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Drosophila fear of intimacy Encodes a Zrt/IRT-like Protein (ZIP) Family Zinc Transporter Functionally Related to Mammalian ZIP Proteins*

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Zinc is essential for many cellular processes, and its concentration in the cell must be tightly controlled. The Zrt/IRT-like protein (ZIP) family of zinc transporters have recently been identified as the main regulators of zinc influx into the cytoplasm (1); however, little is known about their *in vivo* roles. Previously, we have shown that *fear of intimacy* (*foi*) encodes a putative member of the ZIP family that is essential for development in *Drosophila* (2). Here we demonstrate that FOI can act as an ion transporter in both yeast and mammalian cell assays and is specific for zinc. We also provide insight into the mechanism of action of the ZIP family through membrane topology and structure-function analyses of FOI. Our work demonstrates that *Drosophila* FOI is closely related to mammalian ZIP proteins at the functional level and that *Drosophila* represents an ideal system for understanding the *in vivo* roles of this family. In addition, this work indicates that the control of zinc by ZIP transporters may play a critical role in regulating developmental processes.

Zinc is an essential ion for many diverse cellular processes and can be a co-factor or structural component for many types of proteins. The regulation of cellular zinc concentration is crucial, because both zinc deficiency and excess can be detrimental to cells. In humans, a zinc deficiency can cause major health problems such as growth retardation, and defects in the immune system, central nervous system, and gastrointestinal tract function (3). Ion transporters belonging to either of two protein families regulate cellular zinc levels: the Zrt/IRT-like protein (ZIP)[1] family (1) and the cation diffusion facilitator (CDF) family (4). ZIP proteins are involved in zinc influx to the cytoplasm from the plasma membrane or intracellular organelles, whereas the complementary CDF proteins efflux zinc from the cytoplasm to membrane-bound compartments or outside the cell.

The ZIP family was named after its first identified members Zrt1 (5) and Zrt2 (6), the main zinc transporters in *Saccharomyces cerevisiae*, and IRT1 (7), the main iron transporter in roots of *Arabidopsis*, and is conserved from bacteria to humans. Several features define members of the ZIP family, including a conserved transmembrane structure and a highly conserved "signature sequence," also known as the HELP domain in some family members (see Fig. 1*B*). Some ZIP proteins have been shown to transport zinc specifically (5, 6, 8–11), whereas others, such as IRT1, have broader substrate specificity (12).

Currently, little is known about the *in vivo* roles of these zinc transporters, particularly in the animal kingdom. Recent work indicates that careful regulation of zinc homeostasis is likely to be essential for proper embryonic development and in disease prevention. Human ZIP4 (SLC39A4) has been identified as the gene mutated in the genetic disorder acrodermatitis enteropathica (13, 14), which results in epidermal lesions, gastrointestinal defects, and infant mortality. These symptoms mimic those associated with dietary zinc deficiencies and can be treated with high doses of dietary zinc. The mouse homolog mZIP4 can act as a zinc transporter (10), and when mutations found in acrodermatitis enteropathica patients are introduced into mZIP4 they affect the subcellular localization or transport function of the protein (15). Thus, defective zinc transport by hZIP4 is likely the cause of acrodermatitis enteropathica, although it remains unclear how these defects in zinc transport contribute to the physiological symptoms observed in acrodermatitis enteropathica patients.

The first ZIP family member identified as critical during development is encoded by the *Drosophila* gene, *fear of intimacy* (*foi*). *foi* is required for the proper formation of the embryonic gonad, which gives rise to either the testis or ovary, and the tracheal system, which forms the respiratory system of *Drosophila* larvae (2). In both of these processes, FOI is essential for establishment of proper tissue architecture but is not required for cell identity. Thus, during *Drosophila* development, *foi* appears to play a specific role in the morphogenesis of these tissues. Recently, another ZIP family member was also shown to play an essential developmental role; Zebrafish LIV-1 is essential for the proper cell movements during gastrulation (16). Therefore, two known *in vivo* roles of ZIP family members involve the regulation of tissue morphogenesis during embryonic development. If ZIP family members in these model systems function similarly to their mammalian counterparts, they will provide an excellent resource for understanding how zinc transporters act *in vivo* to regulate development and physiology.

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[1] The abbreviations used are: ZIP, Zrt/IRT-like protein; Endo H, endoglycosidase H; IRT, iron-responsive transporter; TM, transmembrane; Zrt, zinc-responsive transporter; HA, hemagglutinin; HEK, human embryonic kidney; PBS, phosphate-buffered saline; h, human; m, mouse; UAS, upstream activating sequence.

To determine whether *Drosophila* FOI is a *bona fide* ZIP protein capable of transporting zinc, we conducted an extensive characterization of the FOI protein. In this paper, we investigate the membrane topology of FOI under conditions where we can verify that the protein retains full biological activity. We demonstrate that FOI can function as a zinc transporter in both yeast and mammalian cells and has similar ion specificity to its mammalian counterparts. Finally, we present a structure-function analysis of FOI that provides insight into the mechanism of action of the ZIP family. Our work indicates that *Drosophila* ZIP family members are likely to be highly similar in function to mammalian ZIP proteins.

EXPERIMENTAL PROCEDURES

Plasmids—Yeast plasmids pFL61, pMC5 (pFL61-yZRT1), and pA6 (pFL61-aZIP1) are as in Ref. 7. A 3× hemagglutinin-epitope (HA) tag was inserted into three positions in FOI (pKS2.4z, (2)), the N terminus (after amino acid 186), between TM3 and TM4 (after amino acid 531), and the C terminus (after amino acid 704). These constructs were subcloned into pUAST using the EcoRI and NotI restriction sites for expression in *Drosophila* S2 cells and embryos. The C-terminal HA-FOI construct was subcloned into pCMV-sport6 (Invitrogen) using EcoRI and NotI for expression in mammalian cells and into pFL61 (17) via a PCR strategy to generate NotI restriction sites in FOI for expression in yeast. The following site-directed mutations were introduced into the C-terminal HA-FOI construct using QuikChange (Stratagene): D308A, H554A, E584A/E588A/D591A, and Y646A. The ΔN mutation was generated by introducing two unique AscI sites in the N terminus of FOI using QuikChange to delete amino acids Asp^{23}-Asp^{254} (Q22GRAK255). All of the mutated versions of FOI were subcloned into pCMV-sport6 and pFL61 for expression in mammalian and yeast cells, respectively. All cloning strategies are available upon request.

Fly Stocks and Transgenic Animals—ru st faf lacZ e ca flies were used as wild-type. Transgenic *Drosophila* were generated for each of the three UAS-HA-FOI constructs described above using standard genomic transformation methods (18). UAS-HA-FOI constructs were expressed using the Gal4 transcriptional activator localized to either the mesoderm (*twist*-Gal4 (19)) or trachea (*breathless*-Gal4 (20)). Rescue experiments were conducted with two independent UAS-HA-FOI transgenic lines in a genetic background lacking endogenous *foi* activity ($foi^{20.71}$), as described previously (2). At least 40 hemi-embryos were analyzed for each genotype.

Computer Analysis of Sequence Data—Multiple sequence alignments and a phylogenetic tree were constructed using Clustal in the Lasergene software (DNAStar, Inc). We considered residues conserved within a subfamily or group if 80% of its members share an identical amino acid not found in proteins of the other subfamily or group. Transmembrane domain predictions were performed using the programs listed in Table I, *N*-linked glycosylation predictions were computed using PROSITE (21), and signal sequence predictions were computed with SignalP V2.0 (22) and PSORT II.

