NRFL-1, the C. elegans NHERF Orthologue, Interacts with Amino Acid Transporter 6 (AAT-6) for Age-Dependent Maintenance of AAT-6 on the Membrane

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Abstract

The NHERF (Na+/H+ exchanger regulatory factor) family has been proposed to play a key role in regulating transmembrane protein localization and retention at the plasma membrane. Due to the high homology between the family members, potential functional compensations have been a concern in sorting out the function of individual NHERF numbers. Here, we studied C. elegans NRFL-1 (C01F6.6) (nherf-like protein 1), the sole C. elegans orthologue of the NHERF family, which makes worm a model with low genetic redundancy of NHERF homologues. Integrating bioinformatic knowledge of C. elegans proteins into yeast two-hybrid scheme, we identified NRFL-1 as an interactor of AAT-6, a member of the C. elegans AAT (amino acid transporter) family. A combination of GST pull-down assay, localization study, and co-immunoprecipitation confirmed the binding and characterized the PDZ interaction. AAT-6 localizes to the luminal membrane even in the absence of NRFL-1 when the worm is up to four-day old. A fluorescence recovery after photobleaching (FRAP) analysis suggested that NRFL-1 immobilizes AAT-6 at the luminal membrane. When the nrf-1 deficient worm is six-day or older, in contrast, the membranous localization of AAT-6 is not observed, whereas AAT-6 tightly localizes to the membrane in worms with NRFL-1. Sorting out the in vivo functions of the C. elegans NHERF protein, we found that NRFL-1, a PDZ-interactor of AAT-6, is responsible for the immobilization and the age-dependent maintenance of AAT-6 on the intestinal luminal membrane.

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Introduction

Proper localization and maintenance of transmembrane proteins in the plasma membrane are essential for appropriate cellular function. Transmembrane proteins often participate in a functional macromolecular complex with other transmembrane or membrane-associated proteins. One of the mechanisms regulating such protein localization and complex formation is via scaffold protein localization and retention at the plasma membrane. Due to the high homology between the family members, potential functional compensations have been a concern in sorting out the function of individual NHERF numbers. Here, we studied C. elegans NRFL-1 (C01F6.6) (nherf-like protein 1), the sole C. elegans orthologue of the NHERF family, which makes worm a model with low genetic redundancy of NHERF homologues. Integrating bioinformatic knowledge of C. elegans proteins into yeast two-hybrid scheme, we identified NRFL-1 as an interactor of AAT-6, a member of the C. elegans AAT (amino acid transporter) family. A combination of GST pull-down assay, localization study, and co-immunoprecipitation confirmed the binding and characterized the PDZ interaction. AAT-6 localizes to the luminal membrane even in the absence of NRFL-1 when the worm is up to four-day old. A fluorescence recovery after photobleaching (FRAP) analysis suggested that NRFL-1 immobilizes AAT-6 at the luminal membrane. When the nrf-1 deficient worm is six-day or older, in contrast, the membranous localization of AAT-6 is not observed, whereas AAT-6 tightly localizes to the membrane in worms with NRFL-1. Sorting out the in vivo functions of the C. elegans NHERF protein, we found that NRFL-1, a PDZ-interactor of AAT-6, is responsible for the immobilization and the age-dependent maintenance of AAT-6 on the intestinal luminal membrane.

The mammalian NHERF (Na+/H+ exchanger regulatory factor) family, which consists of NHERF1, NHERF2, PDZK1 and IKEPP, is a family of PDZ proteins. NHERF1 and NHERF2 have two PDZ domains in tandem, whereas PDZK1 and IKEPP have four tandem PDZ domains. They have overlapping tissue and subcellular distributions; the four members are found in the brush border membrane of the intestine and the renal proximal tubule [4]. The highly homologous primary structures of their PDZ domains allow them to share some of the target proteins such as CFTR (cystic fibrosis transmembrane conductance regulator) [5–7], NHE3 (sodium-hydrogen exchanger 3) [8–10] and organic solute transporters [11–13]. This redundancy in expression profile and interaction, consequently yielding potential functional compensations between the family members, has made it difficult to separate the in vivo functions of individual NHERF family proteins.
Indeed, deletion of *nherf* genes in mouse associates with mild phenotypic changes; NHERF1-null male mice develop healthy but females show increased mortality or weakness [14], [15]; NHERF2 or PDZK1-deficient mice appear normal [16], [17]. Only recently, researchers have started addressing this issue by generating multiple-gene knockout animals. Broere et al. [16] and Singh et al. [10] suggested that the NHERF family members play differential, rather than compensatory, roles in CFTR regulation. This observation seems inconsistent with findings from the single-knockout studies as the knockout animals would demonstrate more noticeable phenotypes if no or little compensations take place.

To better understand the in vivo functions of scaffold proteins of NHERF family members, we looked at *C. elegans* NRFL-1 (C01F6.6) (nherf-like protein 1). Because NRFL-1 is the single worm orthologue of NHERF family, studies in *C. elegans* should be less susceptible to the redundancy problem that we encounter in the mammalian NHERF family. In the present study, NRFL-1 was identified as a binding partner of AAT-6 (T11F9.4) (amino acid transporter 6). AAT-6 is one of the transporters with PDZ-binding motif in the *C. elegans* AAT (amino acid transporter) family that consists of nine genes. This family is closely homologous to the mammalian SLC7 family of amino acid transporters [19], [20]. *C. elegans* is a transparent model organism amenable to genetic manipulation and live-animal imaging. Taking advantage of these properties, we examined the role of PDZ interaction in the localization of AAT-6 in the plasma membrane. Similar to NHERF-mediated interactions in polarized cell lines such as OK cells and MDCK cells [21], [22], we show that NRFL-1 scaffolds NHERF-mediated interactions in polarized cell lines such as OK (Fig. 1B).

## Results

### Identification of NRFL-1 as an AAT-6 Interactor

The C-terminus of AAT-6 protein ends in threonine, arginine, and methionine (-T-R-M), which makes up a class I PDZ binding motif (-S/T-X-Φ-COOH, where X denotes any residue and Φ denotes a hydrophobic residue) [23]. In search for proteins that associate with AAT-6 via PDZ interactions, *C. elegans* proteins were first screened for PDZ domain by a molecular architecture research database, SMART [24]. This process found 72 PDZ domain-containing proteins. Next, the proteins were screened for intestinal expression because our preliminary expression analysis suggested that AAT-6 is expressed in the intestine. Expression data sets were provided by WormBase and NEXTDB (The Nematode Expression Data Base; nematode.lab.mig.ac.jp/). Sixteen proteins were selected as intestinal PDZ proteins (Table 1). For candidates with multiple splice variants such as C01F6.6, we sub-grouped the variants in terms of PDZ domain identity. The variants are classified into the same sub-group if the variants are identical with respect to the number and the primary structures of their PDZ domains (e.g., “single-domain” and “double-domain” groups in Fig. 1B). For each sub-group, one of the variants was tested for potential interaction. Each of the sixteen candidates (prey) was examined for potential interaction with the AAT-6 C-terminus tail ( bait) in the yeast two-hybrid system [25]. Through the matching process, C01F6.6a appeared strongly positive and so was C01F6.6b but to a lesser degree (Table 1). C01F6.6a (NRFL-1) was, thus, subjected to further investigation.

