In Situ Dimerization of Multiple Wild Type and Mutant Zinc Transporters in Live Cells Using Bimolecular Fluorescence Complementation

Zinc transporters (ZnTs) facilitate zinc efflux and zinc compartmentalization, thereby playing a key role in multiple physiological processes and pathological disorders, presumed to be modulated by transporter dimerization. We recently proposed that ZnT2 homodimerization is the underlying basis for the dominant negative effect of a novel heterozygous G87R mutation identified in women producing zinc-deficient milk. To provide direct visual evidence for the in situ dimerization and function of multiple normal and mutant ZnTs, we applied here the bimolecular fluorescence complementation (BiFC) technique, which enables direct visualization of specific protein-protein interactions. BiFC is based upon reconstitution of an intact fluorescent protein including YFP when its two complementary, non-fluorescent N- and C-terminal fragments (termed YN and YC) are brought together by a pair of specifically interacting proteins. Homodimerization of ZnT1, -2, -3, -4, and -7 was revealed by high subcellular fluorescence observed upon co-transfection of non-fluorescent ZnT-YC and ZnT-YN; this homodimer fluorescence localized in the characteristic compartments of each ZnT. The validity of the BiFC assay in ZnT dimerization was further corroborated when high fluorescence was obtained upon co-transfection of ZnT5-YC and ZnT6-YN, which are known to form heterodimers. We further show that BiFC recapitulated the pathogenic role that ZnT mutations play in transient neonatal zinc deficiency. Zinquin, a fluorescent zinc probe applied along with BiFC, revealed the in situ functionality of ZnT dimers. Hence, the current BiFC-Zinquin technique provides the first in situ evidence for the dimerization and function of wild type and mutant ZnTs in live cells.

Zinc is a critical trace element necessary for multiple physiological processes, including DNA synthesis and repair, transcription, intracellular signaling, protein translation, enzyme activity, cell proliferation, differentiation, apoptosis, autophagy, and motility, hence playing a key role in normal growth and development, cellular integrity, and immune function (1, 2). Cellular zinc concentration is tightly regulated through zinc transport activity of two zinc transporter families: (a) the SLC30A gene family, encoding the proteins ZnT1–10, which mediates zinc efflux and compartmentalization in intracellular organelles from the cytosol (3), and (b) the SLC39A gene family, encoding the proteins ZIP1–14, which mediates zinc influx from the extracellular milieu into cells (1, 4). Transporters of the ZnT family can be classified into four subgroups based on phylogenetic analysis (5) and sequence similarities: (a) ZnT1 and ZnT10; (b) ZnT2, ZnT3, ZnT4, and ZnT8; (c) ZnT5 and ZnT7; and (d) ZnT6. ZnT1 is widely expressed and primarily located in the plasma membrane (6), whereas ZnT10 is localized in early/recycling endosomes in vascular smooth muscle cells (3) and is also expressed in brain, retina, and liver (3). ZnT2, ZnT3, ZnT4, and ZnT8 are expressed in a cell- and tissue-specific manner; ZnT2 and ZnT4 are highly expressed in the mammary gland and small intestine. ZnT2 is predominately localized in cytoplasmic vesicles, such as late endosomes (7, 8), and secretory vesicles in specialized secretory epithelia, such as the mammary gland, prostate, and pancreas (9). ZnT5, ZnT7, and ZnT6 (10) are widely expressed and localized in the early secretory pathway, including the endoplasmic reticulum (ER), Golgi, and cytoplasmic vesicles (11–13).

Qualitative (mutations and SNPs) and quantitative alterations (decreased levels) in various ZnTs were found to be associated with several pathological disorders; for example, ZnT1 is involved in epidermodysplasia verruciformis, a rare autosomal recessive genodermatosis associated with a high risk of developing skin carcinoma (14). Mutations in ZnT2 were found to be associated with low zinc levels in mother’s milk, thereby resulting in transient neonatal zinc deficiency (TNZD) in exclusively breast-fed infants (15–17). Low expression of ZnT3 results in
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TABLE 1

| Zinc transporter | Forward primer | Sequence (5′–3′) | Reverse primer | Sequence (5′–3′) |
|------------------|----------------|-----------------|----------------|-----------------|
| ZnT1             | ZnT1-KpnI      | ATGGTACCATGGGGGTGTTGGGTCG | ZnT1-XhoI      | ATATCTCGAGGCAAGAATGATGCAGTCGG |
| ZnT2             | ZnT2-EcoRI     | ATGGGATCCAGAGCCACGGGCTCCTTGGGGTCG | ZnT2-EcoRI     | ATAGAATTCGTATGCAGTCGG |
| ZnT3             | ZnT3-EcoRI     | ATGGTACCATGGGGAGGCTCCTTGGGGTCG | ZnT3-XhoI      | ATATCTCGAGGCAAGAATGATGCAGTCGG |
| ZnT4             | ZnT4-BamHI     | ATGAGCTGATGGGAGGCTCCTTGGGGTCG | ZnT4-EcoRI     | ATAGAATTCGTATGCAGTCGG |
| ZnT5             | ZnT5-EcoRI     | ATGGGATCCAGAGCCACGGGCTCCTTGGGGTCG | ZnT5-XhoI      | ATATCTCGAGGCAAGAATGATGCAGTCGG |
| ZnT6             | ZnT6-BamHI     | ATGGGATCCAGAGCCACGGGCTCCTTGGGGTCG | ZnT6-XhoI      | ATATCTCGAGGCAAGAATGATGCAGTCGG |
| ZnT7             | ZnT7-BamHI     | ATGGGATCCAGAGCCACGGGCTCCTTGGGGTCG | ZnT7-EcoRI     | ATAGAATTCGTATGCAGTCGG |

learning and memory defects in Alzheimer disease (18), whereas a non-synonymous SNP in ZnT8 is responsible for increased susceptibility for type 2 diabetes (19).

ZnTs are predicted to have six transmembrane domains with cytoplasmic N- and C-terminal regions (2), except for ZnT5, which has an exceptionally long N terminus consisting of nine putative transmembrane domains fused to six conserved transmembrane domains of the C-terminal region (12). Human ZnTs are believed to form homodimers based on the homodimeric crystal structure of YiiP, a bacterial ZnT homologue (20, 21). Although dimerization of the bacterial YiiP has a critical role in modulating zinc transport activity (20), little is known about homodimerization and heterodimerization of human ZnTs. In this respect, only a few ZnTs, including ZnT2 and ZnT3, were studied to some extent and were suggested to form homodimers (16, 17, 22), whereas ZnT5 and ZnT6 were found to undergo heterodimerization (23). We recently identified a novel heterozygous G87R mutation in ZnT2 (16), in two Ashkenazi Jewish mothers; this mutation resulted in the production of zinc-deficient milk, leading to the development of zinc-deficient milk, leading to the development of learning and memory defects in Alzheimer disease (18), whereas a non-synonymous SNP in ZnT8 is responsible for increased susceptibility for type 2 diabetes (19).

ZnTs are believed to form homodimers based on the homodimeric crystal structure of YiiP, a bacterial ZnT homologue (20, 21). Although dimerization of the bacterial YiiP has a critical role in modulating zinc transport activity (20), little is known about homodimerization and heterodimerization of human ZnTs. In this respect, only a few ZnTs, including ZnT2 and ZnT3, were studied to some extent and were suggested to form homodimers (16, 17, 22), whereas ZnT5 and ZnT6 were found to undergo heterodimerization (23). We recently identified a novel heterozygous G87R mutation in ZnT2 (16), in two Ashkenazi Jewish mothers; this mutation resulted in the production of zinc-deficient milk, leading to the development of TNZD in their exclusively breast-fed infants. We further found that the G87R mutation had a dominant negative effect on the WT ZnT2 as reflected in WT ZnT2 mislocalization and loss of function, presumably due to ZnT2 homodimerization. In order to provide direct visual evidence for the in situ dimerization of normal and mutant ZnTs in live cells at their established organelles, we applied here the bimolecular fluorescence complementation (BiFC) technique (24). BiFC enables the straightforward in situ visualization of specific protein-protein interactions, including dimerization in live cells (24). BiFC was previously utilized to explore the dimerization of several membrane transporters and receptors, such as the β-adrenergic receptor, breast cancer resistance protein (BCRP/ABCG2), and adiponectin receptor 1 (25–28). The BiFC technique relies on the principle that specifically interacting proteins tagged with molecular fragments of a fluorescent protein (e.g. YFP) enable the non-fluorescent fragments of YFP to associate and refold, thereby leading to the acquisition of YFP fluorescence (24). A positive BiFC signal implies a distance of less than 15 nm between the two interacting proteins (24). Typically, the N-terminal fragment encodes the first 7–8 β-strands of YFP (called YN), whereas the C-terminal fragment encodes for the latter 3–4 β-strands (called YC) (28, 29). Here we assessed whether or not the BiFC approach could be employed in order to determine the in situ dimerization and function of multiple ZnTs in live cells. Co-transfection of ZnT1–4 and ZnT7 after their C-terminal tagging with non-fluorescent YC and YN, as well as co-transfection of tagged ZnT5 and ZnT6, which form heterodimers (23), resulted in high YFP fluorescence that localized in previously established subcellular compartments of each ZnT. Zinquin, a viable cell-permeant fluorescent zinc probe applied along with BiFC revealed the in situ functionality of ZnT dimers. Hence, the current results provide the first direct evidence for the in situ homodimerization of ZnT1–4 and ZnT7 in live cells, indicating that BiFC can pave the way for the direct visualization of the interactions between WT and mutant ZnTs associated with various zinc deficiencies.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—The DNA dyes DAPI, Hoechst 33342, and propidium iodide (PI) were purchased from Sigma-Aldrich. The DNA dye RedDot and the zinc probe Zinquin ethyl ester were purchased from Biotium (Hayward, CA), whereas zinc sulfate was obtained from Merck.