In Vitro Translation and Glycosylation Assays—pKS2.4z (FOI) and ΔN-FOI were transcribed and translated *in vitro* in rabbit reticulocyte lysate (Promega) in the presence or absence of canine microsomal membranes (Promega) according to manufacturer's instructions. The protein was labeled with [^{35}S]Met (Amersham Biosciences) and run on SDS-polyacrylamide gels. Samples were treated with endoglycosidase H (Endo H) (Calbiochem), as done previously (38), to remove *N*-linked sugars. Briefly, 15 μl of lysate, 4 μl of 5× reaction buffer (250 mM sodium phosphate buffer, pH 7.0), and 1 μl of Denaturing solution (2% SDS, 1 M β-mercaptoethanol) were heated at 100 °C for 5 min and cooled to room temperature. 1 μl of Endo H was added prior to incubation overnight at 37 °C. S2 cell extracts were prepared by homogenizing the cells in 17 μl of denaturing buffer and heating at 100 °C for 5 min. Prior to incubating overnight at 37 °C, 2 μl of reaction buffer and 1 μl of Endo H were added. After spinning the lysate at 14,000 for 5 min, 30–35 μg of protein was loaded onto an SDS polyacrylamide gel, and a Western blot was performed essentially as described previously (39).

Cell Culture and Transient Transfection—*Drosophila* S2 cells were cultured at room temperature in Schneider's medium (Invitrogen) containing 10% fetal bovine serum and transfected as described previously (2). Briefly, UAS-HA-FOI constructs were co-transfected with an *actin*-Gal 4 (gift from K. Howard) using Cellfectin (Invitrogen) according to the manufacturer's instructions. HEK293 cells were cultured under 5% CO_2 in high glucose Dulbecco's modified Eagle's medium (Invitrogen) containing 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 10% fetal bovine serum. Cells (2×10^5) were seeded in 24-well poly-L-lysine-coated plates and transfected with the pCMV-Sport6 vector or pCMV-Sport6 expressing FOI (wild-type or mutant). Transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Transfection efficiencies were typically 60%. Between 36 and 48 h after transfection, the cells were used for zinc uptake assays or Western blots (15).

Immunofluorescence Microscopy—Immunolabeling of S2 cells with permeabilization was done essentially as described previously (2). For immunolabeling of HEK293 with permeabilization, cells were fixed in 4% paraformaldehyde for 30 min at room temperature. After washing with 0.25% NH_4Cl in PBS, the cells were permeabilized in PBS containing 0.1% Triton X-100. The cells were incubated with primary antibody in PBS containing 0.1% Triton X-100 and 0.5% bovine serum albumin for 1 h at room temperature, washed in PBS, and incubated with secondary antibody in PBS containing 0.1% Triton X-100 and 0.5% bovine serum albumin for 1 h at room temperature. For immunolabeling without permeabilization, S2 and HEK293 cells were incubated with primary antibody in PBS containing 0.1% bovine serum albumin for 30 min at 4 °C prior to fixation. Cells were then washed in PBS, and fixation and secondary antibody labeling was conducted as with permeabilized cells. Primary antibody and dyes used include mouse anti-HA (Roche Diagnostics, 0.8 μg/ml) and the nuclear dyes 4,6-diamidino-2-phenylindole (2 μg/ml) and OliGreen (Molecular Probes, 1:10,000). Alexa-Fluor-conjugated secondary antibodies (Molecular Probes) were used at a 1:500 dilution according to manufacturer's instructions. Cells were mounted in 70% glycerol containing 2.5% 1,4-diazabicyclo[2.2.2]octane (Sigma) and analyzed by either a Deltavision Deconvolution or Zeiss LSM 510 Meta Confocal microscope.

Yeast Complementation Assays—The following yeast strains were used, ZHY3 (*MATα zrt1*::*LEU2 zrt2*::*HIS3 ade6 can1 his3 leu2 trp1 ura3*) and DY1459 (6). Cultures were selectively grown overnight in liquid minimal medium to an A_{600} of 0.6–1.0 and diluted to an A_{600} of 0.25. Serial 1:5 dilutions were made in rich medium and were spotted onto selective minimal solid medium. Plates were incubated for 3 days at 30 °C prior to analysis.

^{65}Zinc Uptake Assays—Zinc assays were performed essentially as described previously (10). Parallel experiments were conducted with empty vector (pCMV-sport6) to measure endogenous ^{65}Zn uptake rate, which was subtracted from the rate of ^{65}Zn uptake in cells expressing FOI (wild-type or mutant) to obtain net zinc uptake values. Cell-associated radioactivity was measured with a Packard Auto-Gamma 5650-counter. Cells grown in parallel to those used for uptake experiments were washed three times with ice-cold uptake buffer, resuspended in PBS buffer containing 0.1% SDS and 1% Triton X-100 for cell lysis, and then assayed for protein content using a Bradford assay kit (Bio-Rad). Zinc accumulation and uptake rates were normalized to the protein concentrations of these cell lysates. Metal salts were obtained from Sigma and prepared as described previously (10). Michaelis-Menten constants were determined by nonlinear interpolation of the data, using Prism (version 4.0a for Windows, GraphPad Software, San Diego, CA).

RESULTS

FOI Is a Member of the ZIP Family of Ion Transporters—To determine the relationship of FOI to the ZIP family, we compared the FOI protein sequence to other family members. Eukaryotic members of the ZIP family can be divided into the ZIP I/II and LIV-1 subfamilies (Fig. 1*A* (40)) based on several conserved features, including the extended, histidine-rich N terminus of the LIV-1 subfamily (Fig. 1*B*). These subfamilies have further diverged into individual groups, the ZIP I/II subfamily into the ZIP I and ZIP II groups (40) and the LIV-1 subfamily into the LIV-1A and LIV-1B groups (Fig. 1*A*). These divergences are most obvious within the signature sequence domain, also referred to as the HELP domain in the LIV-1 subfamily, where individual groups can be defined by conservation of characteristic amino acids (Fig. 1*C*). Additional group-specific residues are found in other domains, such as in transmembrane domains (TM) 2 and 7 (Fig. 1*C*). Interestingly, *Drosophila* ZIP family members are found within each group that contains a mammalian ZIP family member (Fig. 1*A*). Individual *Drosophila* proteins are more closely related to their mammalian homologs than to other *Drosophila* ZIP family members (Fig. 1, *A* and *B*). This indicates that the distinct groups within