### Table 1. Yeast two-hybrid matching.

| PDZ protein | Gene | LEU2/GFP |
|-------------|------|----------|
| C01F6.6a    | nrl-1| +/-      |
| C01F6.6b    | nrl-1| +/-      |
| C09H6.2a    | lin-10| -/-    |
| C25F6.2a    | dig-1| -/-    |
| C25F6.2b    | dig-1| -/-    |
| C25G4.6     | C25G4.6| -/-    |
| C27A2.6     | C27A2.6| -/-    |
| C35D10.2    | C35D10.2| -/-    |
| F26D11.11a  | let-413| -/-    |
| F30A10.8b   | stn-1| -/-    |
| F30F8.3     | F30F8.3| -/-    |
| F44D12.1    | F44D12.1| -/-    |
| F44D12.4    | F44D12.4| -/-    |
| T14G10.2a   | pex-1| -/-    |
| T21G5.4     | T21G5.4| -/-    |
| T26E3.3a    | par-6| -/-    |

Each of the sixteen intestinal PDZ proteins (prey) was subjected to a yeast two-hybrid assay with AAT-6 as a bait. Pairs were assessed for LEU2 and GFP reporter genes: + and −, positive and negative for LEU2 or GFP, respectively. doi:10.1371/journal.pone.0043050.t001

The *nrl-1* gene encodes C01F6.6a in ten exons. The *nrl-1* mRNA is trans-spliced to the spliced leader SL1 with two bases of cytosine between the spliced leader and the start codon (ykl663b04, NEXTDB). The 3'UTR contains a putative polyadenylation signal, AATAAA, 15 nucleotides upstream of the poly-A tail (yk1651h10, NEXTDB; Fig. 1A). The gene products of *nrl-1* contain five variants (Fig. 1B). All the variants share the C-terminal PDZ domain (PDZ II). Three of the variants have the N-terminal PDZ domain (PDZ I) is structurally related to PDZ II with 45% identity and 59% similarity.

A BLAST [26] search against human proteins using the NRFL-1 protein sequence as a query revealed the NHERF family (NHERF1, NHERF2, PDZK1, and IKEPP) with the closest homology. In the family, NHERF1 and NHERF2 appeared particularly similar to NRFL-1. Reciprocal BLAST searches against worm proteins using the NHERF family protein sequences as queries also found NRFL-1 as the most related *C. elegans* protein. This bidirectional homology assessment suggested that NRFL-1 is a *C. elegans* orthologue of the NHERF family proteins. Individual NRFL-1 PDZ domains were compared with the domains of NHERF1 and NHERF2. In terms of PDZ domain, NHERF2 appeared slightly more related to NRFL-1; for NRFL-1 PDZ I, NHERF2 PDZ I was the most similar with 49% identity (68% similarity), and for NRFL-1 PDZ II, both of the NHERF2 PDZ domains with 43% identity (57–58% similarity) (Fig. 1C). PDZK1 and IKEPP showed somewhat modest yet considerable relatedness to NRFL-1 (Table S1).

### NRFL-1 Mainly Binds AAT-6 with PDZ II Domain

To analyze further the interaction between AAT-6 and NRFL-1 found in the yeast two-hybrid matching, we first tested the involvement of AAT-6 C-terminal PDZ binding motif (-T-R-M) in the interaction. The yeast two-hybrid and the GST pull-down assay demonstrated that the deletion of the PDZ binding motif (-T-R-M) abolished the interaction of AAT-6 with...
NRFL-1 (Fig. 2A and B), showing its participation in the interaction. Next, to identify which PDZ domain of NRFL-1 is responsible for the interaction, we disrupted the PDZ domains by introducing mutations into the conserved carboxylate-binding loop: G26A/Y27A for PDZ I, E154A/F155A for PDZ II [27]. When both domains were mutated, the mutant was unable to bind with GST-AAT-6, further confirming the involvement of PDZ interaction (Fig. 2C). The mutation in PDZ I alone retained the binding, whereas the mutation in PDZ II alone greatly impaired the binding (Fig. 2C). Collectively, the C-terminus of AAT-6 preferentially binds to the PDZ II domain of NRFL-1.

Figure 1. NRFL-1, the worm orthologue of NHERF family. A, Schematic illustration of nrfl-1 transcript (modified from WormBase). Black boxes, exons; grey box, spliced leader 1 (SL1); CC, two bases of cytosine constituting the 5'UTR; ATG, the start codon; TAA, the stop codon; AATAAA, a putative polyadenylation signal; poly-A, poly adenosine tail. The alleles tm3501 and ok2292 lack 953-basepair and 1117-basepair of the genomic DNA, respectively, indicated by the horizontal lines. B, NRFL-1 protein. The nrfl-1 gene products contain either a single or double PDZ domains with PDZ II shared by all. The variants C01F6.6a, c, and e have PDZ I and PDZ II (double-domain group). PDZ I is structurally related to PDZ II with 45% identity and 59% similarity. C, Pairwise domain comparison between NRFL-1 and two human NHERF proteins: NHERF 1 and NHERF 2. PDZ domains were specified by ExPaSy Prosite [67] and compared by BLAST [26]. For each pair, identity/similarity (%) is assigned. The red lines indicate the most remarkable pair(s) for each of NRFL-1 PDZ domains with respect to sequence identity.

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NRFL-1 is Expressed in the Intestine and Phosphorylated 
in vivo

A gfp reporter gene was fused to nrfl-1 by employing the fosmid recombineering technology [28], which enables to incorporate larger genomic DNA into the construct and to cover as many cis-regulatory elements as necessary to reproduce accurate expression in vivo. gfp::nrfl-1 was expressed in the excretory canal (Fig. 3A and B), the worm counterpart of the mammalian renal tubules, and the intestine (Fig. 3B, pharynx-anterior intestine; 3C, middle intestine; 3D, posterior intestine). Although previous large-scale gene expression profiling efforts using nrlf-1 promoters::gfp constructs observed GFP signals in the pharynx, intestine, excretory cells, and some tail cells [29], [30], our gfp::nrfl-1 was exclusively expressed in the intestine and the excretory system. This pattern stayed consistent throughout the larval and adult stages. Using a specific antibody raised against the NRFL-1 (C01F6.6a) protein, endogenous NRFL-1 was detected in the luminal side of the intestine (Fig. 3E, and Fig. S1). However, the excretory canal was not stained with the antibody. NRFL-1 is found apical to IFB-2 (Intermediate Filament, B family-2) which localizes just beneath the intestinal microvilli [31], suggesting the enrichment in the intestinal microvilli (Fig. 3E).

Immunoblotting revealed a ~72 kDa band which was not detected in the nrfl-1 null lysate (Fig. 3F). E. coli-derived recombinant C01F6.6a protein and C01F6.6b protein were used as markers to determine which of the variants would correspond to the ~72 kDa band (Fig. 3F). However, neither of the variant markers matched. This observation motivated us to consider the possibility that the NRFL-1 protein is post-translationally modified. To test the idea, we used phosphatase inhibitors to inhibit endogenous phosphatases and examined the expected band-shift due to dephosphorylation by incubating the fresh worm lysate at 37°C in the presence or absence of the phosphatase inhibitors. In the absence of the inhibitors, the bands gradually shifted to a position which corresponded to that of the recombinant C01F6.6a marker toward the end of incubation, while the bands largely stayed at the original position in the presence of the inhibitors (Fig. 3G). This migration shift likely reflected progressive dephosphorylations of NRFL-1 and/or removal of modifications of NRFL-1 which is mediated by a separate protein in vivo physical interaction. The endogenous phosphatases and examined the expected band-shift due to dephosphorylation by incubating the fresh worm lysate at 37°C in the presence or absence of the phosphatase inhibitors. In the absence of the inhibitors, the bands gradually shifted to a position which corresponded to that of the recombinant C01F6.6a marker toward the end of incubation, while the bands largely stayed at the original position in the presence of the inhibitors (Fig. 3G). This migration shift likely reflected progressive dephosphorylations of NRFL-1 and/or removal of modifications of NRFL-1 which is mediated by a separate protein in vivo physical interaction.

