more severe phenotypes than the single mutants**

To further dissect the relationship between HAF-4 and HAF-9 using genetic interaction analyses, we established haf-4 and haf-9 double deletion mutants, and compared their granular phenotype with those of the single mutants. The haf-4 and haf-9 deletion mutants have a decreased number of intestinal granules \(\geq 1 \mu m\) in diameter, which are characteristic of HAF-4- and HAF-9-positive organelles. As shown in Figure 3(A) and Supplementary Figure S4 (at http://www.biochemj.org/bj/452/bj4520467add.htm), the number of these granules in the haf-4 single mutants, the haf-9 single mutant, and the haf-4 and haf-9 double mutants did not differ significantly. This may be because the HAF-4- and HAF-9-positive large organelles are already completely absent from the single mutants, as supported by the disturbed localizations of HAF-4::GFP and HAF-9::mCherry in the haf-9 and haf-9 deletion mutants respectively (Figure 2).

We then focused on the other physiological phenotypes observed in the haf-4 and haf-9 single deletion mutants: growth rate, brood size and defecation cycle [26]. As shown in Figures 3(B) and 3(C), growth speed and brood size in the haf-4 and haf-9 double mutants are comparable with those in haf-9(gk23) \((P > 0.05)\). As for the prolongation of the defecation cycle, the mean defecation periods in the double mutants were more prolonged than those in the single mutants; however, these differences were not statistically significant (Figure 3D). Therefore the haf-4 and haf-9 double mutants exhibit phenotypes that are essentially the same as the single mutants.

**Physical interaction between HAF-4 and HAF-9**

Genetic interaction analyses suggest a co-operative function of HAF-4 and HAF-9, and reinforced the possibility that they form a heterodimer. To detect the formation of a HAF-4 and HAF-9 heterodimer, we performed co-immunoprecipitation experiments using HAF-4::GFP- and/or HAF-9::mCherry-expressing transgenic worms. Since it was not clear whether a pair of fluorescent protein-tagged proteins could form a functional dimer, we performed rescue experiments of the haf-4 and haf-9 double deletion mutants. Introduction of both haf-4::GFP and haf-9::mCherry into haf-4(ok1042) haf-9(gk23) and haf-4(gk240) haf-9(gk23) restored the intestinal granules to which both HAF-4::GFP and HAF-9::mCherry localize (Supplementary Figure S5 at http://www.biochemj.org/bj/452/bj4520467add.htm). The results from the present study indicate that both HAF-4::GFP and HAF-9::mCherry are functional in the absence of endogenous HAF-4 and HAF-9; therefore it is assumed that they exist as a functional form.
HAF-4 and HAF-9 stabilize each other

For some, but not all, half transporters, a lack of their partner half transporter leads to mislocalization or destabilization. For example ABCG5, which normally localizes to the plasma membrane, is confined to the endoplasmic reticulum in the absence of its partner ABCG8 [5]. TAP2 requires the formation of a complex with TAP1 for the maintenance of its expression [35]. Pxa1p, an ABCD1 homologue of Saccharomyces cerevisiae, is unstable in the absence of Pxa2p, a homologue of ABCD3 [36]. As the organelles to which HAF-4 and HAF-9 localize are not formed in the absence of HAF-9 and HAF-4 respectively, we examined the expression of HAF-4 and HAF-9 in the haf-9 and haf-4 deletion mutants by Western blot analysis. HAF-4 expression was below the limit of detection not only in the haf-4 deletion mutants, but also in haf-9(gk23) (Figure 5A), even though the diffused HAF-4::GFP fluorescence was still observed in the haf-9 mutant under a confocal microscope (Figure 2H). No additional signals suggestive of partial degradation or alternative modification of HAF-4 were detected in the haf-9 mutant except non-specific signals in both the soluble and membrane fractions (Supplementary Figure S7A at http://www.biochemj.org/bj/452/bj4520467add.htm), indicating that HAF-4 is not stable in the absence of HAF-9. HAF-4 expression in the haf-9 mutant was rescued by the introduction of fluorescent protein-tagged HAF-9 (Figure 5A). HAF-4 expression did not decrease in the haf-2 deletion mutant, which is another closely related half ABC transporter protein [26], or that of lmp-1, which shows non-identical, but similar, intestinal granular defects [26,37]. The former suggests that the instability of HAF-4 is HAF-9-specific among the half ABCB transporter proteins. The latter suggests that the stability of HAF-4 and HAF-9 is independent of normal formation of HAF-4- and HAF-9-positive organelles.