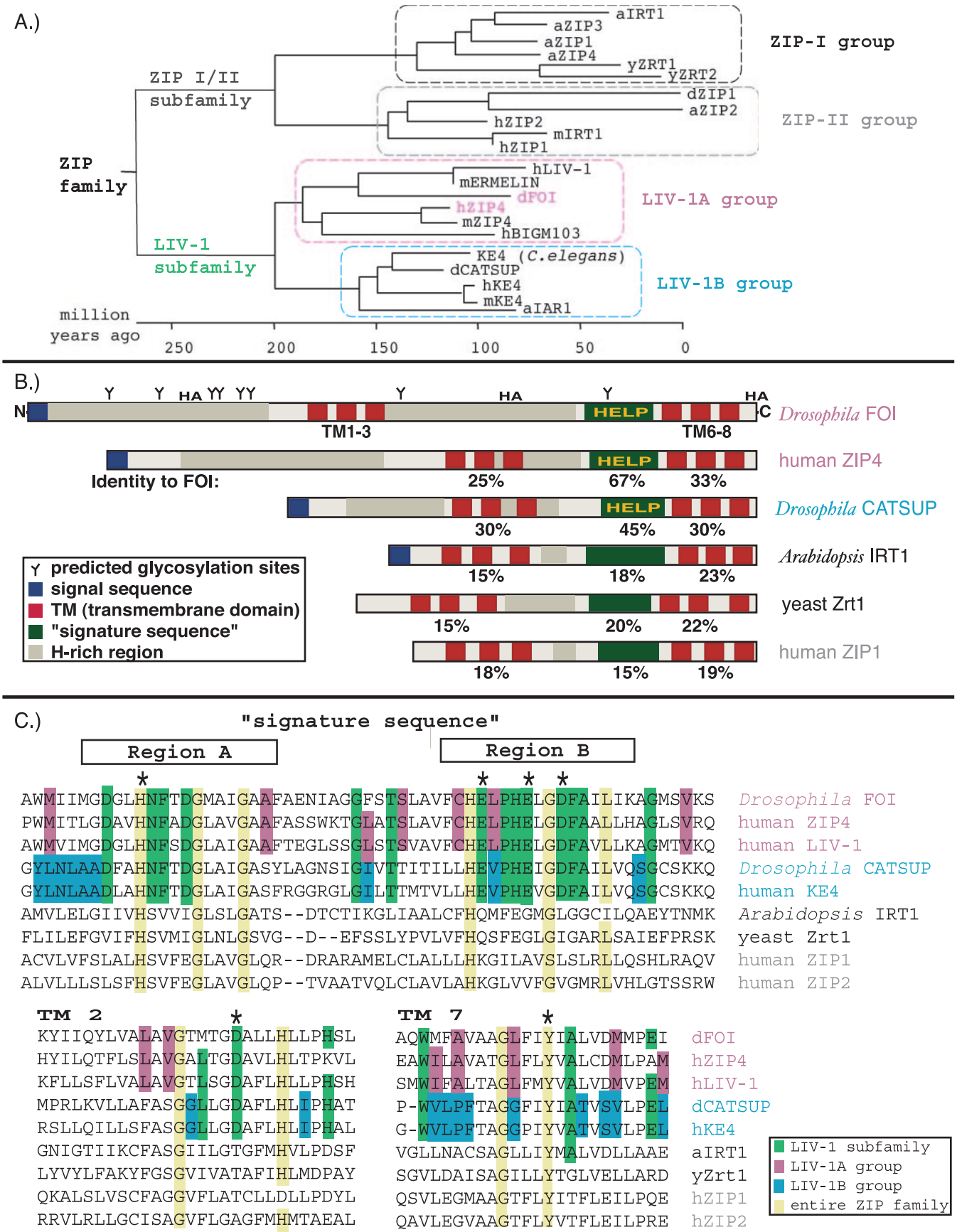


FIG. 1. **FOI, a ZIP protein, belongs to the LIV-1 subfamily.** *A*, phylogenetic tree of several ZIP family members indicating that the family is divided into two subfamilies, ZIP I/II and LIV-1, which can be further divided into distinct groups. Note that FOI belongs to the LIV-1A group. *B*, diagram of several characterized ZIP proteins from different groups. Conserved protein domains are color-coded as indicated in the key. Percentage of sequence identity to FOI within TM1–3, the signature sequence domain or TM6–8 is indicated. The location of predicted N-glycosylation sites (*Y*) and inserted HA-tags (*HA*) are indicated on the FOI protein. *C*, sequence alignment of highly conserved domains (as indicated) of representative ZIP proteins from each group. Residues conserved in the entire ZIP family (*yellow*), the LIV-1-subfamily (*green*), LIV-1A group (*pink*), and LIV-1B group (*blue*) are indicated. *Asterisks* indicate the location of mutations tested for zinc transport ability in FOI.

TABLE I
Transmembrane domain predictions

TM domain prediction programs	FOI		hZIP-1	
	Signature sequence	Total	Signature sequence	Total
SUSUI (23)	0	6	2	8
DAS (24)	1	8	2	8
HMMTOP 2.0 (25, 26)	1	6	2	8
TMPred	1	7	2	8
TMHMM 2.0 (28)	0	6	0	6
SMART (29, 30)	0	6	1 or 2	8 or 9
TMAP (31, 32)	2	8	2	7
PSORT II	0	5	2	7
PRED-TMR (34)	1	6	1	5
Split 3.1 Split 3.1 (35)	0	6	2	8
TM-Finder (36)	1	6	2	7
TopPred 2 (37)	2	8	2	8

the ZIP family existed prior to the divergence of vertebrate and invertebrate lineages. FOI is a member of the LIV-1A group within the LIV-1 subfamily, and is closely related to the human and mouse LIV-1 and ZIP4 proteins.

Divergence in the signature sequence domain observed between the two ZIP subfamilies leads to differences in the predicted membrane topology in this region. Although computer algorithms generally agree about the predicted TM character of TM1–3 and TM6–8 throughout the ZIP family (Table I), the topology of the signature sequence remains unclear. This domain is variably predicted to form 0, 1, or 2 TM domains, depending on the protein and prediction program used (for consistency with the literature, we have counted this domain as TM4 and TM5). Generally, members of the ZIP I/II subfamily are predicted to have 2 TM domains within their signature sequence domain. In human ZIP1, this region is calculated to form 2 TM domains by ten of twelve computer algorithms used (Table I). TM predictions in this region are much less consistent for members of the LIV-1 subfamily, such as hLIV-1 (41) and FOI. For FOI, five algorithms predict 0 TM domains, five predict 1 TM domain, and two predict 2 TM domains in this region. Thus, it is critical to determine the actual membrane topology of ZIP family members *in vivo*.

FOI Has Extracellular N and C Termini—We wanted to experimentally address the membrane topology of FOI using a full-length functional protein. To do this, a 3× HA-epitope tag was inserted into the N terminus, middle (after TM3), or C terminus of FOI (Fig. 1*B*), and these constructs were transfected into cultured *Drosophila* S2 cells. Immunolabeling with an anti-HA antibody revealed that N-terminal HA-FOI could be detected in both permeabilized and non-permeabilized cells (Fig. 2*A*). Because only extracellular epitopes can be immunolabeled in non-permeabilized cells, this indicates that the N terminus of FOI is extracellular. The middle region of FOI was detected only after permeabilization, confirming that this large, unconserved loop is cytoplasmic (Fig. 2*A*). The predictions for the localization of the C terminus of FOI vary depending on the number of TMs in the signature sequence. If this region contains a single TM, and TM6–8 are as predicted, the C terminus of FOI should be intracellular. If the signature sequence contains 0 or 2 TMs, the C terminus should be extracellular. We found that the C-terminal HA-FOI construct could be detected in both permeabilized and non-permeabilized cells, indicating that it is extracellular. Thus, the signature sequence is unlikely to have a single TM and likely has either 0 or 2 TM domains (Fig. 2*B*).