Physical Interaction in vivo

To examine the interaction of AAT-6 and NRFL-1 in vivo, we first determined the precise subcellular localization of AAT-6 in the intestine. Because gfp::aat-6 was not expressed and the product of aat-6::gfp was not localized on the plasma membrane, we instead inserted gfp into the region corresponding to the position between glutamine 517 and phenylalanine 518 in the C-terminal cytoplasmic tail of AAT-6 with the PDZ-binding motif intact. The reporter gene aat-6::gfp was expressed and its product was localized to the luminal surface in the intestine (Fig. 4A). No other organs showed positive GFP signals. Immunofluorescence staining using anti-NRFL-1 antibody detected NRFL-1 on the intestinal luminal membrane (Fig. 3E). Combining the immunostaining of
Figure 3. Expression and protein profile of NRFL-1 (C01F6.6) in C. elegans. A–D, Expression of NRFL-1 in worm. gfp::nrfl-1 expression was detected in excretory canal (arrow in A and B) and luminal membrane of intestinal epithelial cells (singlearrowhead) in the anterior (B), middle (C) and posterior (D) intestine. gfp::nrfl-1 was not detected on the basal side (double-arrowhead in B, C and D). In A, a worm with gfp::nrfl-1 expression restricted to the excretory system was imaged for clarity. Such worms occasionally occurred in the transgenic population. In C and D, cytosolic dispersion of NRFL-1 was seen. Scale bars, 25 μm. Ten worms examined for each. E, Luminal enrichment of NRFL-1. Endogenous NRFL-1 was detected.
endogenous NRFL-1 and the confocal imaging of the AAT-6::GFP::AAT-6 translational fusion, NRFL-1 and AAT-6 were detected with fluorescence overlapping, suggesting that they share their luminal localizations (Fig. 4B and C). To examine whether NRFL-1 and AAT-6 are in a protein complex in vivo, we immunoprecipitated AAT-6::GFP::AAT-6 from the presence of phosphatase inhibitors using anti-GFP monoclonal antibody. We detected NRFL-1 in the sediment (Fig. 4D), confirming that NRFL-1 and AAT-6 are physically interactive in vivo.

The Loss of NRFL-1 Deteriorates the Membrane Localization of AAT-6 Over Age

Studying consequences of the nfrl-1 deletion to the NRFL-1/AAT-6 complex, we transferred an extrachromosomal array carrying aat-6(tm3501);::GFP::AAT-6 to nfrl-1 mutants. We crossed aat-6(tm3501);::GFP::AAT-6 and nrfl-1(tm3501);::GFP::AAT-6, obtaining an extrachromosomal-array-carrying heterozygote: nfrl-1/+;aat-6. The heterozygote was selfed, yielding homozygous siblings carrying aat-6(tm3501);::GFP::AAT-6. For nfrl-1(ok2292) carrying aat-6(tm3501);::GFP::AAT-6, similarly, a sibling strain carrying both intact nfrl-1 and the reporter gene was prepared as control. Siblings were used for analysis since they were thought to have minimal genetic differences. No noticeable differences in gross anatomy and growth within the sibling strains were observed (data not shown).

The impact of the absence of NRFL-1 on the AAT-6 localization was assessed by epifluorescence imaging. In both nfrl-1 and nfrl-1;aat-6 genetic backgrounds, AAT-6 tagged with GFP [AAT-6::GFP::AAT-6] localized to the luminal surface of the intestinal tube till four-day old, at which the transgenic worms began to lay eggs (Fig. 5A). However, six-day old worms with nfrl-1;aat-6 background failed to retain AAT-6 at the luminal membrane, whereas in aat-6, AAT-6 fluorescence remained along the luminal membrane (Fig. 5A). The luminal fluorescence intensity of AAT-6::GFP::AAT-6 reached a peak at day 4 with significant difference between aat-6 and nfrl-1;aat-6. The fluorescence intensity of whole intestine showed a similar pattern to the luminal intensity (Fig. 5B). The luminal intensity of aat-6 at day 6 was not significantly higher than that of nfrl-1;aat-6. Localization index is defined as luminal membrane intensity divided by whole intestine intensity, quantifying the degree of membrane localization. High index values imply tight membranous localization whereas the value of 1 means complete diffusion. At day 6, AAT-6 was tightly localized to the luminal membrane in aat-6 in contrast to the weak membranous localization in nfrl-1;aat-6 (localization index: 2.08 vs. 1.29; p<0.05) (Fig. 5C). There were no significant differences in localization index at day 2 and 4. Consistent with the fluorescence intensities of the whole intestine at day 6, immunoblot analysis for six-day old worms showed no significant difference in the protein amount of AAT-6 between aat-6 and nfrl-1;aat-6 (Fig. 5D). Combined with the lower localization index, these data suggest that the intracellular fluorescence in the six-day old nfrl-1;aat-6 worm is due to AAT-6 diffused in the cytoplasm in the absence of NRFL-1.

A second mutant nfrl-1(ok2292) also demonstrated a similar decay in luminal membrane localization over age: the membranous localization of AAT-6 was maintained at day 2 and day 4 and deteriorated at day 6 (Fig. S2A). The luminal intensity was significantly lower in nfrl-1(ok2292) at day 6 as well as the whole intestine intensity (Fig. S2B). Compared with the pair of aat-6 and nfrl-1(tm3501);aat-6, non specific gut-granules appeared particularly more evident along the basal intestinal membrane in this pair (Fig. S2A). Localization index was significantly lower in nfrl-1(ok2292) at day 4 and day 6 (Fig. S2C). The index for nfrl-1(ok2292) at day 6 was as low as 1.07, suggesting a nearly complete diffusion.

The Loss of NRFL-1 Accelerates the Decay of Membranous Localization of AAT-6 Over Age

Worms were followed up to ten-day old. The membraneous localization of AAT-6 completely decayed by day 10 both in aat-6 and nfrl-1(tm3501);aat-6 (Fig. S3A). In the other experimental pair (nfrl-1(ok2292) and its control), similarly, the localization became blurry in control strain (Fig. S3B). However, about 20% of the control strain still retained AAT-6 on the membrane (Fig. S3B, bottom). Regardless of the presence of NRFL-1, AAT-6 appeared to fail to localize to the membrane in very advanced age. Such decay in the luminal localization is accelerated in mutants lacking nfrl-1 (Fig. 5, S2 and S3).

NRFL-1 Limits the Mobility of AAT-6 on the Membrane

The dynamic status of AAT-6 in the membrane might be dependent on NRFL-1. To examine the effect of NRFL-1 on the mobility of AAT-6 in the membrane, we carried out a fluorescence recovery after photobleaching (FRAP) analysis in four-day old worms in which AAT-6 is still on the membrane with or without NRFL-1. AAT-6 was mostly immobile when NRFL-1 was present (Fig. 6A and B). In the absence of NRFL-1, a FRAP experiment revealed an exchange of the AAT-6 molecules within the intestinal luminal membrane, ~30% recovery of fluorescence intensity in 300 seconds (Fig. 6B). The increased fluorescence recovery is consistent with the static maintenance of AAT-6 in the luminal membrane of the intestine by NRFL-1.