Generation of Constructs for BiFC Analysis—The construction of vectors encoding fragments of Venus YFP, which were kindly provided by Prof. I. D. Kerr (University of Nottingham), has been described previously (28, 30). YN refers to YFP residues 1–173 (β strands 1–8), YC refers to YFP residues 155–238 (β strands 8–11) (30, 31), whereas YFP represents the entire YFP protein (32). The coding regions of the long isoforms of ZnT1, -3, -4, -5, and -7 were amplified using pcDNA3.1/V5-His TOPO vector containing C-terminally HA-tagged ZnT2 as a template (15). PCR was performed using the proofreading enzyme PWO and primers containing restriction sites (Table 1). The PCR products were then analyzed on 16% polyacrylamide gels and bands were excised from the gel. The DNA was purified from the gel using a Promega Wizard SV gel and PCR cleanup system. The purified PCR products were digested using agarose gel electrophoresis, following which the DNA was purified from the gel using a Promega Wizard SV gel and PCR cleanup system. The purified PCR products were digested with the appropriate restriction enzymes and cloned into the BiFC constructs in a vector/insert ratio of 1:6 using the same restriction enzymes. The ligation was performed for 30 min at 16 °C, and ligation products were transformed into heat shock-competent Escherichia coli DH5α. Positive colonies were selected using PCR. The fidelity of the insert and the tag were confirmed by direct sequencing (Technion, Rappaport School of Medicine, DNA Sequencing Facility, Haifa, Israel). The G87R, S296L, and W152R mutations were introduced into the...
YFP/YC/YN-tagged ZnT2 expression plasmids using *Pfu* Turbo DNA polymerase (QuikChange kit, Stratagene, La Jolla, CA) and primers encoding the proper amino acid substitution as follows. The G87R mutation was introduced into YFP/YC/YN-tagged ZnT2 plasmids using the following primers: forward primer, 5′-CTGCCCTGGTTGCTGTTACATGCTCGAGGAGTGGATGC-3′, reverse primer, 5′-GATCTCAACGACTTGCTGTGGAGATC-3′ (described previously (33) using a mouse anti-ZnT2 monoclonal antibody (at 1:2000 dilution, overnight incubation at 4 °C; generously provided by Prof. T. Kambe, Kyoto University). Blots were then washed three times in Tris-buffered saline (TBS; 150 mM NaCl, 0.5% Tween 20, and 10 mM Tris/Cl at pH 8) for 10 min each at room temperature and detected with horseradish peroxidase-conjugated goat anti-mouse IgG (1:20,000 dilution; Jackson Immunoresearch Laboratories, West Grove, PA) for 1 h at room temperature. After three 10-min washes in TBS at room temperature, enhanced chemiluminescence detection was performed according to the manufacturer’s instructions (Biological Industries, Beth-Haemek, Israel). Similarly, actual protein loading onto the SDS-polyacrylamide gel was confirmed by blot reprobing with a rabbit polyclonal antibody against the α subunit of Na⁺/K⁺ ATPase (KETTY at 1:3000 dilution; kindly provided by Prof. S. J. D. Karlish, Weizmann Institute of Science, Rehovot, Israel) and detected with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:15,000 dilution; Jackson Immunoresearch Laboratories).

Fluorescence Microscopy—Twenty-four hours after transfection, the growth medium was removed, and monolayer cells were washed with PBS and then maintained in PBS containing 1 mM MgCl₂, 1 mM CaCl₂ and 10 mM glucose at pH 7.4. Hoechst 33342 (2 μg/ml) and RedDot were used for nuclear DNA staining. Live cells were imaged using an inverted confocal microscope (Zeiss LSM 700) at a magnification of ×63 under immersion oil. Representative images from at least three independent experiments are shown. Green YFP fluorescence signal indicates dimer formation (YC-YN fluorescence). Hoechst 33342 (blue fluorescence) was used to stain nuclei. RedDot (red fluorescence) was used to stain membranes with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:15,000 dilution; Jackson Immunoresearch Laboratories).

Flow Cytometric Analysis of YFP Fluorescent Cells—Transiently transfected MCF-7 cells were analyzed by flow cytometry for both the percentage of YFP-positive cells and YFP fluorescence levels. Cells were washed three times with PBS and then harvested in sterile Eppendorf tubes. Cells were spun for 5 min at 1000 × g at 4 °C and were then pelleted in a 10-kDa centrifugal filter device (Millipore). The cell pellets were then stained with 500 μl of FACS buffer (0.5% BSA, 0.01% NaN₃, and 0.1% EDTA in PBS) and incubated with 2 μl of the appropriate YFP antibody for 10 min at room temperature. After three 10-min washes in TBS at room temperature, fluorescence was analyzed using a BD FACSCanto II (BD Biosciences). 

### Table 2

Primers used for RT PCR of ZnT1–ZnT7 ORF

| Primer | ORF | Length |
|--------|-----|--------|
| ZnT1-F | ZnT1-R | 127 bp |
| ZnT2-F | ZnT2-R | 131 bp |
| ZnT3-F | ZnT3-R | 119 bp |
| ZnT4-F | ZnT4-R | 134 bp |
| ZnT5-F | ZnT5-R | 97 bp |
| ZnT6-F | ZnT6-R | 145 bp |
| ZnT7-F | ZnT7-R | 118 bp |

*Note: The table includes primers used for the amplification of ZnT1–ZnT7 ORFs.*
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down by centrifugation at 150 × g for 5 min, resuspended in 0.5 ml of PBS, and transferred into flow cytometer test tubes at a cell density of ~2.4 × 10^6 cells/ml. Ten thousand cells were analyzed for each transfection.

Three-dimensional Modeling and Structure-Analysis of ZnT2—The monomers of human ZnT2 were aligned against the recently revealed 13 Å resolution, cryo-electron microscopy structure of a YiiP homologue from the bacterium Shewanella oneidensis (Protein Data Bank entry 3I1Z) (34) using HHpred, a template-based structure prediction server (35). The models of ZnT2 monomers were generated using Modeller (36). To minimize steric clashes in the human ZnT2 structure model, we used the Chiron server (37), which employs an iterative protocol aimed at minimizing the steric clashes of a given protein until it attains an “acceptable clash score” (any atomic overlap resulting in Van der Waal’s repulsion energy less than 0.3 kcal/mol except in the following cases: (a) when the atoms are bonded, (b) when the atoms form a hydrogen bond, or (c) when the atoms involved are backbone atoms and have separation of two residues. The van der Waals repulsion energy is calculated using CHARMM non-bonded parameters, which are identical to CNS parameters except for carboxyl oxygens. Chiron minimizes clashes while at the same time it causes minimal perturbation in the protein backbone. The resulting protein structure has a normalized clash score that is comparable with those of high resolution protein structures (<2.5 Å). The model of the ZnT2 homodimer was obtained by superimposing the two identical monomers into each of the chains of the YiiP homologue (PDB entry 3I1Z chains P and Q), using the MatchMaker tool of Chimera UCSF (38).