To confirm that the HA-tagged versions of FOI retain biological activity, we tested the ability of each of these constructs to supply FOI function *in vivo* in a transgenic rescue assay (2). *foi* mutant embryos have defects in gonad coalescence, in which the cells of the gonad fail to properly associate with one another, and in trachea formation, in which some tracheal branches fail to connect correctly with neighboring branches (2, 42). Expression of each HA-tagged version of FOI in the appropriate tissue was able to fully rescue the gonad and tracheal defects normally observed in *foi* mutants (Fig. 2, *C* and *D*). Thus, insertion of the HA-tag in the N terminus, middle, or C terminus of FOI does not disrupt the ability of the protein to function *in vivo*, indicating that the tagged proteins accurately represent the membrane topology of the wild-type FOI protein.

The N Terminus of FOI Is Glycosylated—FOI contains eight consensus *N*-glycosylation sites, six on the N terminus, one between TM3 and TM4, and one in the signature sequence domain (Fig. 1*B*). To determine which, if any, of these sites are glycosylated, we translated FOI *in vitro* in the presence or absence of canine microsomal membranes, which allow for co-translational processing of the protein. In the presence of microsomes, FOI was detected at both the predicted, unprocessed size and at a higher molecular mass (Fig. 3*A*). The larger protein was Endo H-sensitive (Fig. 3*A*) indicating that FOI can be glycosylated *in vitro*. To confirm that FOI is a glycoprotein *in vivo*, we expressed HA-tagged FOI in *Drosophila* S2 cells. Again, FOI was observed at two molecular masses, the larger of which is Endo H-sensitive (Fig. 3*B*) confirming that FOI is a glycoprotein (in contrast to mammalian cells, mature glycoproteins in insects are often Endo H-sensitive (43)). To determine whether glycosylation occurs on the N terminus of FOI, we deleted the entire N terminus (ΔN) and translated the resulting protein *in vitro*. The ΔN protein was detected at approximately the same molecular mass in both the presence and absence of microsomes and was not Endo H-sensitive (Fig. 3*C*). These data suggest that the N terminus is the only region of FOI that is glycosylated, further indicating that this domain is extracellular and that the unglycosylated region of the protein between TM3 and TM4 is cytoplasmic.

FOI Functions as a Zinc Transporter in Both Yeast and Mammalian Cells—We next wanted to determine whether FOI can function as a zinc transporter and is therefore a true member of the ZIP family. Mammalian LIV-1 subfamily members have been shown to affect intracellular zinc accumulation (41, 44), and for mZIP4 and mZIP5, this has been demonstrated to be due to an increased rate of zinc uptake (10, 11). We first utilized a yeast cell assay (7) to determine whether FOI exhibits the characteristics of a zinc transporter. Expression of HA-FOI in a zinc transporter-deficient yeast strain (*zrt1*Δ*zrt2*Δ) rescued the growth defects of these mutants in low zinc conditions. Although FOI was not able to complement the growth defects of the *zrt1*Δ*zrt2*Δ strain as efficiently as the endogenous yeast transporter, yZrt1, its rescue was similar to that of the *Arabidopsis* zinc transporter, aZIP1 (7) (Fig. 4*A*) suggesting that FOI can act as a zinc transporter in yeast.

To assay the function of FOI as a zinc transporter more directly, we expressed HA-FOI in mammalian cultured cells (HEK293) and assayed for ^{65}Zn accumulation. FOI-expressing cells (Fig. 4*B*) accumulated significantly more ^{65}Zn than untransfected control cells over the course of the assay (Fig. 4*C*). FOI effected ^{65}Zn accumulation by affecting the rate of ^{65}Zn uptake (Fig. 4*D*); cells expressing FOI exhibited a similar K_m (2.21 ± 0.12 μM) with the control cells (2.16 ± 0.19 μM) but a higher V_{max} (7.69 ± 0.11 *versus* 4.16 ± 0.10 pmol of zinc/min/mg of protein).

To measure the ion specificity of FOI, we assessed the ability of different metal cations to compete for FOI-dependent ^{65}Zn uptake. FOI is largely zinc-specific; no cation tested could compete for ^{65}Zn uptake as efficiently as zinc itself (Fig. 4*E*). A 10-fold excess of silver or nickel exhibited moderate competi-