Discussion

In spic variants of NRFL-1, the double-domain isoform was identified as the dominant nfrl-1 gene product in this study (Fig. 3F and G). We found that AAT-6, a member of the C. elegans AAT (amino acid transporter) family (an orthologue of the mammalian SLC7 family), binds to the PDZ II domain of NRFL-1. In mammals, it has been shown that the PDZ binding motif -D-S/T-X-L has particularly high affinity to the PDZ 1 domains of NHERF1 and NHERF2 [32]. In contrast, the PDZ binding motif -C/T-R-M of AAT-6 preferentially binds to the PDZ II domain of NRFL-1 which is the less homologous to the PDZ domains of
mammalian NHERF1 and NHERF2 (Fig. 1A and Table S1). It is interesting that the PDZ II domain of NRFL-1 may be more adapted to ligands relatively uncommon for mammalian NHERFs such as amino acid transporters.

Immunoblot analysis revealed that NRFL-1 is multiply-phosphorylated (Fig. 3G), similar to NHERF1 which is constitutively phosphorylated [33]. Phosphorylation of NHERF1 has various consequences in its function and localization. Phosphorylated NHERF1 is more localized in the cytoplasm rather than to the plasma membrane and has weaker affinity to ligands such as CFTR, platelet-derived growth factor (PDGF) receptor, and β2-adrenergic receptor [34]. Phosphorylation of NHERF1 regulates the intramolecular interaction in which the C-terminus of NHERF1, which also constitutes a PDZ-binding motif, binds to the second PDZ domain [35]. While the significance of phosphorylation in NRFL-1 is left to future studies, it is possible that phosphorylation and dephosphorylation of NRFL-1 have some functional relevance through the regulation of protein–protein interactions and macromolecule complex building.

The advantage of C. elegans NRFL-1 in the study of PDZ scaffolds is that NRFL-1 is the single worm orthologue of NHERF family, so that the studies in C. elegans should be less susceptible to the redundancy problem which we encounter in mammals. In Drosophila melanogaster, Sip1 is the sole fly orthologue of NHERF protein [4], [36]. Sip1 has a single PDZ domain with 54% identity and 66% similarity to PDZ I of NRFL-1, and 48% identity and

Figure 4. Interaction of AAT-6 with NRFL-1 in vivo. A. Expression of AAT-6 in worms. AAT-6's localization to the luminal surface (single arrowhead) but not to the basal side (double arrowhead) of the intestinal epithelial cells. Scale bar, 25 μm. Non-specific fluorescence on gut granules was seen. More than twenty worms were analyzed. B. Co-localization of AAT-6 and NRFL-1. GFP fluorescence from AAT-6::GFP::AAT-6's top was co-localized with immunostaining of NRFL-1 by anti-NRFL-1 antibody visualized by Cy3-labeled secondary antibody (middle). Bottom image is merged from top and middle images. Confocal images of a representative intestine section (whole worm) are shown. Scale bar: 25 μm. More than five worms were analyzed. C. Intensity profile along the line A–B in the merged image shows an overlapping of the peaks of the NRFL-1 and the AAT-6 signal. D. Immunoprecipitation of NRFL-1/AAT-6 complex from worm lysate. The worm lysate was immunoprecipitated with anti-GFP monoclonal antibody (mouse) and immunoblotted using anti-NRFL-1 antibody. Top: input (2.5%). Middle and bottom: immunoprecipitant was immunoblotted using anti-NRFL-1 antibody and anti-GFP antibody (chicken), respectively. In the middle blot, two major bands of NRFL-1 detected (arrowheads), probably reflecting partial dephosphorylation during immunoprecipitation process. A representative blot of two separate experiments is shown.

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61% similarity to PDZ II of NRFL-1. In fly embryo, Sip1 is found along the luminal side of the epithelium in the intestine, wing imaginal disc, and follicle cells [36]. Despite the similar protein profile of Sip1 with NRFL-1, the Sip1-null fly fails to hatch to larva or die shortly after advancing to the larval stage with remarkably impaired epithelium integrity [36], [37]. Sip1 regulates development of the embryonic epithelium by interacting with actin, moesin, and sterile-20 kinase Slik [36]. In contrast, the nrfl-1 mutants are viable with normal gross appearance, consistent with findings from genome wide RNAi screenings in which the interferences of nrfl-1 do not elicit morphological or lethal phenotypes [38–40]. The viability of the nrfl-1 mutants is an obvious advantage because we are able to study a NHERF-related protein throughout development in a system with low genetic redundancy.

**Figure 5.** Maintenance of AAT-6 on the intestinal luminal membrane by NRFL-1. A. The localization of AAT-61517::GFP::AAT-6518–523 was compared between aat-6 and nrfl-1(tm3501);aat-6 genetic backgrounds. Epifluorescence images of the distribution of AAT-6 in the intestine are shown for worms two, four and six days after hatching. In six-day old worm, the membraneous localization of AAT-6 decayed in nrfl-1(tm3501);aat-6, whereas AAT-6 was retained on the luminal membrane in six days in the presence of NRFL-1 (aat-6). Scale bars: 100 μm. Representative pictures from more than ten worms analyzed for each are shown. B. Fluorescence intensity was measured to quantify the age-dependent regulation. The intensity of the intestinal luminal surface was peaked at day four with significantly stronger signal in aat-6 (gray column) compared with nrfl-1(tm3501);aat-6 (white column). The intensity at day six did not differ significantly between the strains (luminal membrane). C. The fluorescence intensity of the whole intestine showed a similar pattern (whole intestine). The localization index, luminal intensity divided by whole intestine intensity, quantifies the membraneous localization. The six-day old nrfl-1(tm3501);aat-6 worm had a significantly lower score, showing age-dependent decay in luminal localization (localization index). Gray column, aat-6; White column, nrfl-1(tm3501);aat-6. Values are presented with mean ± S.E. (n = 5). D Immunoblot and densitometric analysis of AAT-61517::GFP::AAT-6518–523 in six-day old worm. Densitometric analysis followed by anti-GFP antibody immunobloting exhibited no significant difference in band intensity between the genetic backgrounds. A representative blot was presented with actin as a loading control. The bar graph indicates the relative band intensities of the respective sample. Values are presented with mean ± S.E. (n = 4).

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**Figure 6.** Immobilization of AAT-6 on the intestinal apical membrane by NRFL-1. A FRAP analysis of the AAT-6 dynamics was performed in 4-day old worms. Confocal images of AAT-6 (AAT-61517::GFP::AAT-6518–523) was compared between aat-6 and nrfl-1;aat-6 genetic backgrounds. Top pictures indicate representative images prior to photobleaching (pre-bleach), immediately after photobleaching (post-bleach), and 300 sec after photobleaching (recovery). B. Graph depicts the time course of recovery of AAT-6 fluorescence for nrfl-1;aat-6 (●) and aat-6 (□). Recovery was measured with the pre-bleach fluorescence intensity being 100% and the post-bleach intensity being 0%. The recovery curves were generated from 5 separate experiments and the values were expressed as mean ± S.E. (n = 5). Scale bars: 2 μm.