Hydrophobicity Calculations—The Kyte and Doolittle hydrophobicity scale is based on the hydrophilic and hydrophobic properties of each of the 20 amino acid side chains (39). The scale spans from a value of −4.5 for Arg to 4.5 for Ile.

Graphic Imaging—Molecular graphics and analyses in Fig. 6 were performed using the UCSF Chimera package. Chimera was developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIGMS, National Institutes of Health, Grant P41-GM103311) (38).

Analysis of Vesicular Zinc Accumulation in Viable Cells—In order to assess vesicular zinc accumulation in transfected cells, we used the viable zinc probe, Zinquin ethyl ester, which is a lipophilic fluorescent derivative of Zinquin that is cell-permeant. Zinquin ethyl ester is selective for zinc, and upon zinc binding, its fluorescence is enhanced. In order to determine the optimal concentration of extracellular ZnSO_4 that can provide high Zinquin fluorescence intensity as well as a high percentage of cells displaying vesicular accumulation of Zinquin fluorescence while retaining a minimal percentage of dead cells, MCF-7 cells were co-transfected with equal amounts of ZnT2-YC and ZnT2-YN plasmid DNA. Untransfected cells were used as a control for endogenous vesicular zinc accumulation in MCF-7 cells. Twenty-four hours after transfection, cells were incubated in RPMI 1640 growth medium containing increasing concentrations of ZnSO_4 (0, 0.1, 0.3, 1, 3, 10, 30, 75, and 100 μM) for 2 h at 37 °C. Cells were then rinsed twice with PBS and incubated in growth medium containing 40 μM Zinquin ethyl ester (Biotium, Hayward, CA) for 1 h at 37 °C. Finally, cells were washed three times with PBS and transferred into 0.5 ml of PBS. In order to examine the percentage of dead cells, cells were incubated with 0.5 μg/ml PI for 2 min at room temperature (40). Then, cells were analyzed for Zinquin fluorescence or PI fluorescence using flow cytometry. Cells showing fluorescence that was above the autofluorescence level were considered Zinquin-positive cells or PI-positive cells. Based on our zinc titration results and previously published vesicular Zinquin accumulation experiments (7, 13, 37), an extracellular concentration of 75 μM ZnSO_4 was used in order to evaluate vesicular zinc accumulation in WT and mutant ZnT2 MCF-7-transfected cells using flow cytometry and Zinquin fluorescence microscopy.

Statistical Analysis—Results are presented as means ± S.D. Statistical comparisons were performed using Student’s t test (Prism Graph Pad, Berkeley, CA), and a significant difference was demonstrated when p was < 0.05. Results from at least three independent experiments are shown.

RESULTS

BiFC Analysis with C-terminally Tagged ZnTs Harboring Non-fluorescent YFP Fragments—Our previous study suggested that ZnT2 homodimerization is the underlying basis for the dominant negative effect of a novel heterozygous G87R mutation identified in women producing zinc-deficient milk (16). In order to provide direct in situ evidence for the specific interaction between ZnTs, including homodimerization and heterodimerization in live cells at their established subcellular compartment, we here applied the BiFC technique (29). BiFC provides a native and physiological environment for the direct visualization of intracellular protein-protein interactions in viable cells, by maintaining the conditions for correct protein folding and function (29). The BiFC technique is based on specific protein-protein interactions, hence relying on the ability of non-fluorescent fragments of YFP (called YC and YN) to reassociate and refold into a fully fluorescent protein. Intimate physical association between the non-fluorescent YFP fragments can be easily achieved when these two fragments are fused to the domain(s) of interaction between proteins (41). Thus, non-fluorescent YFP fragments were fused to the C terminus of various ZnTs. The choice of C-terminal tagging is based on several lines of evidence indicating that the C termini of ZnTs are necessary for protein-protein interaction, dimer formation, zinc binding, and zinc sensing (20–23, 42–44). Moreover, ZnT1 to ZnT7 were also C-terminally tagged with the full-length YFP in order to provide an intrinsic fluorescent control for their expression levels and subcellular localization, which does not depend on transporter dimerization. Table 3 describes several structural and topological properties of various zinc transporters, including transporter size, the predicted number of transmembrane helices, and the distance between YC/YN/YFP and the end of the last transmembrane helix. The mammalian epithelial MCF-7 carcinoma cells were used for transient transfections of BiFC-ZnT constructs. RT-PCR using targeted primers to the open reading frame (ORF) of ZnT1–7 (Table 2) revealed that MCF-7 cells had high endogenous expression levels of ZnT1, ZnT6, and ZnT7 (Fig. 1). ZnT4 and
ZnT7 were found to be expressed at moderate levels, whereas ZnT2 and ZnT3 were found to be expressed at low levels. Evaluation of MCF-7 transfecants was performed using flow cytometry and fluorescence microscopy. When indicated, ZnT-YC-YN represents co-transfection of equal amounts of both ZnT-YC and ZnT-YN.

Western Blot Analysis Reveals Equal Expression of ZnT2-YC and ZnT2-YN—In order to confirm the comparable expression levels of the tagged ZnT2 proteins, MCF-7 cells were transiently transfected with equal amounts of either ZnT2-YC, ZnT2-YN, or ZnT2-YFP plasmid DNA (7 μg of DNA). In addition, cells were co-transfected with equal amounts of both ZnT2-YC and ZnT2-YN (i.e. 3.5 μg of each of the two constructs). Transfection of untagged ZnT2 (7 μg of DNA) was used as a positive control, whereas immunoblots of untransfected cells or cells transfected with YFP empty vector (7 μg of DNA) were used as a negative control. Western blot analysis after SDS-PAGE under denaturing conditions was performed using an anti-ZnT2 antibody, whereas equal protein loading was verified using an antibody against the α subunit of Na⁺/K⁺ ATPase (~100 kDa) (Fig. 2). This Western blot analysis demonstrated equal protein expression levels of ZnT2-YC and ZnT2-YN when transfected alone as well as upon co-transfection. Interestingly, although samples were run under denaturing conditions, ZnT2-YC-YN homodimer (Fig. 2A, see asterisk in the second lane from the left) with an approximate expected molecular mass of ~110 kDa was detected when ZnT2-YC and ZnT2-YN were co-transfected, hence suggesting that ZnT2-YC and ZnT2-YN form metastable homodimers, presumably due to the refolding into a fully fluorescent YFP protein (29). ZnT2-YFP was found to be expressed at somewhat higher levels than ZnT2-YC or ZnT2-YN, possibly due to some diminished stability of proteins that are tagged with partially unfolded protein fragments of YFP (i.e. YC and YN) and due to their increased protease sensitivity as shown previously (26). In this previous study, lower molecular mass breakdown products were obtained with YC- and YN N-terminally tagged ABCG2 (26). Remarkably, however, although ZnT2-YC and ZnT2-YN exhibited lower expression levels compared with ZnT2-YFP, the percentage of ZnT2-YC-YN-transfected cells showing BiFC fluorescence (i.e. the percentage of ZnT2 homodimerization) was higher than 50% of the fluorescence of ZnT2-YFP-transfected cells displaying fluorescence (Fig. 3B), as could be expected due to the apparent enhanced stability of the ZnT2-YC-YN homodimer. Hence, these results provide further support for the validity of the BiFC analysis with YC- and YN-tagged ZnTs.