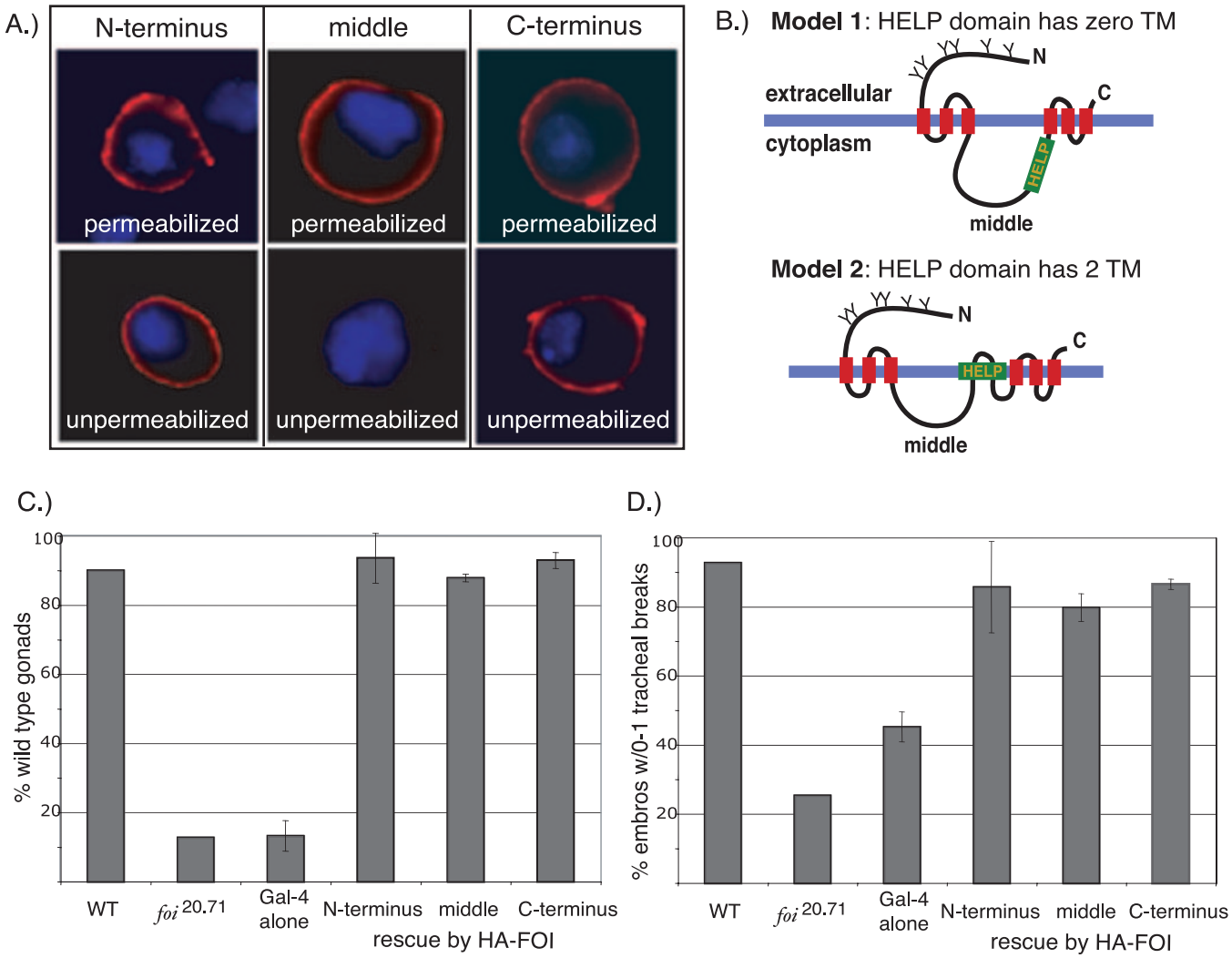


FIG. 2. **Membrane topology of FOI.** *A*, deconvolution images of *Drosophila* S2 cells transfected with the three HA-FOI constructs as indicated, and incubated with anti-HA antibody (*red*) before or after membrane permeabilization. Extracellular regions of FOI are recognized with anti-HA in both permeabilized and non-permeabilized cells, whereas intracellular regions are detected only after permeabilization. DNA is stained with DAPI (4,6-diamidino-2-phenylindole (*blue*). *B*, models for the membrane topology of FOI in which the signature sequence domain contains either 0 or 2 transmembrane domains. *Y* indicates putative glycosylation sites. *C* and *D*, *in vivo* rescue assay of transgenic *Drosophila* expressing HA-FOI in the mesoderm (*C*, *twist*-Gal4) or trachea (*D*, *breathless*-Gal4) of embryos mutant for endogenous *foi*. *Error bars* represent the S.D. obtained from the average percent rescue from two independent insertion lines. *C*, percentage of wild-type gonads. *D*, percentage of wild-type hemi-embryos (defined by having only 0 or 1 defective tracheal branches).

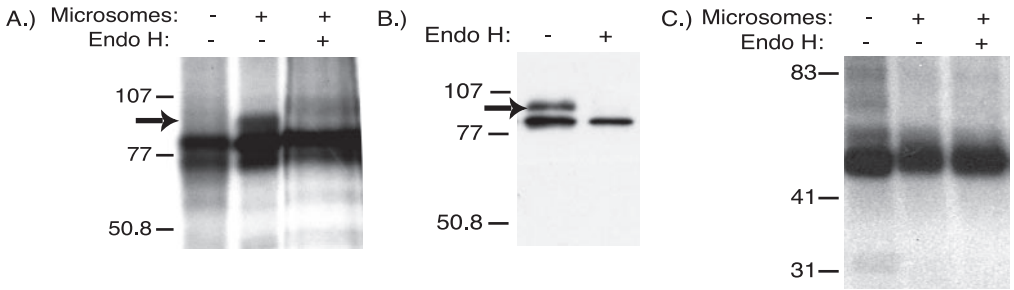


FIG. 3. **FOI is a glycoprotein.** *A*, *in vitro* translation of FOI [^{35}S]Met-labeled protein in the presence and absence of microsomal membranes. Glycosylation was determined by comparing the molecular mass of the protein produced in the presence (~80 and ~95 kDa, *lane 2*) and absence of microsomes (~80 kDa, *lane 1*). The sample was treated with Endo H to remove *N*-linked glycosylation (*lane 3*). Note that following Endo H treatment, FOI is detected at only ~80 kDa. *Arrow* indicates glycosylated protein. *B*, Western blot of cell lysates from *Drosophila* S2 cells transfected with HA-FOI and probed with anti-HA to detect FOI. Lysate in *lane 2* was treated with Endo H. HA-FOI is detected at ~80 and ~95 kDa in untreated samples (*lane 1*) but only at ~80 kDa after Endo H treatment (*lane 2*). *Arrow* indicates glycosylated protein. *C*, *in vitro* translation of the ΔN [^{35}S]Met-labeled protein in the presence and absence of microsomal membranes. ΔN is detected at ~50 kDa in the presence (*lane 2*) and absence of microsomal membranes (*lane 1*), as well as after Endo H treatment (*lane 3*).

tion that was not significantly increased at 50-fold excess. Some other cations showed a modest competition at 50-fold excess, but none were as potent as a 10-fold excess of zinc (Fig. 4*E*). The competition profile for FOI-dependent ^{65}Zn uptake is different from the competition profile observed with the endogenous uptake system present in control HEK293 cells (10). This

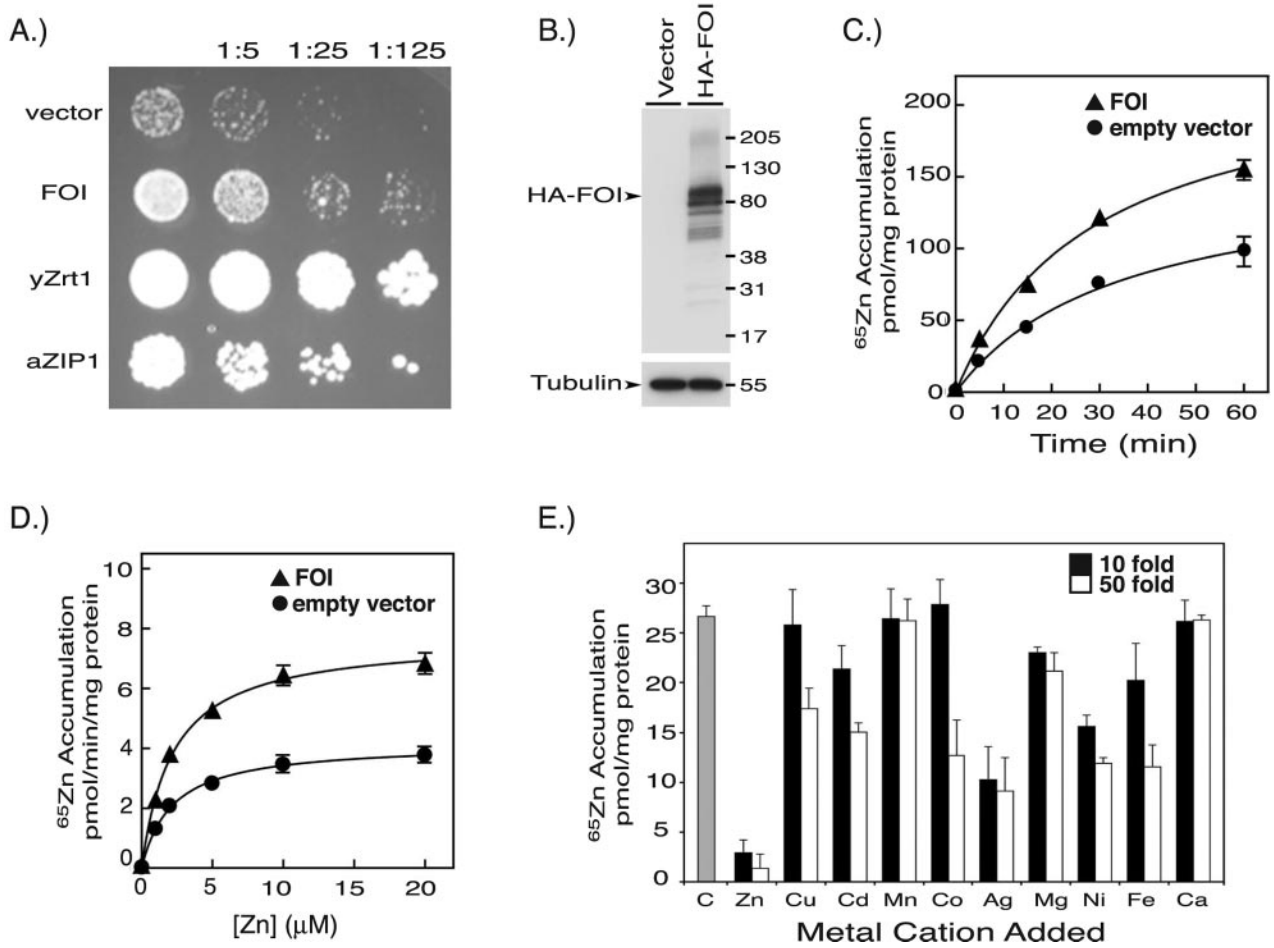
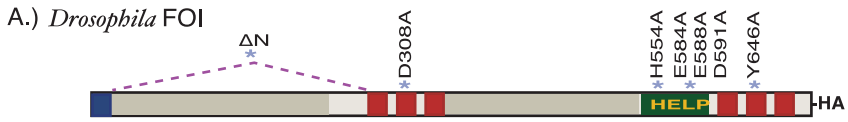