In the haf-4 deletion mutants, HAF-9 expression decreased considerably, although it is still detectable (Figure 5B). HAF-4 expression was also rescued by the introduction of HAF-4::GFP. As with HAF-4, no additional signals were detected in the haf-4 mutants (Supplementary Figure S7B) and expression does not decrease in both haf-2(gk13) and lmp-1(nr2045). Therefore HAF-9 is unstable in a HAF-4-deficiency-dependent manner.

As the haf-4 and haf-9 deletion mutants are defective for HAF-4- and HAF-9-positive intestinal organelles, there is a possibility that not only HAF-4 and HAF-9, but also other membrane proteins localizing on the same organelles are affected. However, as shown in Figure 5(C), LMP-1 was detected in the haf-4 and haf-9 mutants at the same levels as in the wild-type. Therefore mutual stabilization of HAF-4 and HAF-9 is a rather specific event. Similar results were obtained using total worm lysate (Supplementary Figure S8 at http://www.biochemj.org/bj/452/bj4520467add.htm), indicating that the mutual stabilization is not an artefact during fractionation, but an event inside worms. The results of the present study strongly suggest that HAF-4 and HAF-9 form a heterodimer.

We observed a similar result by RNAi. The haf-4 knockdown and the haf-9 knockdown down-regulated HAF-4::GFP expression (Figures 5G–5K and 5V). Meanwhile, the haf-4 knockdown decreased HAF-9::GFP expression (Figures 5L–5P and 5W). LMP-1::mRFP expression in the intestinal cells did not decrease following haf-4 and haf-9 knockdown, but in fact it increased (Figures 5Q–5U and 5X).

DISCUSSION

We showed genetic and physical interactions between the half ABC transporters HAF-4 and HAF-9, which colocalize to specific intestinal organelles in C. elegans. Genetic interaction analyses indicated that HAF-4 and HAF-9 cannot substitute for each other, but instead function co-operatively. The findings of the present study lead to two possible models, although they are not mutually exclusive. One model is that HAF-4 and HAF-9 form a...
Interaction of half ABC transporters HAF-4 and HAF-9

Figure 5  HAF-4 and HAF-9 are mutually required for stability

(A–F) Western blot analysis of HAF-4 and HAF-9. Membrane fractions were prepared from synchronized young adult hermaphrodites, and 5 µg of protein was subjected to Western blot analysis. Anti-HAF-4 (A), anti-HAF-9 (B), anti-LMP-1 (C), anti-DLG-1 (D) (as a loading control for the membrane fractions), anti-GFP (E) and anti-RFP (F, which cross-reacts with mCherry) antibodies were used. Signals for HAF-4 (~90 kDa), HAF-4::GFP (120 kDa), HAF-9 (80 kDa), HAF-9::GFP (110 kDa), HAF-9::mCherry (110 kDa), LMP-1 (35 kDa) and DLG-1 (120 kDa) are indicated by the arrows. HAF-9::mCherry was not detectable in the anti-HAF-9 antibody blot (B), which was probably due to its low expression; HAF-9::mCherry expression can be confirmed in the anti-RFP antibody blot (F). Tag-cleaved proteins were also detected in the anti-HAF-4 (A) and anti-HAF-9 (B) antibody blots of the transgenic worms, which have molecular masses indistinguishable from the endogenous proteins. Blots of soluble fractions are shown in Supplementary Figure S7 (at http://www.biochemj.org/bj/452/bj4520467add.htm). (G–X) Effect of haf-4 and haf-9 knockdown on the expression of HAF-4::GFP, HAF-9::GFP and LMP-1::mRFP. is[haf-4::GFP] (G–K), is[haf-9::GFP] (L–P) and is[Pges-1::lmp-1::mRFP] (Q–U) were fed E. coli HT115 cells expressing double-stranded RNA corresponding to haf-4 (H, M and R), haf-9 (I, N and S), haf-2 (J, O and T) or lmp-1 (K, P and U) mRNA. Insets show the corresponding bright-field images. The scale bar represents 200 µm. Fluorescence signals were quantified by the ImageJ program with five worms in each RNAi treatment (V–X). Dunnett’s test was used for the multiple comparisons. ***P < 0.001. The error bars represent the S.D.
heterodimer, the other is that they form homodimers and transport different substrates that function co-operatively in the formation of intestinal organelles.