BiFC Analysis Reveals That ZnT1, ZnT2, ZnT3, ZnT4, and ZnT7 Form Homodimers—To provide direct in situ evidence for ZnT2 homodimerization in live cells and to establish the suggested homodimerization of multiple ZnTs, including ZnT1, ZnT3, ZnT4, and ZnT7, these ZnTs were C-terminally tagged with non-fluorescent YFP fragments and transfected independently into MCF-7 cells. The use of flow cytometry enabled us to determine two parameters that reflect specific dimerization of ZnTs. First, we determined the percentage of
ZnT2-YC-YN transfected cells displaying YFP fluorescence, which was above the autofluorescence level obtained with untransfected cells. In order to take ZnT protein expression levels and transfection efficiency into account, the percentage of ZnT2-YC-YN fluorescent cells was normalized to that of YFP-positive cells transfected with ZnT-YFP (i.e. harboring the full-length YFP). Results are presented as percentage of dimerization (Fig. 3A). We then quantified the mean fluorescence of ZnT-YC-YN-positive transfectedants as a measure of the relative strength of monomer interaction and homodimer formation. Second, we also determined the mean fluorescence intensity of ZnT-YFP-positive cell transfectedants in order to evaluate protein expression levels (Fig. 3B). In this respect, the mean percentage of dimerization was significantly higher for independent co-transfections with ZnT1–4-YC-YN or ZnT7-YC-YN compared with the percentage obtained with the co-transfection of ZnT6-YC-YN, which does not form homodimers (23) (Fig. 3A) and Table 4). Consistently, cells co-transfected with ZnT1–4-YC-YN showed the highest fluorescence intensity compared with background fluorescence levels obtained with a negative control (i.e. co-transfection of ZnT2-YC and β2AR-YN) (Fig. 3B). In contrast, cells co-transfected with ZnT7-

FIGURE 3. ZnT1–4 and ZnT7 form homodimers based on the BiFC analysis. A, MCF-7 cells were transiently co-transfected with YC- and YN-tagged ZnT1–7 independently. MCF-7 cells were also transiently transfected with YFP-tagged ZnT1–7 in order to take protein expression levels into consideration. The percentage of dimerization was evaluated using flow cytometry and represents the fraction of YC-YN ZnT-positive cells divided by the fraction of ZnT-YFP-positive cells. The negative control (co-transfection of ZnT2-YC and β2AR-YN) resulted in 5.0 ± 1.3% of cells displaying BiFC fluorescence (not shown due to the inability to calculate the percentage of dimerization for this negative control). *, values shown are significantly different (p < 0.05) when compared with ZnT2-YC-YN. B, the mean YFP BiFC fluorescence of MCF-7 cells transiently transfected with ZnT1-YC-YN or ZnT7-YFP was examined using flow cytometry. *, values obtained are significantly higher (p < 0.05) when compared with co-transfection of ZnT2-YC and β2AR-YN. C, MCF-7 cells were transiently co-transfected with ZnT1-YC and ZnT1-YN (i–iii), ZnT2-YC and ZnT2-YN (iv–vii), ZnT3-YC and ZnT3-YN (ix–xi), ZnT4-YC and ZnT4-YN (xiii–xv), and ZnT7-YC and ZnT7-YN (xvii–xix). Additionally, cells were transiently transfected with ZnT1-YFP through ZnT7-YFP (iv, vii, xi, xiv, and xx, respectively). The green YFP fluorescence signal indicates dimer formation (YC-YN fluorescence). Hoechst 33342 (blue fluorescence) was used to stain nuclei. Error bars shown in A and B represent S.D.
Summary of results obtained in Figs. 3 and 4. The mean percentage of cells displaying ZnT-YC-YN fluorescence obtained from three independent experiments is depicted.

TABLE 4

Characterization of ZnT dimers using BiFC analysis

| Zinc transporter | Homodimer/ Heterodimer Dimerizationa | Cells displaying ZnT-YC-YN fluorescence | Mean YC-YN fluorescence per AU | Strength of interaction between monomersb | Subcellular localization in MCF-7 cells |
|------------------|-------------------------------------|----------------------------------------|--------------------------------|--------------------------------------|---------------------------------------|
| ZnT1             | Homodimer                           | %                                      | %                              | 2358 Strong                           | Plasma membrane                       |
| ZnT2             | Homodimer                           | 76                                     | 35                            | 2142 Strong                           | Cytoplasmic vesicles and plasma membrane |
| ZnT3             | Homodimer                           | 81                                     | 41                            | 1655 Strong                           | Cytoplasmic vesicles                  |
| ZnT4             | Homodimer                           | 63                                     | 30                            | 1692 Strong                           | Cytoplasmic vesicles and plasma membrane |
| ZnT5             | Homodimer                           | 91                                     | 36                            | 647 Moderate                          | Golgi and cytoplasmic vesicles        |
| ZnT6             | No interaction                      | 39                                     | 22                            | 387 Weak                             | Golgi and cytoplasmic vesicles        |
| ZnT5-ZnT6        | Heterodimer                         | 14                                     | 14                            | 727 Weak                             | Golgi and cytoplasmic vesicles        |
| ZnT7             | Homodimer                           | 71                                     | 23                            | 495 Moderate                          | Golgi and cytoplasmic vesicles        |
| βAR-YC + ZnT2-YN | No interaction                      | 5                                      | 5                             | 204 Very weak                         | ER                                    |

a Percentage of ZnT-YC-YN fluorescent cells divided by the percentage of ZnT-YFP fluorescent cells.
b YFP fluorescence values between 0 and 500 indicate a weak interaction between monomers. YFP intensity levels between 500 and 1000 were considered as a moderate interaction between monomers. YFP intensity levels above 1000 were considered as a strong interaction between monomers. This scale is based on the background fluorescence obtained with co-transfection of βAR-YC + ZnT2-YN.

YC-YN showed a moderate fluorescence compared with the background fluorescence levels (Fig. 3B). Importantly, the BiFC fluorescence of cells co-transfected with ZnT-YC-YN localized at the characteristic subcellular compartments of each zinc transporter (Fig. 3C); specifically, ZnT1 localized at the plasma membrane (Fig. 3C, i–iii), as shown previously (6), whereas ZnT2, ZnT3, and ZnT4 predominantly localized at cytoplasmic vesicles, as described previously (Fig. 3C, v–vii, ix–xi, xiii–xv, respectively) (2, 7–9). In addition, ZnT2 and ZnT4 also displayed some plasma membrane localization (2, 7–9). The BiFC fluorescence of ZnT7 was found to be localized in the Golgi apparatus and in cytoplasmic vesicles (Fig. 3C, xvi–xix) (13). The subcellular localization of ZnT-YC-YN homodimers was highly consistent with the subcellular localization of ZnT-YFP (Fig. 3C, iv [ZnT1], vii [ZnT2], xii [ZnT3], xvi [ZnT4], and xx [ZnT7]). Collectively, these results provide the first in situ evidence for the homodimerization of ZnT1, ZnT2, ZnT3, ZnT4, and ZnT7 in live cells at their established subcellular compartments, hence demonstrating that BiFC is a bona fide tool for the analysis of the dimerization of various ZnTs.

BiFC Analysis Reveals that ZnT5 and ZnT6 Form Heterodimers—In order to establish BiFC as a valid tool for the assessment of both homodimerization and heterodimerization of ZnTs, we examined the ability of the established heterodimers ZnT5 and ZnT6 (23) to form a fluorescent signal using the BiFC assay. Flow cytometry and fluorescence microscopy revealed that co-transfection of ZnT6-YC and ZnT6-YN resulted in a relatively low fluorescence in a very small fraction of cells, which was comparable with the background fluorescence level obtained with the negative control (i.e. co-transfection of β2AR-YC along with ZnT2-YN; Fig. 4A). Transfection of ZnT6-YFP indicated that the very low fluorescence of ZnT6-YC-YN was not due to low expression of ZnT6 because 25% of transfected cells exhibited a substantial YFP fluorescence. These findings are in accord with previous studies demonstrating that ZnT6 does not form homodimers (23). Interestingly, however, co-transfection of ZnT5-YC-YN resulted in moderate fluorescence levels in a substantial fraction of cells (22%), suggesting that ZnT5 can form homodimers, at least to some extent (Fig. 4A). These results are different from those of a recent co-immunoprecipitation-based study suggesting that ZnT5 does not form homodimers (23). Here, the percentage of YC-YN-positive transfectants was not normalized to the percentage of cells displaying YFP fluorescence (i.e. transfected with ZnT1-YFP) due to the co-transfection of two different ZnTs (i.e. ZnT5 and ZnT6). Fluorescence microscopy revealed that ZnT5 homodimers (Fig. 4B, i–iii) and ZnT5-YFP (Fig. 4B, iv) localized at their established compartments, including Golgi apparatus and cytoplasmic vesicles, as described previously (12). In contrast, no fluorescence was detected when ZnT6-YC and ZnT6-YN, which do not form homodimers, were co-transfected (Fig. 4B, v–vii). ZnT6-YFP fluorescence was readily detected at cytoplasmic vesicles (Fig. 4B, vii). Expectedly, co-transfection of ZnT5-YC and ZnT6-YN as well as co-transfection of ZnT5-YN and ZnT6-YC resulted in substantial fluorescence levels in a relatively large fraction of cells (29–31%), compared with background fluorescence levels of the negative control (co-transfection of ZnT2-YN along with the β2AR-YC) as well as with fluorescence levels obtained with transfection of ZnT6-YC-YN (Fig. 4A) (23). ZnT5-ZnT6 heterodimers were found to localize at the Golgi apparatus and cytoplasmic vesicles (Fig. 4B, ix–xi and xii–xiv). These results indicate that ZnT6 does not form homodimers, whereas ZnT5 appears to form homodimers, at least to some extent. Moreover, this is the first study in which BiFC analysis allowed the in situ identification of ZnT5-ZnT6 heterodimers as well as ZnT5 homodimer formation in live cells at their established subcellular compartments. Table 4 summarizes the interactions between various ZnT monomers as well as the subcellular localization of ZnT homodimers and heterodimers.