FIG. 4. **FOI functions as a zinc-specific transporter.** *A*, growth of *zrt1*Δ*zrt2*Δ yeast cells expressing empty vector (pFL61), FOI (pFL61-FOI), yZrt1 (pMC5), and aZIP1 (pA6) on low zinc medium grown for 3 days at 30 °C. *B*, Western blot of empty vector (pCMV-sport 6) and HA-FOI (pCMV-FOI) expression in HEK293 cells, probed with anti-HA antibody to detect FOI. *C* and *D*, characterization of zinc uptake kinetics in HEK293 cells transiently transfected with either empty vector (pCMV-sport6) or FOI (pCMV-FOI). Each point represents the mean in a representative experiment ($n = 3$), and the *error bars* indicate ±1 S.D. *C*, zinc accumulation assayed in cells expressing the empty vector or FOI with 5 μM ^{65}Zn at 37 °C. *D*, concentration dependence of zinc uptake activity determined over a range of ^{65}Zn concentrations in cells incubated for 15 min with labeled zinc. *E*, the ion specificity of FOI was assayed via the ability of various metal ions to inhibit ^{65}Zn uptake in transiently transfected HEK293 cells. Either a 10- or 50-fold molar excess of competing ion was added to uptake buffer containing 1.5 μM ^{65}Zn for 15 min. Zinc uptake was compared with cells incubated in the absence of inhibitor (*C*, *hatched bar*). Parallel experiments were conducted using cells transfected with an empty vector to measure endogenous ^{65}Zn uptake rate, which was subtracted from FOI expression vector values to obtain net zinc uptake values. All ions were present as divalent cations. Each *bar* represents the mean in a representative experiment ($n = 3$) and the *error bars* indicate ±1 S.D.

result, combined with the ability of *Drosophila* FOI to function in both yeast and mammalian cells, indicates that FOI is a zinc transporter rather than a modulator of an endogenous zinc transporter.

Conserved Residues in the TM and Signature Sequence Domains Are Essential for Zinc Transport by FOI—We conducted a structure-function analysis of FOI to better understand how ZIP proteins function as zinc transporters. In addition, we wanted to determine whether characteristics that are divergent between different ZIP subfamilies are involved in zinc transport or other subfamily-specific functions. We generated mutations in the N-terminal (ΔN), TM2 (D308A), TM7 (Y646A), and signature sequence (H554A, E584A/E588A/D591A) domains of FOI (Fig. 5*A*) within the C-terminal HA-tagged FOI protein. The expression of each mutant protein was examined in HEK293 cells by immunostaining for the C-terminal HA tag, and all were detected at levels similar to wild-type HA-FOI (Fig. 5*B*). In addition, the D308A, H554A, and E584A/E588A/D591A mutations all exhibited cell surface localization equivalent to the wild-type protein (Fig. 5*B*). Although the ΔN and Y646A mutants were predominantly localized to the cell surface, these proteins also showed increased intracellular staining, suggesting that a portion of the protein was mislocalized (Fig. 5*B*). To examine membrane topology in the mutant proteins, we immunostained for HA in unpermeabilized cells. In each case, the C-terminal HA tag was still detected, similar to the wild-type HA-FOI, indicating that the proper membrane topology (extracellular C terminus) has been maintained (data not shown). Similar results were obtained when these mutated proteins were expressed in *Drosophila* S2 cells (data not shown).

The large N terminus present in FOI exhibits no sequence homology with other LIV-1 subfamily members and is absent in the ZIP I/II subfamily. However, this domain is histidine-rich in LIV-1 subfamily members, a feature that may be functionally important to coordinate zinc ions. The ΔN protein exhibited reduced complementation of the zinc transporter-deficient yeast strain compared with wild-type HA-FOI (Fig. 6*A*). Similarly, zinc influx by the ΔN protein was severely reduced in mammalian cells (Fig. 6*B*), suggesting that the N terminus of FOI is important for zinc influx. The defect in zinc transport appears to be greater than can be accounted for by the small percentage of the protein that is mislocalized in these cells. Because the majority of the ΔN protein is at the plasma membrane in the proper orientation, and this mutation causes a severe reduction in zinc trans-



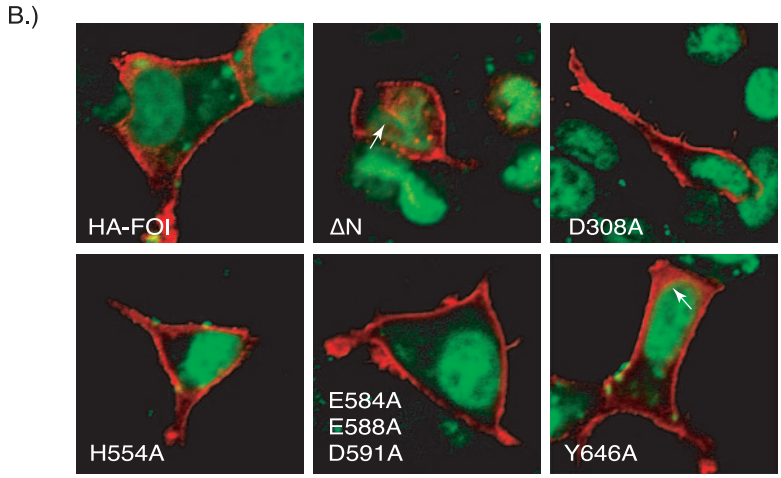


FIG. 5. **Effects of mutations in FOI on its plasma membrane localization.** *A*, location of mutations in the FOI protein whose cellular localization has been tested in cultured cells. *B*, confocal images of HEK293 cells transiently transfected with HA-FOI (wild-type or mutant, as indicated). Permeabilized cells were immunolabeled using anti-HA to reveal FOI (*red*) and Oligreen (*green*) to label DNA. Note the plasma membrane localization of all mutated versions of FOI. *Arrows* indicate small percentage of protein mislocalization in ΔN and Y646A.