Then, we demonstrated the physical interaction between HAF-4 and HAF-9 by co-immunoprecipitation. As these half transporters do not interact with LMP-1, which is also thought to colocalize to these intestinal organelles, the physical interaction between HAF-4 and HAF-9, together the results of the genetic analyses, probably indicates heterodimer formation, which functions in the normal formation of these intestinal granules. However, it does not exclude homodimerization of HAF-4 and HAF-9 or heterodimerization with other unidentified partners. In contrast with HAF-4, which is undetectable in the absence of HAF-9, HAF-9 is still present at detectable levels even in the absence of HAF-4. This difference may reflect the existence of considerable amounts of HAF-9 homodimer that is stabilized without HAF-4. Homodimerization analysis of HAF-4 requires the expression of two differentially tagged HAF-4 proteins; however, establishing the transgenic animals other than Is[haf-4::GFP] has not been successful despite our many efforts. Alternatively, endogenous HAF-4 and HAF-4::GFP could be used for this purpose; however, endoproteolysis of HAF-4::GFP generates a degradation product with the same molecular mass as endogenous HAF-4.

In the face of its mode of association, the interactions between TAP1 and TAP2 subunits via both the transmembrane domains and nucleotide-binding domains have been suggested [38–40]. Recent three-dimensional structural modelling of the core heterodimeric TAP complex, as well as cysteine-scanning and cross-linking approaches, also demonstrated the interface between the TAP1 and TAP2 at the amino acid level [41]. In literature, the X-loop in the cytosolic nucleotide-binding domain is close to CLs (cytosolic loops) 1 and 2 of the opposite subunit, which extend between TM (transmembrane helix) 2 and TM3 and between TM4 and TM5 respectively. In particular, the close interaction between the conserved glutamate residue in the X-loop of TAP2 and specific residues (Glu277 and Ala401) in CL1/2 of TAP1 has been described. Alignment analysis of TAP1/2 and HAF-4/HAF-9 indicates the possibility of an interaction between Glu631 in the X-loop of HAF-4 and Ala280 in the CL2 of HAF-9. Another report on the crystal structure of a heterodimeric ABC transporter from Thermotoga maritima identified several amino acids in the nucleotide-binding domain that are involved in heterodimer formation [42]; however, these amino acids are not well conserved in HAF-4 and HAF-9.

Recently, Demirel et al. [43] reported the interaction of mammalian TAPL with LAMP-1 and LAMP-2 and its contribution to the stability of TAPL. However, our finding showed that LMP-1, a LAMP homologue, does not interact with HAF-4 or HAF-9, the TAPL homologues of C. elegans, neither does it contribute to their stability. The interaction of TAPL with LAMP-1 and LAMP-2 is mediated by the N-terminal domain of TAPL, and this domain does not share significant identity with the N-terminal domains of HAF-4 and HAF-9 [26]. Most likely, the stabilization mechanism and interacting factors of C. elegans HAF-4 and HAF-9 are not quite the same as those of mammalian TAPL.

The dependence of intestinal organelle biogenesis on these half ABCB transporters (HAF-4/HAF-9) parallels that of another intestinal lysosome-related organelle, which requires a full ABCB transporter, PGP-2, for biogenesis [44]. PGP-2 localizes to this organelle, which is a typical lysosome-related organelle in terms of acidification [34]. In contrast, the HAF-4- and HAF-9-positive organelle is a non-canonical lysosome-related organelle because it is not acidified despite the localization of LMP-1::mRFP [26]. Although diverse lysosome-related organelles have been reported [45], C. elegans intestinal cells are prominent since one cell type has two kinds of lysosome-related organelles, and these organelles require different types of ABCB transporters for their biogenesis. The C. elegans intestinal cell will be an excellent model for analysing the role of ABCB transporter proteins in the biogenesis of lysosome-related organelles.