Putative ZnT2 Topology and Three-dimensional Localization of Mutations Associated with TNZD—Recent studies revealed that ZnT2 plays a key role in zinc secretion into milk (8, 9, 46, 47). In this respect, two independent heterozygous mutations as well as a compound mutation in ZnT2 were identified in women producing zinc-deficient milk (15–17). Consequently, their exclusively breast-fed infants developed TNZD manifested by severe dermatitis. The first heterozygous mutation involved the substitution of a conserved histidine 54 with arginine (H54R) (15), thereby resulting in reduced zinc secretion into milk due to perinuclear, aggresomal accumulation of mutant ZnT2. Interestingly, however, H54R ZnT2 did not exert...
a dominant negative effect on WT ZnT2 (15). The second heterozygous mutation, which substituted glycine 87 with arginine (G87R), was recently identified in our laboratory (16); this G87R mutation resulted in perinuclear retention of ZnT2 as well as in a dominant negative effect on the WT ZnT2 presumably due to homodimerization. The dominant negative effect of the G87R mutation resulted in WT ZnT2 mislocalization and loss of zinc transport. Most recently, two novel compound mutations in ZnT2 were identified in a Japanese mother producing very low milk zinc concentrations (>90% reduction), the infant of whom developed severe TNZD (17). The mutation in the first allele resulted in the substitution of a tryptophan by an arginine (W152R), whereas the mutation in the second allele resulted in a substitution of a serine by leucine (S296L). Bio-

![Figure A](image1.png)

**FIGURE 4.** Establishment of BIFC as a novel dissection tool for the analysis of ZnT dimerization of various ZnTs. A, MCF-7 cells were transiently co-transfected with ZnT2-YC-YN as a positive control for dimerization as well as with ZnT2-YC and β2AR-YN as a negative control. Co-transfection of ZnT5-YN, ZnT6-YC-YN, ZnT5-YN with ZnT6-YN as well as ZnT6-YC with ZnT5-YN was performed in order to evaluate ZnT5-ZnT6 heterodimerization using BIFC analysis. Transfection of ZnT2-YFP, ZnT5-YN, and ZnT6-YN was also performed in order to take ectopic protein expression into consideration. Cells were analyzed using a flow cytometer for the percentage of cells showing YFP fluorescence as well as for mean YFP fluorescence. In this case, the percentage of dimerization cannot be calculated due to heterodimerization between two different proteins (i.e. ZnT5 and ZnT6). *, values obtained are significantly higher (p < 0.05) when compared with co-transfection of ZnT2-YC and β2AR-YN. B, MCF-7 cells were transiently co-transfected with ZnT5-YC and ZnT5-YN (i–iii), ZnT6-YC and ZnT6-YN (v–vii), ZnT5-YC and ZnT6-YN (ix–xi), and ZnT6-YC and ZnT5-YN (xii–xiv) or transfected with either ZnT5-YFP (iv) or ZnT6-YFP (viii). Green YFP fluorescence signal indicates dimer formation (YC-YN fluorescence). Hoechst 33342 (blue fluorescence) was used to stain nuclei. Error bars shown in A represent S.D.
chemical characterization indicated that the W152R mutation abolished dimer formation and zinc transport, indicating a loss-of-function mutation. In contrast, mutant S296L ZnT2 retained both abilities but was extremely destabilized. In order to characterize the impact of these TNZD mutations on ZnT2 structure and function, ZnT2 topology and a structural three-dimensional model were generated. According to multiple bioinformatics tools (OCTOPUS, HMMTOP, PHOBIUS, MEMSAT3, and TMHMM), ZnT2 is predicted to contain six transmembrane helices (Fig. 5). The Gly-87 (16) and Trp-152 (17) mutations were found to be localized in the first and the third transmembrane helices, respectively, whereas Ser-296 (17) was found to be localized in the proximal region of the cytoplasmic C terminus. Additionally, we generated an updated three-dimensional model of ZnT2 based on the recent inward facing conformation cryoelectron microscopy structure of *Shewanella oneidensis* YiiP (Protein Data Bank entry 3J1Z) (34). YiiP is a homodimeric bacterial transporter that mediates Zn$^{2+}$/H$^+$ exchange across the inner membrane of *S. oneidensis* (34). Although human ZnT2 shares low sequence identity with YiiP (about 15% identity, based on an aligned region of 290 amino acids), its three-dimensional model allowed us to deduce the probable structure-function impact of the three mutations identified in mothers producing zinc-deficient milk. As expected, most of the residues facing the hydrophobic phospholipid membrane core were found to be highly hydrophobic, whereas most residues lining the cytoplasmic domains facing the solvent were found to be predominantly hydrophilic (Fig. 6, A and B), thereby confirming that the model properly reflects the most basic physical-chemical properties of the structure. Gly-87 (see GLY 87 insets) is located in a highly hydrophobic region and is facing the membrane environment (Fig. 6, A and B). Substitution of the small inert glycine (−0.4 according to the Kyte and Doolittle hydrophathy scale) with a positively charged arginine (−4.5 according to the Kyte and Doolittle hydrophathy scale) most likely disrupted the interaction between the transporter membrane domain and the phospholipid bilayer core surrounding it, thereby leading to ZnT2 misfolding. Trp-152 is located in the heart of the monomer–monomer interface, in a highly hydrophobic region (Fig. 6, A and B; see TRP 152 inset); substitution of this hydrophobic Trp-152 residue (−0.9 according to the Kyte and Doolittle hydrophathy scale) with any residue, especially with a positively charged residue, such as arginine, will necessarily disrupt the interaction between ZnT2 monomers, thereby leading to a profound disruption of homodimer formation. Additionally, it is possible that the W152R mutation impairs the stability of the dimer–membrane interaction, because Trp-152 is partially exposed to the membrane. Ser-296 is located in the cytoplasmic C-terminal tail, facing the solvent (−0.8 according to the Kyte and Doolittle hydrophathy scale) and resides in a small hydrophobic pocket surrounded by hydrophilic residues (Fig. 6, A and B; see SER 296 inset). Its substitution by a highly hydrophobic residue, such as leucine (3.8 according to the Kyte and Doolittle hydrophathy scale), will enhance considerably the hydrophobicity of this region, thereby increasing solvation energy and decreasing domain stability.

**Characterization of ZnT2 Mutations Associated with TNZD Using BiFC Analysis**—Because our current findings indicated that BiFC may serve as a reliable tool for the analysis of ZnT2 homodimerization, we explored the ability of BiFC to recapitulate the dominant negative effect of the G87R mutation on the WT ZnT2 and assessed the impact of the recently identified compound mutations W152R and S296L on ZnT2 homodimerization as well as on vesicular zinc accumulation. Zinc accumulation was assessed using the viable cell-permeant fluorescent zinc probe Zinquin ethyl ester. Zinquin ethyl ester fluorescence is enhanced upon zinc binding. Hence, higher zinc concentration accumulated in compartments, such as intracellular vesicles, due to ZnT2 transport activity will result in higher Zinquin intensity in a higher fraction of cells. In order to determine the optimal concentration of extracellular ZnSO$_4$ that provides high Zinquin fluorescence as
BiFC and in Situ ZnT Dimerization

FIGURE 6. Three-dimensional model of ZnT2 and localization of ZnT2 mutations associated with TNZD. A, i, ribbon representation of the three-dimensional ZnT2 model. The two monomers are colored in sky blue and tan. The mutation positions in each monomer are shown in a CPK representation and colored in dodger blue and orange red. ii, 90° rotation in the y axis of the ZnT2 model. According to our three-dimensional model, the Trp-152 is at the center of the monomer-monomer interface in the transmembrane domain, Gly-87 faces the phospholipid membrane core, and Ser-296 faces the solvent in the cytoplasmic C-terminal domain. B, the homodimer model is shown in a CPK representation and color-coded by the hydrophobicity scale of Kyte and Doolittle. The model is colored from blue (highly hydrophilic) to red (highly hydrophobic) amino acids. The approximate transmembrane domain limits are marked by two parallel horizontal black lines. The insets show a zoom into the location of each mutation. Trp-152 is boxed by a green square, Gly-87 is boxed by a white square, and Ser-296 is boxed by a yellow square.