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In this study we found that NRFL-1 regulates AAT-6 in two aspects: maintenance of its membrane localization (Fig. 5 and S2) and immobilization on the luminal membrane (Fig. 6). Until early breeding stage (up to four-day old), AAT-6 stays on the membrane with or without NRFL-1 (Fig. 5 and S2), and NRFL-1 immobilizes AAT-6 on the membrane (Fig. 6). In late breeding stage (six-day old), in contrast, NRFL-1 is crucial in retaining AAT-6 at the apical membrane: the absence of NRFL-1 causes the internalization of AAT-6. In further advanced age (ten-day old), the deterioration of the membranous localization of AAT-6 occurs in the majority of worms carrying NRFL-1 (Fig. S3), suggesting that such deterioration is a natural course. Localization index, which measures how tightly AAT-6 is membrane-localized, declines with age in the nrlf-1 mutants; it is not the case with the control worms (Fig. 5C and S2C). This suggests that a loss of AAT-6 membranous localization is accelerated with age and that NRFL-1 protects AAT-6 from such aging process. When the interaction with NHERF proteins is disrupted, some membrane proteins are susceptible to internalization and degradation [41–43]. As for NRFL-1/AAT-6 complex, the protein amount of AAT-6 remains unchanged in nrlf-1/aat-6 worm even at six days old, suggesting that the loss of NRFL-1 does not cause the degradation of AAT-6 (Fig. 5D). Without NRFL-1, AAT-6 somehow stays intracellularly without being degraded. While the mechanism underlying age-dependent maintenance of AAT-6 on the intestinal luminal membrane has yet to be clarified, NRFL-1 with two PDZ domains may further bind unidentified proteins essential to keep AAT-6 in the intestinal apical membrane in aged phase.

Taking advantage of the amenability of live-animal imaging in *C. elegans*, a FRAP analysis was applied to the NRFL-1/AAT-6 complex in *vivo* to further examined the role of NRFL-1 in the maintenance of AAT-6 in the membrane (Fig. 6). To our knowledge, this is the first report to apply FRAP analysis in *vivo* to the study of PDZ interactions involving NHERF proteins [21], [44]. In the presence of NRFL-1, the fluorescence recovery of AAT-6 reaches to plateau in 30 s, leaving ~95% of immobilized fraction. The absence of NRFL-1 results in an increased fluorescence recovery of ~35% in 5 min, suggesting that AAT-6 is immobilized by NRFL-1 (Fig. 6). For human parathyroid hormone receptor PTH1R, it was proposed that the receptor in the plasma membrane is immobilized by NHERF1 anchored to the actin cytoskeleton through ERM (Ezrin-Radixin-Moesin) proteins [44]. The distribution of the worm orthologue ERM-1 completely overlaps with NRFL-1: the excretory canal and the luminal surface of the intestine [45], where ERM-1 participates in brush border formation [46]. The physical association between NRFL-1 and ERM-1 in a yeast two-hybrid experiment has been reported [47]. Recently, it was revealed that ERM proteins recognize the C-terminal 13 residues of NHERF1 or 2 for interaction and that the binding sequence is characterized as Motif-1: MDWXXXX(L/I)XXFXL/F, where X denotes any amino acid [48]. NRFL-1 has a C-terminal 13-residue similar to Motif-1: MSLNEKQLVSNM, underpinning the yeast two-hybrid finding. Therefore, it is reasonable to assume that NRFL-1 immobilizes AAT-6 through the interaction with ERM proteins such as ERM-1.

The NHERF-interacting CFTR, whose D-value (diffusion coefficient) was estimated to be 0.99 ± 0.09 × 10^-10 cm^2/s, has a half time recovery of 1–2 min for a bleached circle of ~5 μm diameter in MDCK cells [22]. In contrast, AAT-6 does not reach a 50% recovery in 5 min of observation for ~2 μm bleached circle. AAT-6 shows a much slower recovery in worm than CFTR does in MDCK cells. Our experiment was performed at 25°C, a physiological temperature for *C. elegans*, whereas the CFTR diffusion was measured at 37°C. The temperature is a considerable determinant of molecular motility since the viscosity heavily depends on the temperature. A temperature hike from room temperature (~25°C) to 37°C observes two to four-fold increases in the diffusion coefficients of plasma membrane proteins such as epidermal growth factor receptor in MDCK cells [49]. Differences in the apical surface architecture between MDCK cell and worm intestinal epithelium should also be taken into account. Estimating from cross-section images, worms have 4–6 microvilli in a 1-μm segment of the intestinal brush border [50], whereas in MDCK cells there are as few as 1 or 2 microvilli in 1 μm [51]. The abundance of microvilli in worm is likely a morphological constraint hindering molecular diffusions along the membrane. Otherwise, though it is not mutually exclusive, yet unidentified molecules which limit the motility may be involved in this phenomenon.

In summary, we found that NRFL-1, the sole *C. elegans* orthologue of mammalian NHERF family, is crucial in protecting AAT-6 from the decrement in the intestinal luminal membrane associated with aging as well as in immobilizing the transporter on the membrane. *C. elegans*, which is transparent and has a fast life cycle, facilitated observations of the age-associated change in localization of a transporter supported by scaffold protein. Since NRFL-1 has two PDZ domains, it likely has more binding partners. There might be interactors which interplay with NRFL-1 in an age-dependent manner.

**Materials and Methods**

**Strains and Maintenance**

Strains were seeded with *E.coli* OP50 on NGM agar plates and maintained under standard conditions at 20°C [52]. For large-scale culture, worms were grown in liquid culture as described [53] and were isolated by sucrose flotation [54].

Alleles used were *N2* Bristol wild type, nrlf-1(tm3501), nrlf-1(ok2292), aat-6(tm2881), and nrlf-1(tm517);aat-6(tm2881). After isolated from the TMP/UV library [55], nrlf-1(tm3501) and aat-6(tm2881) were outcrossed eight and five times to *N2*, respectively. The resultant strains were crossed to generate nrlf-1(tm3501);aat-6(tm2881) N2 and nrlf-1(ok2292) was purchased from the Caenorhabditis Genetics Center. The ok2292 strain was outcrossed three times against *N2*. The genotypes were confirmed by single worm PCR [56].

Transgenic lines generated were *N2* Ex[agl::nrlf-1, rol-6(DiEx1006)], *N2* Ex[at-61::gfp::aat-6518–523, rol-6(DiEx1006)], nrlf-1(tm3501) Ex[agl::nrlf-1, rol-6(DiEx1006)], nrlf-1(ok2292) Ex[at-61::gfp::aat-6518–523, rol-6(DiEx1006)], aat-6(tm2881) Ex[at-61::gfp::aat-6518–523, rol-6(DiEx1006)] and nrlf-1(tm517);aat-6(tm2881) Ex[at-61::gfp::aat-6518–523, rol-6(DiEx1006)].

**Yeast Two-hybrid Constructs and Two-hybrid Matching**

The C-terminal end of AAT-6 corresponding to residues 487–523 was obtained by PCR using pcDNA3.1-AAT-6 (full-length) as a template. The fragment was inserted into EcoRI and Xhol sites of the bait vector pEG202 (MoBiTec). For the AAT-6 bait with PDZ-binding motif deletion (ΔTRM), the cDNA fragment of AAT-6 corresponding to residues 487–520 was obtained by PCR and inserted to pEG202.