Finally, we showed that HAF-4 and HAF-9 are unstable in the absence of each other. This phenomenon can be explained by the following two interpretations. One is that HAF-4 and HAF-9 need to form a heterodimer in order to be stable, as is the case for TAP2 and S. cerevisiae Pxa1p (a ABCD1 homologue) [35,36]. The other interpretation is that they are unstable owing to the formation defect in the intestinal organelles to which they localize. The latter possibility is quite unlikely since HAF-4 and HAF-9 are stable even in an lmp-1 deletion mutant, which also exhibits the intestinal organelle defect. Unlike the heterodimeric ABC transporters TAP1/TAP2 and Pxa1p/Pxa2p, in which only TAP2 and Pxa1p, but not TAP1 and Pxa2p, are unstable in the absence of each partner, both HAF-4 and HAF-9 require the other for their stability. Pxa2p can probably efficiently form a homodimer or a heterodimer with another half ABC transporter, whereas both HAF-4 and HAF-9 may predominantly exist as a heterodimer. Uncovering the mechanism underlying the stability and dimerization of HAF-4 and HAF-9 through mutational analysis would provide a novel clue as to how ABC transporters adopt a stable functional form.

At present, we speculate that nutrient peptides are feasible candidates for transport substrates of the HAF-4–HAF-9 complex, since the haf-4 and haf-9 mutants exhibit the slow growth and reduced brood size phenotypes [26] and the HAF-4–positive intestinal organelles decrease when worms are starved for a few hours (T. Tanji, K. Nishikori, H. Shiraishi and A. Ohashi-Kobayashi, unpublished data). Identification of the substrates would help elucidate its physiological function and demonstrate how the transport activity of this transporter affects the biogenesis of the intestinal organelle as well as its other physiological aspects. Comparison of the substrates between the C. elegans probable HAF-4–HAF-9 heterodimer and the mammalian TAPL homodimer may shed light on why C. elegans evolved ancestral TAPL as a heterodimer of paralogues.

AUTHOR CONTRIBUTION
This study was conceived and directed by Ayako Ohashi-Kobayashi. All authors contributed to discussion of experimental design and data analyses. Takahiro Tanji carried out all of the experimental studies unless otherwise indicated. Hirohisa Shiraishi established haf-9(pk23);Is[haf-4::GFP] and performed the RNAi experiments. Takahiro Tanji and Hirohisa Shiraishi performed confocal microscopic analysis of haf-9(pk23);Is[haf-4::GFP]; Takahiro Tanji, Kenji Nishikori and Hirohisa Shiraishi measured the growth rate and performed preliminary experiments for physical interaction analyses. Takahiro Tanji, Kenji Nishikori, Hirohisa Shiraishi and Ayako Ohashi-Kobayashi wrote the paper. Overall supervision of the study was undertaken by Masatomo Maeda and Ayako Ohashi-Kobayashi.

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SUPPLEMENTARY ONLINE DATA

Co-operative function and mutual stabilization of the half ATP-binding cassette transporters HAF-4 and HAF-9 in *Caenorhabditis elegans*

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Figure S1  Schematic representations of the genome structures of the haf-4 and haf-9 genes and their mutants

Shaded boxes on each gene delineate exons. The position and length of deletion and/or insertion found in each mutant are indicated by the bar below the genome structures.

Figure S2  HAF-4 and HAF-9 do not mislocalize to autofluorescent granules in the absence of HAF-4- and HAF-9-positive lysosome-related organelles

Fluorescence in haf-9(gk23);Is[haf-4::GFP] (A–C) and haf-4(ok1042);Is[haf-9::GFP] (D–F) was unmixed using a spectral deconvolution program in the FV1000 software FV10-ASW following the spectral scanning. GFP fluorescence (A and D), autofluorescence (B and E) and the merged images (C and F) are shown. GFP fluorescence and autofluorescence did not overlap in either strains. The scale bar represents 10 μm.