well as a high percentage of Zinquin fluorescent cells while retaining a minimal percentage of dead cells, MCF-7 cells were transfected with equal amounts of ZnT2-YY and ZnT2-YN. As indicated above (Fig. 1), MCF-7 cells were found to endogenously express low levels of ZnT2, thereby introducing low endogenous background while measuring vesicular zinc accumulation mediated by ZnT2. Moreover, untransfected cells were used as a control for endogenous vesicular zinc accumulation in MCF-7 cells (see “Experimental Procedures”). The percentage of Zinquin-positive untransfected cells was negligible when the extracellular zinc concentration was increased from 0.1 to 30 μM and increased in a zinc dose-dependent manner only when the extracellular concentration of ZnSO₄ was increased >30 μM (Fig. 7A), indicating that vesicular accumulation of low zinc concentrations was presumably due to low endogenous expression of ZnT2 (Fig. 1). In contrast, the percentage of Zinquin-positive ZnT2-YY-transfected cells was increased in a dose-dependent manner when the extracellular concentration of ZnSO₄ was increased from 0.3 to 100 μM due to vesicular accumulation of higher zinc concentration by the transfected ZnT2 (Fig. 7A). Similarly, cellular Zinquin fluorescence intensity of ZnT2-YY-YN-transfected cells was increased in a zinc dose-dependent manner at extracellular concentrations of ZnSO₄ >3 μM (Fig. 7B). In contrast, there was no zinc dose-dependent increase in Zinquin fluorescence in untransfected cells (Fig. 7B). To exclude the possibility that toxic concentrations of zinc were used here, we assessed cell death using a PI staining assay (Fig. 7C). The percentage of cell death (i.e. PI-positive cells) in untransfected cells as well as in ZnT2-YY-YN-transfected cells was constant for all extracellular zinc concentrations tested, as reported previously (48). Specifically, the mean percentage of cell death in untransfected cells was ~6–8%, whereas that in the ZnT2-YY-YN transfectants was somewhat higher (~15–18%), due to well established transfection reagent toxicity (Fig. 7C). Thus, in both untransfected and transfectant cells, no toxicity could be attributed to extracellular ZnSO₄ concentrations of 0.1–100 μM. Based on these findings, an extracellular zinc concentration of 75 μM was further used upon flow cytometry and fluorescence microscopy experiments in order to determine vesicular zinc accumulation in WT and mutant ZnT2 transfectants. This very same concentration of ZnSO₄ that was previously used in vesicular Zinquin accumulation experiments (7, 13, 37) was found here to provide (a) a high percentage of Zinquin-positive cells, (b) a high Zinquin intensity, (c) a low percentage of cell death, and (d) a significant difference between vesicular Zinquin accumulation parameters of untransfected cells and ZnT2-YY-YN-transfected cells. All transfections resulted in similar transfection efficiencies, which enabled us to compare zinc accumulation in the various transfectants. Two quantitative parameters were determined in order to assess vesicular zinc accumulation in ZnT2 transfectants. The first parameter was derived by dividing the fraction of YC-YN or YFP-positive cells displaying Zinquin fluorescence by the total fraction of YC-YN or YFP fluorescent cells; this ratio is presented as a percentage of vesicular Zinquin accumulation. The second parameter was the mean Zinquin fluorescence intensity per transfected cell. A high percentage of Zinquin-positive cells as well as high Zinquin fluorescence intensity were considered as high vesicular Zinquin accumulation, whereas a low percentage of Zinquin-positive cells as well as low Zinquin intensity were considered as low vesicular Zinquin accumulation activity. MCF-7 cells were transfected with ZnT2-YFP alone (Fig. 8A, iv–vi) or co-transfected with both WT ZnT2-YY and WT ZnT2-YN (Fig. 8A, vii–ix) as well as co-transfected with half the total amount of WT ZnT2-YY and WT ZnT2-YY plasmid DNA (Fig. 8A, x–xii). The relatively strong interaction between ZnT2 monomers was reflected in high fluorescence levels (Fig. 8B) and the vesicular localization of ZnT2 yielded a high fraction of Zinquin-accumulating cells as well as high Zinquin fluorescence levels (Fig. 8C). In contrast, cells transfected with the mutant G87R ZnT2-YY (Fig. 8A, xiii–xv) as well as cells co-transfected with the mutant G87R ZnT2-YY and G87R ZnT2-YN (Fig. 8A, xvi–xviii) exhibited perinuclear ER retention of ZnT2 homodimers, resulting in low vesicular Zinquin-accumulating cells and low Zinquin fluorescence levels, which were even below the levels observed with empty vector transfectants (Fig. 8C). Moreover, co-transfection of G87R ZnT2-YY and WT ZnT2-YN resulted in homodimer retention at the perinuclear compartment (Fig. 8A, xix–xxi) with consequent low Zinquin flu-
FIGURE 7. Vesicular Zinquin accumulation and cell viability was evaluated as a function of increasing extracellular ZnSO₄ concentrations. Untransfected MCF-7 cells (solid line with filled circles) or MCF-7 cells co-transfected with ZnT2-YC-YN (hatched line with triangles) were incubated in RPMI 1640 growth medium containing increasing concentrations of ZnSO₄ (0.1–100 μM) for 2 h. Then, cells were incubated with the fluorescent zinc probe, Zinquin ethyl ester (40 μM), for 1 h and examined by flow cytometry for the following parameters: the percentage of cells showing Zinquin fluorescence (A); mean Zinquin fluorescence per cell (B) (one representative experiment is presented); and the percentage of cells showing PI staining (see “Experimental Procedures” for details) (C), determined in order to assess the fraction of dead cells. For A and C, asterisks indicate that the values obtained are significantly different when compared with untransfected cells (p < 0.05). Error bars shown in A, B, and C represent S.D.
orescence levels being markedly lower than vesicular Zinquin accumulation obtained upon transfection with half of the total amount of WT ZnT2-YC-YN plasmid DNA (x–xi) along with YC empty vector. In order to examine the putative dominant negative effect of TNZD ZnT2 mutations, MCF-7 cells were further transfected with G87R ZnT2-YFP (xiii–xv), as well as with G87R ZnT2-YC-YN (xvi–xviii) or with G87R ZnT2-YN and WT ZnT2-YN (xix–xxi). YFP (green fluorescence) indicates homodimer formation. RedDot (red fluorescence) was used to stain nuclei. Zinquin (blue fluorescence) accumulation in live cells was visualized after incubating transfected cells with the fluorescent zinc probe, Zinquin ethyl ester (40 μM), for 1 h (blue fluorescence). B, MCF-7 cells transfected with the constructs described along the x axis, were examined for the percentage of cells showing YFP fluorescence (dark bars) as well as for mean YFP fluorescence (gray bars) using flow cytometry. C, MCF-7 cells transfected with the constructs described along the x axis were examined for the percentage of transfected cells displaying Zinquin fluorescence (dark bars) as well as for Zinquin fluorescence levels (gray bars). The percentage of transfected cells displaying Zinquin fluorescence was calculated by dividing the fraction of YFP fluorescent cells (YC-YN or YFP) displaying Zinquin fluorescence by the total fraction of cells displaying YFP fluorescence (YC-YN or YFP). For B and C, asterisks indicate that the values obtained are significantly different (p < 0.05) when compared with WT ZnT2-YN (p < 0.05). Error bars shown in B and C represent S.D.