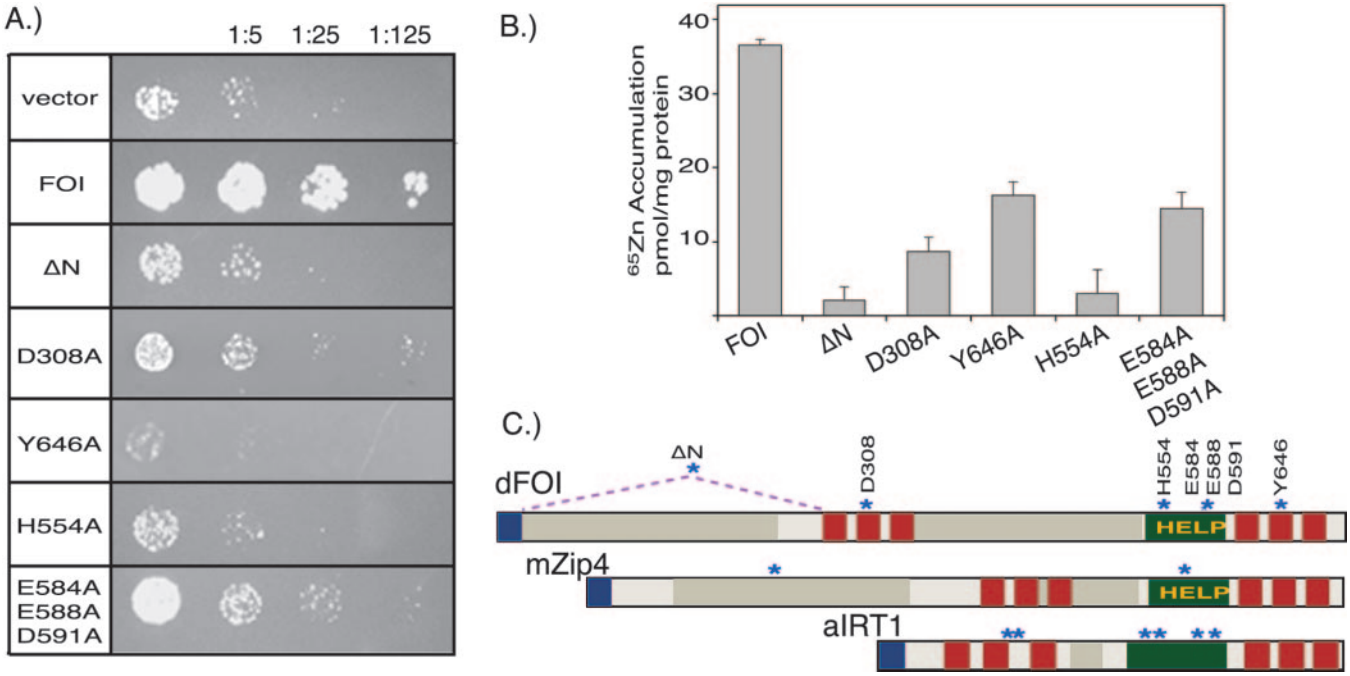


FIG. 6. **FOI mutant proteins are defective in zinc transport.** *A*, growth of *zrt1*Δ*zrt2*Δ cells expressing the empty vector (pFL61) or FOI (wild-type and mutant, as indicated) on low zinc medium after 3 days at 30 °C. Note that all mutations reduce the ability of FOI to complement the growth of *zrt1*Δ*zrt2*Δ. *B*, HEK293 cells transiently transfected with wild-type or mutated FOI. Zinc accumulation was assayed using 5 μM of ^{65}Zn for 15 min at 37 °C. Parallel experiments were conducted with cells transfected with empty vector to measure endogenous ^{65}Zn uptake rate, which was subtracted from the FOI allele values to obtain net zinc uptake values. Each point represents the mean in a representative experiment ($n = 3$) and the *error bars* indicate ±1 S.D. *C*, diagram of FOI, ZIP4, and IRT1 proteins with mutations affecting zinc transport ability indicated with *blue asterisks*. Note that most of the functional mutations are localized to highly conserved domains in the ZIP family.

port ability, we concluded that the N terminus of FOI is functionally involved in zinc transport.

The primary amino acid sequence within ZIP family TM domains is well conserved (Fig. 1, *B* and *C*), including residues that are infrequently found in TM domains (*e.g.* Asp in TM2 of LIV-1 subfamily and Tyr in TM7). This suggests that in addition to creating the proper membrane topology, the primary sequence of the TM domains may be essential for ion transport or some other critical function. Both TM mutants, D308A and Y646A, exhibited reduced complementation of the yeast zinc transporter-deficient strain (Fig. 6*A*) compared with wild-type HA-FOI. Similar results were seen in mammalian cells where D308A and Y646A showed an ~4- and 2.2-fold reduction in zinc accumulation, respectively, compared with wild-type HA-FOI (Fig. 6*B*). The fact that the Y646A mutant exhibited some defects in subcellular localization may account for its effects on zinc transport, either partially or fully. However, the D308A mutant exhibited no such defects in subcellular localization, and so this residue is clearly required for zinc transport activity of FOI.

To characterize the signature sequence domain, we first mutated histidine 554, one of the most highly conserved residues in the entire ZIP family (Fig. 1*C*). Complementation of *zrt1*Δ*zrt2*Δ by H554A was severely reduced on low zinc medium compared with wild-type HA-FOI (Fig. 6*A*), consistent with a crucial role for the signature sequence domain in zinc transport. This histidine residue is also essential for zinc influx into mammalian cells, as H554A exhibited severely reduced zinc transport activity (Fig. 6*B*). Thus, altering this single amino acid can virtually abolish the function of FOI as a zinc transporter. We also mutated the acidic residues in region B of the signature sequence domain (Fig. 1*C*). The acidic nature of this region is largely restricted to the LIV-1 subfamily and is predicted to form an amphipathic α helix. In the yeast assay, E584A/E588A/D591A was partially deficient in complementing the growth defects of the zinc transporter deficient strain (Fig. 6*A*). A similar effect was seen in the mammalian cell assay; influx by E584A/E588A/D591A was reduced by ~2.5-fold compared with wild-type HA-FOI (Fig. 6*B*).

DISCUSSION

Membrane Topology of FOI—We have been able to assess the membrane topology for a ZIP family member within the context of a full-length protein that retains biological function *in vivo*. We find that the N terminus of FOI is extracellular, which is consistent with the presence of a predicted signal peptide and our findings that the N terminus is glycosylated. The middle region of FOI is intracellular, which agrees with computer predictions that there are 3 TM domains between the N terminus and this region and with our observations that a potential glycosylation site in this region is not used. Finally, our finding that the C terminus of FOI is extracellular agrees with previous studies on the LIV-1 subfamily (41, 45) and indicates that there are an odd number of TM domains between the middle region and the C terminus. If the 3 strongly predicted TM domains (TM6–8) are correct, this indicates that the signature sequence domain cannot contain a single TM domain as predicted by many computer algorithms.