The fragments of C01F6.6a (full-length), C09H6.2a (residues 14–594), C25F6.2a (full-length), C27A2.6 (residues 9–518), C35D10.2 (residues 1–327), F26D11.1a (residues 113–679), F30F8.3 (full-length), F44D12.1 (residues 4–1094), F44D12.4 (full-length), T14G10.2a (residues 419–1311), and T21G5.4 (full-length) were obtained by PCR using the cDNA clones Yk1663b04,
Expression and Purification of Recombinant Proteins

The cDNA fragment corresponding to residues 487–525 of AAT-6 was cloned between BamHI and XhoI sites of pGEX-6P-1 (GE Healthcare). The plasmid was transformed into E. coli strain BL21 (DE3). The transformed bacteria were grown in LB medium at 37°C until A_{600} between 0.4 and 0.6. Protein expression was induced by 1 mM of isopropyl-β-D-thiogalactopyranoside for 90 minutes. The bacteria were lysed by sonication in 20 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA and Complete Protease Inhibitor Cocktail (Roche). The lysate was centrifuged at 5,000×g for 15 minutes to remove debris. The supernatant was further ultracentrifuged at 350,000×g for 15 minutes. The supernatant was applied to a chromatography column (Poly-Prep Chromatography Columns, BioRad) packed with Glutathione Sepharose 4B beads (GE Healthcare). Washing and elution procedure followed manufacturer’s instructions. The EG48 yeast strain was transformed with pEG202-AAT-6 (487–525), pGNG1 (a GFP reporter vector, MoBiTec), and pGFP-45 carrying one of the prey proteins. The transformed colonies were first assayed for growth on medium lacking leucine. Growing colonies were further assayed for GFP expression in a dark room under a handy UV lamp (wave length: 365 nm, Model UVGL-25, UVP Inc.).

GST Pull-down Assay

For GST pull-down assay, 2 µg of GST fusion protein and 5 µg of recombinant NRFL-1 wild-type or mutant protein were incubated overnight at 4°C in 50 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA, and 0.1% NP-40. After washing the sediment five times with the incubation buffer, the sample was separated on a 10% polyacrylamide gel, immunoblotted, and probed with anti-FLAG rabbit polyclonal antibody (Sigma; dilution 1:20,000). The membrane was reprobed with anti-GST mouse monoclonal antibody (Santa Cruz; dilution 1:10,000).

Transgenic Lines

Transformation of worms followed the standard protocol of exogenous DNA microinjection into the gonads [57].

The λ-Red-based fosmid recombineering technique was used to make transforming constructs [28]. The recombineering kit was kindly gifted by Dr. Oliver Hobert (Columbia University). In brief, for the nrfl-1 reporter gene, the fosmid WRM063dG02 was electroporated into E. coli strain SW105 [58] (distributed by Biological Resource Branch, NCI). A DNA segment containing gfp, FRT/Flp sites, and galK selectable marker was recombined at the immediate upstream of the start codon by heat shock-inducible λ Red recombinase. Arabinoinducible Flp recombinase removed the galK marker as well as a part of the FRT/Flp, resulting in a translational fusion GFP::NRFL-1. A similar strategy to generate an N-terminal GFP fusion of AAT-6 was attempted. However, no expression was observed. Instead, we inserted gfp into the region corresponding to the cytosolic tail with the C-terminal PDZ-binding motif intact. A GFP tag flanking bi-directional four-glycine linkers (4×glycine-GFP-4×glycine) was inserted between glutamine 517 and phenylalanine 518 using a gfp cassette against the fosmid WRM063dC09, resulting in a translational fusion GFP::NRFL-1. A similar strategy to generate an N-terminal GFP fusion of AAT-6 was attempted. However, no expression was observed. Instead, we inserted gfp into the region corresponding to the cytosolic tail with the C-terminal PDZ-binding motif intact. A GFP tag flanking bi-directional four-glycine linkers (4×glycine-GFP-4×glycine) was inserted between glutamine 517 and phenylalanine 518 using a gfp cassette against the fosmid WRM063dC09, resulting in a translational fusion GFP::NRFL-1. A similar strategy to generate an N-terminal GFP fusion of AAT-6 was attempted. However, no expression was observed. Instead, we inserted gfp into the region corresponding to the cytosolic tail with the C-terminal PDZ-binding motif intact. A GFP tag flanking bi-directional four-glycine linkers (4×glycine-GFP-4×glycine) was inserted between glutamine 517 and phenylalanine 518 using a gfp cassette against the fosmid WRM063dC09, resulting in a translational fusion GFP::NRFL-1. A similar strategy to generate an N-terminal GFP fusion of AAT-6 was attempted. However, no expression was observed. Instead, we inserted gfp into the region corresponding to the cytosolic tail with the C-terminal PDZ-binding motif intact. A GFP tag flanking bi-directional four-glycine linkers (4×glycine-GFP-4×glycine) was inserted between glutamine 517 and phenylalanine 518 using a gfp cassette against the fosmid WRM063dC09, resulting in a translational fusion GFP::NRFL-1.
For each construct, multiple transmissible lines were established and one representative line was used for the analysis. *nrfl-1(tm3501);aat-6(tm2081) Ex[aat-6\(^{517+-}\):gfp::aat-6\(^{518-523}\), rol-6\(^{su1006}\)] was generated by crossing *aat-6* (tm2081) *Ex[aat-6\(^{517+-}\):gfp::aat-6\(^{518-523}\), rol-6\(^{su1006}\)] to *nrfl-1* (tm3501); *aat-6* (tm2081). A *nrfl-1* (ok2292) *Ex[aat-6\(^{517+-}\):gfp::aat-6\(^{518-523}\), rol-6\(^{su1006}\)] was outcrossed against N2, yielding *nrfl-1* (ok2292) *Ex[aat-6\(^{517+-}\):gfp::aat-6\(^{518-523}\), rol-6\(^{su1006}\]) and a sibling strain which carries the intact *nrfl-1* and the *gfp* reporter genes.

Protein Extraction and Dephosphorylation-inhibition Assay

Protein extraction was performed following Gendrel et al. [59] with minor modification. Around 70 µl of frozen worm pellet was ground in a mortar and pestle immersed in liquid nitrogen and thawed in 300 µl of ice-cold homogenization buffer (50 mM HEPES pH 7.6, 50 mM KCl, 2 mM MgCl\(_2\), 250 mM sucrose, 1 mM EDTA, and 6 µl of Complete Protease Inhibitor Cocktail (Roche) solution (two tablets in 0.5 ml of H\(_2\)O)). After brief sonication, the sample was cleared at 20,000 g for 15 min. The supernatant was equally split into an experimental group and a control group. The experimental group was supplemented with 20 µl of PhosSTOP phosphatase inhibitor cocktail (Roche) solution (two tablets in 1 ml of H\(_2\)O), while the control group was left untreated. The samples were incubated at 37°C for 90 min at 4°C and briefly sonicated. Anti-NRFL-1 antibody was prepared in PBST (PBST, supplemented with 1% bovine serum albumin, 0.3% Triton X-100, and 1 mM EDTA) at a dilution of 1:25. Anti-NRFL-1 antibody in PBST was incubated with the fixed *nrfl-1* (tm3501) worm overnight at 4°C. After centrifuge, the supernatant was used as the primary antibody.

To study the subcellular localization of NRFL-1 relative to IFB-2, anti-IFB-2 monoclonal antibody (MH33) [61] was used at 1:50. For co-localization study, *aat-6* (tm2081) *Ex[aat-6\(^{517+-}\):gfp::aat-6\(^{518-523}\), rol-6\(^{su1006}\]) worms were fixed as described above. The fixed worms were processed as described elsewhere [62]. The worms were then, treated overnight with the primary antibody followed by Cy3-labeled donkey anti-rabbit IgG (H+L) (Jackson ImmunoResearch Laboratories) as a secondary antibody (dilution: 1:200) at 4°C. The sample was prepared for observation as described [63].