FIGURE 8. Characterization of the G87R mutant ZnT2 using BiFC analysis and vesicular Zinquin accumulation. A, MCF-7 cells were transiently transfected with WT and mutant ZnT2 and examined for dimerization formation as well as for vesicular Zinquin accumulation. Cells were transfected with YFP empty vector (i–iii) as a negative control as well as with ZnT2-YFP (iv–vi) or with ZnT2-YN (vii–ix) as a positive control. Cells were also transfected with half of the total amount of WT ZnT2-YC-YN plasmid DNA (x–xi) along with YC empty vector. In order to examine the putative dominant negative effect of TNZD ZnT2 mutations, MCF-7 cells were further transfected with G87R ZnT2-YFP (xiii–xv), as well as with G87R ZnT2-YN (xvi–xviii) or with G87R ZnT2-YN and WT ZnT2-YN (xix–xxi). YFP (green fluorescence) indicates homodimer formation. RedDot (red fluorescence) was used to stain nuclei. Zinquin (blue fluorescence) accumulation in live cells was visualized after incubating transfected cells with the fluorescent zinc probe, Zinquin ethyl ester (40 μM), for 1 h (blue fluorescence). B, MCF-7 cells transfected with the constructs described along the x axis, were examined for the percentage of cells showing YFP fluorescence (dark bars) as well as for mean YFP fluorescence (gray bars) using flow cytometry. C, MCF-7 cells transfected with the constructs described along the x axis were examined for the percentage of transfected cells displaying Zinquin fluorescence (dark bars) as well as for Zinquin fluorescence levels (gray bars). The percentage of transfected cells displaying Zinquin fluorescence was calculated by dividing the fraction of YFP fluorescent cells (YC-YN or YFP) displaying Zinquin fluorescence by the total fraction of cells displaying YFP fluorescence (YC-YN or YFP). For B and C, asterisks indicate that the values obtained are significantly different (p < 0.05) when compared with WT ZnT2-YN (p < 0.05). Error bars shown in B and C represent S.D.
form homodimers at moderate levels, hence allowing for high vesicular zinc accumulation as reflected in high Zinquin accumulation (Fig. 9C). The third mutant transfectant, W152R ZnT2-YFP, localized at the ER (Fig. 9A, xiii–xv) and exhibited low vesicular Zinquin accumulation (Fig. 9C), which was similar to the levels obtained with G87R ZnT2 (Fig. 9C). The low YFP fluorescence levels of the cotransfection of the mutant W152R ZnT2-YC and W152R ZnT2-YN indicated a very weak interaction between mutant W152R ZnT2 monomers (Fig. 9, A (xvi–xviii) and B). Consequently, W152R ZnT2 homodimers displayed very low vesicular Zinquin accumulation levels (Fig. 9C). Interestingly, however, although the interaction between W152R ZnT2 and WT ZnT2 was relatively weak (Fig. 9, A (xix–xxi) and B), it was somewhat stronger than that observed with W152R ZnT2 homodimers (Fig. 9B); this consistently resulted in a somewhat elevated vesicular Zinquin accumulation in W152R and WT ZnT2 homodimers compared with W152R homodimers (Fig. 9C). Finally, to recapitulate the compound mutations occurring in the Japanese mothers harboring both S296L and W152R, we co-transfected both W152R ZnT2-YN (Fig. 9A, xxii–xxiv); this resulted in very low YFP fluorescence levels (Fig. 9B), indicating a very weak interaction between these mutant monomers that are associated with very poor zinc levels in the mothers’ milk. The low vesicular Zinquin accumulation levels obtained in this case (Fig. 9C) are presumably a result of the residual zinc accumulation activity of S296L ZnT2. The results of all these WT and mutant ZnT2 transfections described above are summarized in Table 5.

DISCUSSION

It has recently been proposed that oligomerization of zinc transporters plays a key role in modulating their function and intracellular trafficking (49). To provide direct visual evidence
for the in situ protein-protein interactions (i.e. homodimerization and heterodimerization) presumably occurring between various ZnTs in live cells, we here applied the BiFC technique. BiFC was previously used to probe for the interaction between various receptors and transporters, including the calcium ion channel receptor-like receptor (50), the α1b-adrenergic receptor (51), the multidrug efflux transporter ABCG2 (26), and other transmembrane proteins (52, 53). Herein, tagging the C terminus of the multidrug efflux transporter ABCG2 (26), and other transmembrane proteins with non-fluorescent halves of YFP allowed us to follow the formation of YFP fluorescence signal in live cells at their established subcellular compartments, upon co-transfection of both vectors into cells. In contrast, only background fluorescence localized at the characteristic compartments of ZnT5 resulted in proteins that were cytotoxic, presumably due to the formation of nonfunctional complexes (23, 54).

(b) Consistently, this C-terminal region of ZnT5 is important for recognizing ZnT6 as a partner for heterodimerization (23). (c) The corresponding region of ZnT3 appears to regulate intracellular trafficking and zinc transport activity by forming a covalent dityrosine bond in response to oxidative stress (22). (d) The C-terminal region was further found to be important for protein-protein interactions between ZnT1 and the protein kinase Raf-1 (44). (e) Two crystal structures of the cytoplasmic domain of the bacterial ZnT homologues revealed that this cytoplasmic domain is essential and sufficient for stable dimer formation and that the structure of this domain is highly conserved despite a high degree of sequence variability (42, 43).

In our current study, co-transfection of non-fluorescent ZnT5-YC and ZnT6-YN, which were shown to form heterodimers but not homodimers (23), also resulted in the formation of YFP fluorescence levels that were much higher than those obtained after transfection of ZnT6 alone. The fluorescence localized at the characteristic compartments of ZnT5 and ZnT6, including Golgi apparatus and cytoplasmic vesicles. In contrast to previous findings obtained using co-immunoprecipitation, which showed that ZnT5 does not form homodimers (23), our present results with the very sensitive BiFC technique suggest that ZnT5 does form homodimers, at least to some extent. This is in line with a previous study showing that heterologous expression of ZnT5 was sufficient to trigger zinc transport in vesicles isolated from human ZnT5-transfected HeLa cells (12). Furthermore, endogenous ZnT5 was found to be localized in cytoplasmic vesicles, such as COPII-coated vesicles in various cells (55), and was associated with the insulin granule membrane in pancreatic β-cells (12). In contrast, endogenous ZnT6 mainly localized in the trans-Golgi network (11, 56). These differences may also suggest that each transporter (i.e. ZnT5 and ZnT6) has a specific function when it is not co-ex-

### Table 5: Characterization of ZnT2 mutations associated with TNZD using BiFC analysis

| Transfection | Subcellular localization of ZnT2 | Cells displaying YC-YN fluorescence & YFP fluorescence | Mean YFP fluorescence per cell | Strength of interaction between monomers | Zinquin accumulation | Mean Zinquin fluorescence per cell |
|--------------|---------------------------------|-------------------------------------------------------|-------------------------------|------------------------------------------|---------------------|-----------------------------------|
| YFP empty vector | Cytoplasm | % | AU | 2529 | NA | 54 | 391 |
| ZnT2-YFP | Cytoplasmic vesicles | 65 | 5670 | NA | 78 | 530 |
| ZnT2-YC-YN | Cytoplasmic vesicles | 60 | 3913 | Strong | 81 | 483 |
| ZnT2-YC-YN + empty vector | Cytoplasmic vesicles | 48 | 3440 | Strong | 79 | 409 |
| G87R ZnT2-YFP | ER | 63 | 7903 | NA | 27 | 329 |
| G87R ZnT2-YC-YN | ER | 56 | 2582 | Moderate | 28 | 335 |
| G87R ZnT2-YC-YN + ZnT2-YN | ER | 66 | 2791 | Moderate | 33 | 324 |
| S296L ZnT2-YFP | ER and cytoplasmic vesicles | 60 | 5924 | NA | 70 | 416 |
| S296L ZnT2-YC-YN | ER and cytoplasmic vesicles | 51 | 1509 | Weak | 54 | 354 |
| S296L ZnT2-YN + ZnT2-YN | ER and cytoplasmic vesicles | 57 | 2471 | Moderate | 75 | 418 |
| W152R ZnT2-YFP | ER | 64 | 4844 | NA | 27 | 317 |
| W152R ZnT2-YC-YN | ER | 54 | 1637 | Weak | 35 | 318 |
| W152R ZnT2-YC-YN | ND | 63 | 2037 | Weak | 47 | 293 |
| ZnT2-YN | ND | 50 | 987 | Very weak | 32 | 336 |
| ZnT2-YC-YN | ER | 3 | 455 | Very weak | NA | NA |

* YFP fluorescence values between 0 and 1000 indicate a very weak interaction between monomers. YFP fluorescence levels between 1000 and 2000 indicate a weak interaction between monomers. YFP fluorescence levels between 2000 and 3000 indicate a moderate interaction between monomers. YFP fluorescence levels above 3000 indicate a strong interaction between monomers.