It remains an open question as to whether the signature sequence domain of FOI contains 0 or 2 TM domains. Most computer predictions favor 0 TM domains for FOI, but the homology with the ZIP I/II family, in which TM4 and TM5 are more strongly predicted, favors the presence of 2 TM domains. In an attempt to address this, we first inserted a single glycosylation consensus site between the two predicted amphipathic helices (Regions A and B) of the signature sequence domain of the N-terminal deleted FOI, which is not otherwise glycosylated (Fig. 3*C*). The engineered site can be glycosylated only if the signature sequence domain contains 2 TM domains, making the loop between the helices extracellular (Fig. 3*B*, Model 2). However, no evidence for glycosylation of this site was found in the *in vitro* translation/glycosylation assay.[2] We also inserted a proline in place of the threonine in Region A (T556P) of the signature sequence domain within the full-length HA-FOI protein. If Region A normally forms a TM domain, then this proline might disrupt its helical nature and alter the membrane topology or subcellular localization of FOI, as was observed when a proline was inserted into TM3 of mZIP4 (15). However, the FOI T556P protein localizes normally to the cell surface of cultured S2 cells and has an extracellular C terminus.[2] Although these negative results are insufficient for concluding that the signature sequence domain does not contain TM character, they do raise the possibility that the two predicted amphipathic helices of this region might remain cytoplasmic or that they do not fully traverse the membrane, such as proposed for the re-entrant membrane loop of the ionotropic glutamate receptor (46).

Structure-Function Analysis of FOI as a Zinc Transporter—ZIP proteins are defined by their family-specific homology in conserved domains and common protein structure (1). Because the conserved domains and overall protein structure are likely to be functionally important, we tested the importance of some of these features in FOI and found that they are crucial for zinc transport. The signature sequence domain, the most highly conserved domain in the ZIP family, contains two histidine residues that have been conserved throughout the entire family. We have demonstrated that mutating a single one of these residues (H554A) has a drastic effect on zinc transport by FOI in both yeast and mammalian zinc transport assays. Mutation of the homologous residue in *Arabidopsis* IRT1 (H197A, Figs. 1*C* and 6*C*), a ZIP I/II family member with broad cation specificity, blocked the function of this protein in yeast assays designed to test transport of different cations (47). Together these data suggest that this histidine is crucial for ion transport by all ZIP family members and thus is likely involved in coordinating ions through a membrane pore. Mutation of the second highly conserved histidine also blocked the function of IRT1 (H224A) (47), indicating that both of these histidines are essential for ion transport.

Other structural features and conserved residues are specific for individual subfamilies within the larger ZIP family. We were particularly interested in determining whether these features are also involved in zinc transport or reflected other subfamily-specific functions. One feature unique to LIV-1 proteins is their large N terminus that is glycosylated and rich in histidines. Our work indicates that this domain is critical for zinc transport by FOI. Additionally, several disease-causing mutations in human acrodermatitis enteropathica patients have been mapped to the N terminus of hZIP4 (13, 14, 48), and one of these mutations, P200L, causes reduced zinc influx by mouse ZIP4 (15). This histidine-rich domain may function as a zinc-binding domain to induce protein conformational changes required for ion transport or to increase local zinc concentrations. The importance of the N terminus in LIV-1 proteins indicates a functional divergence between ZIP subgroups, perhaps affecting the mechanism of ion specificity, transport, or regulation.

In the LIV-1 subfamily, the signature sequence domain has many more conserved charged residues than are present in the ZIP I/II subfamily and is predicted to form two amphipathic α helices. In Region B, LIV-1 subfamily members have three conserved acidic residues, whereas ZIP I/II subfamily members have one or none (Fig. 1*C*). Mutation of these three acidic residues (E584A/E588A/D591A) reduces zinc transport levels by FOI, suggesting that the amphipathic nature of this region is essential for zinc transport. Interestingly, the mutation of a single acidic residue in this region of IRT1 (E228A) also affected function in yeast transport assays (47) (Figs. 1*C* and 6*C*). Together the data suggest that both the amphipathic nature of the signature sequence domain and its specific histidine residues are crucial for ion transport function. Differences between the ZIP subfamilies also are found in TM1–3 and TM6–8. In TM2, the LIV-1 subfamily has a conserved aspartic acid, whereas the ZIP I/II subfamily usually has threonine in this position (Fig. 1*C*). Mutation of this residue (D308A) has a strong effect on the ability of FOI to act as a zinc transporter. Thus, the conserved primary sequence within the TM domains of the ZIP family is clearly important for transport function in addition to providing the necessary TM structure.

We have now examined a number of features of FOI that represent characteristic differences between the LIV-1 and ZIP

[2] W. Mathews and M. Van Doren, unpublished observations.

I/II subfamilies. In each case, alteration of these features affects the ability of FOI to function as a zinc transporter. We concluded that these differences do not reflect other subfamily-specific functions for these proteins that are independent of zinc transport and that the different subfamilies are likely to share similar functions. These differences may instead reflect subtle changes in ion specificity or in the mechanism of zinc transport between the subfamilies.

In Vivo Roles of the ZIP Family—Because ZIP family members play an important role in both development and human disease, it is crucial to understand the *in vivo* functions of these proteins. Our work demonstrates that *Drosophila* FOI is a zinc transporter with similar ion specificity to other LIV-1 subfamily members. Essential features and sequence identities are conserved between FOI and mammalian ZIP family members, and these features are critical for ion transport. Thus, FOI functions in a similar or identical manner to other family members and represents an excellent opportunity to study the biological roles of this family in a genetically tractable system. In addition, FOI strongly prefers zinc over other cations tested, indicating that zinc plays an important role in regulating developmental processes.

Two ZIP family members have known developmental roles, *Drosophila* FOI and Zebrafish LIV-1. These proteins may act, at least in part, by affecting the expression or function of the calcium-dependent cell adhesion molecule E-cadherin. Like FOI, E-cadherin is required in *Drosophila* for both gonad and trachea morphogenesis (2, 49), and expression of E-cadherin is strongly reduced in *foi* mutants (42). Thus, FOI may affect morphogenesis primarily by affecting E-cadherin. Similarly, the role of zLIV-1 in Zebrafish gastrulation may be because of effects on E-cadherin expression. zLIV-1 regulates the activity of the zinc-finger transcription factor Snail, which is a known regulator of E-cadherin (16). This indicates that ZIP family members can regulate E-cadherin at the transcriptional level by modulating intracellular zinc concentration. It is possible that zinc can also regulate E-cadherin in other ways, for example by directly binding to the E-cadherin protein to affect its function, or by regulating the activity of metalloproteinases that are known to cleave the extracellular domain of E-cadherin. One possibility that our work excludes is that FOI affects the calcium-dependent function of E-cadherin by acting as a calcium transporter to regulate extracellular levels of calcium. Even a 50-fold molar excess of calcium failed to compete with zinc for transport by FOI in our experiments. Thus, FOI likely affects E-cadherin function primarily by affecting the intracellular and/or extracellular concentration of zinc.

Interestingly, the roles of ZIP family members in disease may also be due in part to effects on cell-cell adhesion and cadherin function. hLIV-1 is strongly expressed in breast cancer cell lines, and hLIV-1 expression has been correlated with the potential of breast cancer tumors to metastasize (50). E-cadherin is a critical regulator of metastasis in breast and other cancers (reviewed in Ref. 33). Furthermore, E-cadherin and other cadherin family members are critical for integrity and function of the epidermis (27). Thus, the dermatological lesions that are prevalent in acrodermatitis enteropathica patients with mutations in hZIP4 may represent effects on cadherin function. Although this is highly speculative, the regulation of cadherin function by ZIP proteins represents the type of biological role for this family that can be identified and studied in model organisms like *Drosophila*.

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