Confocal Microscopic Observation of Worms

Observations were made using a LSM 510 Meta laser scanning confocal microscope with a Plan-Apochromat 63 × 1.4 oil immersion objective lens (Zeiss). For expression pattern analysis, worms anesthetized in 0.4% NaN\(_3\) in M9 were examined under a 488 nm laser line for GFP excitation. Co-localization imaging was carried out under a 488 nm laser line for GFP and a 543 nm laser line for Cy3, respectively. Intensity profile was analyzed by ImageJ (National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997–2012).

Epifluorescence Imaging

Two-day, four-day, six-day, and ten-day old worms were pick up from a synchronized colony of *aat-6* (tm2081) *Ex[aat-6\(^{517+-}\):gfp::aat-6\(^{518-523}\), rol-6\(^{su1006}\)], *nrfl-1* (tm3501); *aat-6* (tm2081) *Ex[aat-6\(^{517+-}\):gfp::aat-6\(^{518-523}\), rol-6\(^{su1006}\)], *nrfl-1* (ok2292); *Ex[aat-6\(^{517+-}\):gfp::aat-6\(^{518-523}\), rol-6\(^{su1006}\]), or the sibling strain of *nrfl-1* (ok2292) *Ex[aat-6\(^{517+-}\):gfp::aat-6\(^{518-523}\), rol-6\(^{su1006}\]). Epifluorescence imaging was carried out by an Olympus BX61 microscope equipped with the MetaMorph version 6.1 software (Molecular Devices) [64]. Worms were anesthetized as above for imaging. All the worms were imaged under identical imaging parameters: UPlanApo 10× objective; exposure time 30 msec for *tm3501* and 70 msec for ok2292; 1×1 binning.

For quantification of the fluorescence intensity, ImageJ was used for demarcation of the region of interest and measurement of the intensity and the area. The intestine and the luminal surface were demarcated. The intensity and the area of the demarcated regions were measured. After subtracted by the background intensity, the mean intensity (the raw intensity divided by the area) was calculated for each sample. The localization index was defined as the mean luminal surface intensity divided by the mean intestine intensity. Data was processed by Prism. The data are expressed as the means ± S.E.
Fluorescence Recovery after Photobleaching (FRAP) Analysis
To prepare worms with minimal movement during observation, four-day-old worms bearing up to five eggs were anesthetized by immersing in 0.4% Na2S in M9 for 30 min [63]. FRAP experiments were carried out in a room air-conditioned at 25°C, using a LSM 510 Meta laser scanning confocal microscope with a Plan-Apochromat 63x/1.4 oil immersion objective lens. An intestinal epithelium cells around the vulva were subjected to photobleach invasion. A circular spot of ~2 µm on the luminal surface of the intestine was photobleached by 20 iterations of the 488 nm laser with 100% laser power transmission. The subsequent time-lapse images were acquired at 0.05% transmission (a 30-second interval up to 300 seconds). Images were collected at a 12-bit intensity resolution over 512x512 pixels at a pixel dwell time of 3.2 µsec. For each strain, five immobilized worms were studied. All the images were acquired under the identical microscope and camera setting. The fluorescence recovery of the region of interest was calculated as \( f_{\text{post}}/f_{\text{pre}} \), where \( f_{\text{pre}} \) denotes the fluorescence intensity before photobleaching, \( f_{\text{post}} \) denotes the fluorescence intensity immediately after photobleaching, and \( t \) denotes the fluorescence intensity at \( t \) seconds after photobleaching [66]. Data was processed by Prism. The data are expressed as the means ± S.E.

Supporting Information
Figure S1 Specific immunostaining of NRFL-1 in C. elegans. Anti-NRFL-1 antibody stained the luminal membrane of intestinal tubes (arrow heads) of wild type worms, whereas it failed to stain the intestine of nrfl-1(tm3501) with occasional non-specific stains in ruptured body wall (arrows). The staining along the body wall in the wild type worm in the right panel is considered non-specific. (TIF)

Figure S2 Recapitulation of the decay in the apical localization of AAT-6 in a second mutant nrfl-1(ok2292). A. As observed in nrfl-1(tm3501);aat-6, the membranous localization of AAT-6 became blurr in nrfl-1(ok2292). The control strain is a sibling of the experimental nrfl-1(ok2292) strain which carries intact nrfl-1. Compared with nrfl-1(tm3501);aat-6, gut granules were more evident particularly in old worms in nrfl-1(ok2292) and its control. Scale bars: 100 µm. Representative pictures from more than ten worms analyzed for each are shown. B. Significantly stronger fluorescence was observed on the intestinal luminal surface at day six in the control (gray column) compared with nrfl-1(ok2292) (white column) (luminal membrane). The intestinal fluorescence was also stronger at day six (whole intestine). C, The localization indexes, luminal intensity divided by intestinal intensity, were higher at day four and six, recapitulating a similar pattern observed in nrfl-1(tm3501);aat-6 (localization index). Gray column, control. White column, nrfl-1(ok2292). Values are presented with mean ± S.E. (n = 5). (TIF)

Figure S3 The membrane retention of AAT-6 in ten-day old worm. A. The distribution of AAT-6 was followed up to day ten to determine whether the loss is a normal occurrence. In the aat-6 worm, the membranous localization of AAT-6 was completely lost by day ten. Scale bars: 100 µm. Representative pictures from more than ten worms analyzed for each are shown. B. In nrfl-1(ok2292) and its control, typically, AAT-6 disappeared from the membrane (top). In ~20% of the 10-day old control worm, the membrane retention was still preserved (arrowed, bottom). Scale bars: 100 µm. Representative pictures from more than nine worms analyzed for each are shown. (TIF)

Table S1 Comparison between NRFL-1 and, PDZK1 and IKEPP. Two PDZ domains of NRFL-1 was compared with PDZ domains of human PDZK1 (519 amino acids) and human IKEPP (305 amino acids). PDZK1 and IKEE have four PDZ domains in tandem. For each domain comparison, identity/similarity (%/%) was assigned as described in Figure 1. BLAST searches using PDZK1 and IKEE as query also converged to NRFL-1. However, the identity/similarity values are lower than those assigned to NHERF1 and 2 (Fig. 1C). (DOC)

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Author Contributions
Conceived and designed the experiments: KH S. Nagamori YMU YK. Performed the experiments: KH YK. Analyzed the data: KH S. Nagamori YMU RO HT DM KHN EKN SM KN YK. Contributed reagents/materials/analysis tools: KH YMU EKN DM S. Nakagomi KHN KN. Wrote the paper: KH YK.