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Figure S3  *haf-4* and *haf-9* complement each other

DIC images of intestinal cells near the vulva in N2 (A), *haf-4(ok1042)/haf-4(+)* (B), *haf-4(ok1042)/haf-4(ok1042)* (C), *haf-9(gk23)/haf-9(+)* (D), *haf-9(gk23)/haf-9(gk23)* (E) and *haf-4(ok1042)*, *haf-9(gk23)/haf-9(gk23)* (F) nematodes are shown. The scale bar represents 10 μm. (G) Quantification of intestinal granules. The number of granules ≥1 μm in diameter within a 300-μm² area on DIC images was manually counted. Five worms of each strain were analysed. The granule number decreased significantly only in *haf-4(ok1042)/haf-4(ok1042)* and *haf-9(gk23)/haf-9(gk23)* (P <0.001). The error bars represent the S.D.
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Figure S4  DIC images of intestinal cells near the vulva in single and double haf-4 and haf-9 mutants at the young adult stage

(A) Wild-type N2, (B) haf-4(ok1042), (C) haf-4(gk240), (D) haf-9(gk23), (E) haf-4(ok1042) haf-9(gk23) and (F) haf-4(gk240) haf-9(gk23). Intestinal cells are tightly packed with granules in the wild-type animals, whereas the haf-4 and haf-9 deletion mutants have lost numerous such granules. The double mutants were indistinguishable from the single mutants. The scale bar represents 10 μm.

Figure S5  HAF-4::GFP and HAF-9::mCherry are functional in the absence of endogenous HAF-4 and HAF-9

haf-4(ok1042) haf-9(gk23);Is[haf-4::GFP];Is[haf-9::mCherry] (A–D) and haf-4(gk240) haf-9(gk23);Is[haf-4::GFP];Is[haf-9::mCherry] (E–H) were established and then observed under a confocal microscope. GFP fluorescence (A and E, green), mCherry fluorescence (B and F, magenta), merged images (C and G) and corresponding DIC images (D and H) are shown. Intestinal cells are filled with granules to which HAF-4::GFP and HAF-9::mCherry co-localize. The scale bar represents 10 μm.

Figure S6  HAF-9::GFP and HAF-9::mCherry can form a complex

Membrane fractions extracted from haf-9(gk23);Is[haf-9::GFP] (worms 1), haf-9(gk23);Is[haf-9::mCherry] (worms 2), and haf-9(gk23);Is[haf-9::GFP];Is[haf-9::mCherry] (worms 3) were subjected to immunoprecipitation (IP) with an anti-RFP monoclonal antibody (clone 5F8 which cross-reacts with mCherry). Arrows indicate the specific signals for HAF-9::GFP and HAF-9::mCherry. The anti-RFP antibody slightly co-immunoprecipitated HAF-9::GFP in the presence of HAF-9::mCherry [IP(RFP), worms 3]. WB, Western blot.
The lysate protein (5 μg per lane) was subjected to Western blot analysis. The whole blot images with anti-HAF-4 (A) and anti-HAF-9 (B) sera are shown. Signals for HAF-4 (approximately 90 kDa) and HAF-9 (80 kDa) are indicated by arrows on the right-hand side. Signals for HAF-4::GFP and HAF-9::GFP are indicated by arrowheads on the right. HAF-4 and HAF-9 decreased in haf-9(gk23) and the haf-4 mutants respectively. However, no additional signals were detected, except non-specific signals in both the soluble and membrane fractions. Signals detected in all strains were considered non-specific as indicated by asterisks.
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Figure S8 Western blot analysis of HAF-4 and HAF-9 using the total worm lysate

The lysates (lane 1, N2; lane 2, haf-4(ok1042); lane 3, haf-4(gk240); lane 4, haf-9(gk23); lane 5, haf-2(gk13); and lane 6, lmp-1(nr2045)) were prepared from synchronized young adult hermaphrodites, and 5 μg of protein was subjected to Western blot analysis. Anti-HAF-4 (A), anti-HAF-9 (B), anti-LMP-1 (C) and anti-DLG-1 (D, as a loading control) antibodies were used. Signals for HAF-4 and HAF-9 are indicated by arrows on the left-hand side. HAF-4 and HAF-9 decreased in the haf-9(gk23) and the haf-4 mutants. Signals detected in all strains were considered non-specific as indicated by asterisks.

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