References
1. Harris BZ, Lim WA (2001) Mechanism and role of PDZ domains in signaling complex assembly. J Cell Sci 114: 3219–3231.
2. Zhang Y, Yeh S, Appleton BA, Held HA, Kausalya PJ, et al. (2006) Convergent and divergent ligand specificity among PDZ domains of the LAP and zona occludens (ZO) families. J Bio Chem 281: 22299–22311.
3. Tonkian K, Zhang Y, Naujoks SL, Corell R, Yeh JH, et al. (2008) A specificity map for the PDZ domain family. PLoS Biol 6(9): e239. doi:10.1371/journal.pbio.0060239.
4. Donowitz M, Cha B, Zachou NC, Brett CL, Sharma A, et al. (2005) NHERF family and NHE3 regulation. J Physiol 567: 3–11.
5. Wang S, Raab RW, Schatz PJ, Guggino WB, Li M (1998) Peptide binding consensus of the NHERF PDZ1 domain matches the C-terminal sequence of cystic fibrosis transmembrane conductance regulator (CFTR). FASEB J 12: 1003–1010.
6. Sun F, Hug MJ, Lewarchik CM, Chirs Yun CH, Bradbury NA, et al. (2000) E1KAPP mediates the association of Earrin and protein kinase A with the cystic fibrosis transmembrane conductance regulator in airway cells. J Biol Chem 275: 29539–29546.
7. Wang S, Yue H, Derin RB, Guggino WB, Li M (2000) Accessory protein facilitated CFTR-CFTR interaction, a molecular mechanism to potentiate the chloride channel activity. Cell 103: 169–179.
8. Weinman EJ, Seplock D, Wang Y, Senolik S (1995) Characterization of a protein cofactor that mediates protein kinase A regulation of the renal brush border Na+/H+ exchanger. J Clin Invest 95: 2143–2149.
9. Yun CH, Oh S, Zizak M, Seplock D, Tiao S, et al. (1997) cAMP-mediated inhibition of the epithelial brush border Na+/K+ pump. Nature 387: 29539–29546.
10. Weinman EJ, Seplock D, Wang Y, Senolik S (1995) Characterization of a protein cofactor that mediates protein kinase A regulation of the renal brush border Na+/H+ exchanger. J Clin Invest 95: 2143–2149.
11. Weinman EJ, Seplock D, Wang Y, Senolik S (1995) Characterization of a protein cofactor that mediates protein kinase A regulation of the renal brush border Na+/H+ exchanger. J Clin Invest 95: 2143–2149.
12. Weinman EJ, Seplock D, Wang Y, Senolik S (1995) Characterization of a protein cofactor that mediates protein kinase A regulation of the renal brush border Na+/H+ exchanger. J Clin Invest 95: 2143–2149.
13. Weinman EJ, Seplock D, Wang Y, Senolik S (1995) Characterization of a protein cofactor that mediates protein kinase A regulation of the renal brush border Na+/H+ exchanger. J Clin Invest 95: 2143–2149.
14. Weinman EJ, Seplock D, Wang Y, Senolik S (1995) Characterization of a protein cofactor that mediates protein kinase A regulation of the renal brush border Na+/H+ exchanger. J Clin Invest 95: 2143–2149.
15. Weinman EJ, Seplock D, Wang Y, Senolik S (1995) Characterization of a protein cofactor that mediates protein kinase A regulation of the renal brush border Na+/H+ exchanger. J Clin Invest 95: 2143–2149.
16. Weinman EJ, Seplock D, Wang Y, Senolik S (1995) Characterization of a protein cofactor that mediates protein kinase A regulation of the renal brush border Na+/H+ exchanger. J Clin Invest 95: 2143–2149.
activity of renal urate-anion exchanger URAT1 via its C terminus. J Biol Chem 274: 1595–1602.
2. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215: 403–410.
3. Doyle DA, Lee A, Lewis J, Kim E, Sheng M, et al. (1996) Crystal structures of a complexed and peptide-free membrane protein-binding domain molecule: structural basis of peptide recognition by PDZ. Cell 85: 509–520.
4. Terasaki S, Maesaki R, Hakoshima T (2006) Structural basis for NHERF-1/2-mediated regulation of renal urate-anion exchanger URAT1 via its C terminus. J Biol Chem 281: 5607–5614.
5. Francis GR, Waterston RH (1985) Muscle organization in Caenorhabditis elegans. Nature 318: 75–81.
6. Merrett M, Raper J, Rosewell K (2000) Expression profiling of cells, tissues, and developmental stages of the nematode C. elegans. In: Enzymology 412: 256–282.
7. Gendrel M, Rapti G, Richmond JE, Bessereau JL (2009) A secreted covalent inhibitor of PI3K suppressor EXT genes, is indispensable for heparan sulfate synthesis and embryogenesis. Nature 461: 935–939.
8. Brenner S (1974) The genetics of Caenorhabditis elegans. Genetics 77: 71–94.
9. Gloerich M, ten Klooster JP, Vliem MJ, Koorman T, et al. (2012) Rap2A links cytoskeleton as determined by FRAP. J Cell Sci 117: 3353–3365.
10. Songyang Z, Fanning AS, Fu C, Xu J, Marfatia SM, et al. (1997) Recognition of an amino acid transporters. J Exp Biol 200: 159–165.
11. Gloerich M, ten Klooster JP, Vliem MJ, Koorman T, et al. (2012) Rap2A links cytoskeleton as determined by FRAP. J Cell Sci 117: 3353–3365.
12. Songyang Z, Fanning AS, Fu C, Xu J, Marfatia SM, et al. (1997) Recognition of an amino acid transporter family of PDZ proteins. J Biol Chem 272: 30832–30838.
13. Hall RA, O'Farrell PH (2004) The lateral mobility of Na+ channels in the apical membrane of renal epithelial OK cells is limited by the PDZ domain proteins NHERF1/2, but is dependent on an intact actin cytoskeleton as determined by FRAP. J Cell Sci 117: 3353–3365.
14. Hall RA, O’Farrell PH (2004) The lateral mobility of Na+ channels in the apical membrane of renal epithelial OK cells is limited by the PDZ domain proteins NHERF1/2, but is dependent on an intact actin cytoskeleton as determined by FRAP. J Cell Sci 117: 3353–3365.
15. Hall RA, O’Farrell PH (2004) The lateral mobility of Na+ channels in the apical membrane of renal epithelial OK cells is limited by the PDZ domain proteins NHERF1/2, but is dependent on an intact actin cytoskeleton as determined by FRAP. J Cell Sci 117: 3353–3365.
16. Hall RA, O’Farrell PH (2004) The lateral mobility of Na+ channels in the apical membrane of renal epithelial OK cells is limited by the PDZ domain proteins NHERF1/2, but is dependent on an intact actin cytoskeleton as determined by FRAP. J Cell Sci 117: 3353–3365.
17. Hall RA, O’Farrell PH (2004) The lateral mobility of Na+ channels in the apical membrane of renal epithelial OK cells is limited by the PDZ domain proteins NHERF1/2, but is dependent on an intact actin cytoskeleton as determined by FRAP. J Cell Sci 117: 3353–3365.
18. Hall RA, O’Farrell PH (2004) The lateral mobility of Na+ channels in the apical membrane of renal epithelial OK cells is limited by the PDZ domain proteins NHERF1/2, but is dependent on an intact actin cytoskeleton as determined by FRAP. J Cell Sci 117: 3353–3365.
19. Hall RA, O’Farrell PH (2004) The lateral mobility of Na+ channels in the apical membrane of renal epithelial OK cells is limited by the PDZ domain proteins NHERF1/2, but is dependent on an intact actin cytoskeleton as determined by FRAP. J Cell Sci 117: 3353–3365.
20. Hall RA, O’Farrell PH (2004) The lateral mobility of Na+ channels in the apical membrane of renal epithelial OK cells is limited by the PDZ domain proteins NHERF1/2, but is dependent on an intact actin cytoskeleton as determined by FRAP. J Cell Sci 117: 3353–3365.
21. Hall RA, O’Farrell PH (2004) The lateral mobility of Na+ channels in the apical membrane of renal epithelial OK cells is limited by the PDZ domain proteins NHERF1/2, but is dependent on an intact actin cytoskeleton as determined by FRAP. J Cell Sci 117: 3353–3365.
22. Hall RA, O’Farrell PH (2004) The lateral mobility of Na+ channels in the apical membrane of renal epithelial OK cells is limited by the PDZ domain proteins NHERF1/2, but is dependent on an intact actin cytoskeleton as determined by FRAP. J Cell Sci 117: 3353–3365.