* Percentage of cells displaying YC-YN or YFP fluorescence as well as Zinquin fluorescence, divided by the percentage of cells displaying YC-YN or YFP fluorescence.
pressed along with its cognate transporter and thus does not form ZnT5–ZnT6 heterodimers. Consistently, transfection of ZnT5 alone, which was found to harbor a zinc binding site that is apparently absent in ZnT6, resulted in an accelerated removal of zinc from the cytoplasm and increased vesicular zinc sequestration; this was concomitantly associated with alkalization of the trans-Golgi network, hence consistent with the H⁺ exchange mechanism of zinc transport activity mediated by ZnT5 (56). The moderate interaction between ZnT5 monomers indicated by the moderate YFP fluorescence levels possibly allows their flexibility in generating heterodimers with ZnT6 as well. These findings are in accord with the possible formation of functional ZnT5 homodimers mediating zinc transport via a proton exchange mechanism. However, in the mutant chicken cell line DT-40, only coexpression and oligomerization of ZnT5 and ZnT6 was sufficient to activate the zinc-dependent phosphatase TNAP, whereas individual expression of ZnT5 or ZnT6 did not substantially activate TNAP (57, 58). In this respect, it has been proposed that the failure of ZnT5 alone to support TNAP activation may indicate a slower zinc transport, presumably mediated by ZnT5 homodimers when compared with the full zinc transport activity of ZnT5/ZnT6 heterodimers (56). Clearly, however, further studies are warranted to experimentally address this hypothesis. The apparent discrepancy between the previous co-immunoprecipitation results suggesting that ZnT5 does not form homodimers (23) and our current BiFC results indicating that ZnT5 does form homodimers, at least to some extent, may be attributed to the increased sensitivity of the BiFC assay in detecting interactions between specific proteins. Indeed, the BiFC technique enables the interacting protein partners to form a complex with a long half-life or a high occupancy, and unassociated fusion proteins do not interfere with detection of the complex. Moreover, it has been shown previously that BiFC allows the detection of interactions at lower protein concentrations (24). In contrast to traditional fluorescence assays, including immunofluorescence or tagging ZnT5 or ZnT6 with a fluorescent protein, the BiFC approach enabled the direct in situ identification of ZnT5–ZnT6 heterodimers in live cells for the first time.

Interestingly, YFP-tagged ZnT1, -2, -3, and -4 showed higher mean fluorescence levels compared with the mean YFP fluorescence levels of YFP-tagged ZnT5, -6, and -7. In addition, the apparent interaction between ZnT1–4 monomers was found to be stronger than the interaction between ZnT5 and ZnT6 monomers as well as ZnT7 monomers based on YFP fluorescence intensity. This difference may be explained by the distinct localization pattern of ZnTs, which can affect YFP fluorescence intensities; specifically, whereas ZnT1–4 are distributed all over the cell in vesicles and/or in the plasma membrane, ZnT5–7 are restricted to the Golgi apparatus and cytoplasmic vesicles. It may be further suggested that zinc transporters, including those that belong to the secretory pathway, undergo post-translational regulation when overexpressed.

Oligomerization of ZnTs was found to be associated with several pathologic disorders. Recently, co-immunoprecipitation and Western blot analysis revealed that ZnT3 forms cova-

lent homodimers mediated by intermolecular dityrosine bonds, which are induced by oxidative stress (22); these covalent dityrosine bonds were found to modulate subcellular localization and zinc transport activity of ZnT3 (22). Herein we showed that homodimers of ZnT3 localize at intracellular vesicles, consistent with its vesicular localization in the brain (45). The ability to assess and visualize ZnT3 homodimerization in situ using BiFC in live cells provides an advantage in examining the role of oxidative stress in ZnT3 homodimerization. In addition, a previous study showed that a non-synonymous SNP in ZnT8, which introduced an arginine in place of tryptophan at amino acid 325, at the dimer interface of the cytoplasmic C-terminal region, is associated with increased susceptibility to type 2 diabetes (19). Based on three-dimensional homology modeling of ZnT8, Weijers et al. (59) predicted that both Arg-325 and Trp-325 are shielded by the planar surface of the three cytoplasmic β-strands and hence are unable to affect the sensing capacity of the C-terminal domain. In this respect, BiFC will be a useful tool in the assessment of the non-direct effect of the R325W SNP on ZnT8 homodimerization. Moreover, the relatively high sequence homology between ZnT2, ZnT3, and ZnT8 (17) suggests that these transporters share a common mechanism, including intermolecular dityrosine bonds as well as the involvement of compatible residues at the dimer interface for modulating their homodimerization.

Recently, we identified a novel G87R mutation in ZnT2 in two women producing zinc-deficient milk; as a result, their exclusively breast-fed infants developed TNZD (16). Gly-87 was predicted to point toward the membrane lipid core according to a ZnT2 model that was generated based on the crystal structure of E. coli YiiP (16). Our current updated ZnT2 three-dimensional model, which was generated herein based on the recent inwardly facing conformation cryoelectron microscopy structure of S. oneidensis YiiP (Protein Data Bank entry 3J1Z) (34) also suggested that Gly-87 is located in a highly hydrophobic region and is facing the membrane environment. Consequently, substitution of a small glycine residue with a positively charged arginine (G87R) resulted in ER retention and loss of zinc transport in MCF-7 and HC-11 cell transfectants (16). Our previous findings also revealed that the G87R mutation had a dominant negative effect on the WT ZnT2, reflected in an ER retention of WT ZnT2 and loss of zinc transport, presumably due to homodimerization of ZnT2 (16). Herein, BiFC analysis allowed us to visualize directly the dominant negative effect of the G87R mutation on the subcellular localization of the WT ZnT2 in live cells, which was accompanied by the loss of vesicular zinc accumulation. The relatively moderate interaction between mutant G87R ZnT2 and WT ZnT2 monomers indicated by the BiFC analysis explains the dominant negative effect of G87R ZnT2 on WT ZnT2. Moreover, whereas dermatitis manifested already at an age of 2–2.2 months after birth in the case of the G87R mutation, it appeared at an age of 3–6 months in the case of the H54R mutation, which does not inflict a dominant negative effect (15). The prominent dominant negative effect inflicted by the G87R mutation may provide the molecular explanation for the relatively rapid onset of dermatitis in the exclusively breast-fed infants. Nonetheless, the heterozygous G87R mutation resulted in a 75% reduction in milk zinc.
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collection, suggesting that the fraction of WT ZnT2 homodimers is sufficient to preserve as much as 25% of normal milk zinc concentration. Moreover, other mammary gland ZnTs, including ZnT1 and ZnT4, may also play a contributing role in zinc secretion into milk (60). Hence, it will be of interest to determine whether or not ZnT2 forms heterodimers with other mammary gland ZnTs, thereby enhancing our understanding of the molecular mechanisms underlying mammary gland zinc transport and zinc homeostasis. Two novel compound mutations in the ZnT2 gene were recently identified in a Japanese mother producing very low milk zinc concentrations (>90% reduction), the infant of whom developed severe TNZD already at the age of 13 days (17). The mutation in the first allele resulted in the substitution of a tryptophan by an arginine (W152R), whereas the mutation in the second allele resulted in a substitution of serine by leucine (S296L). Biochemical characterization indicated that W152R was a loss of function mutation because it abolished dimer formation and zinc transport activity. In contrast, the second S296L mutation retained both abilities; however, the mutant protein was highly unstable (17). Herein, BiFC analysis enabled us to readily detect the in situ subcellular localization of S296L and W152R ZnT2 homodimers in live cells. S296L ZnT2-YFP was found to be localized at the ER and cytoplasmic vesicles. The substitution of a polar serine 296, which is predicted to be localized at the cytoplasmic C terminus of ZnT2, by a hydrophobic leucine was previously suggested to alter the topology of ZnT2 by creating an additional transmembrane domain (17). Our current updated three-dimensional model of ZnT2 also suggests that native polar Ser-296 is facing the solvent (−0.8 according to the Kyte and Doolittle hydropathy scale) and resides in a small hydrophobic pocket surrounded by hydrophilic residues. Its substitution by a highly hydrophobic residue, such as leucine (3.8 according to the Kyte and Doolittle hydropathy scale), will enhance considerably the hydrophobicity of this region, thereby increasing solvation energy and decreasing domain stability. Hence, this suggestion may explain the apparent misfolding of S296L and the observed ER retention. Furthermore, it is possible that mutations in the C terminus of ZnT2, which is important for protein-protein interactions, will interfere with ZnT2 homodimerization, thereby leading to ER retention. The weak interaction between mutant S296L ZnT2 monomers directly caused by mutant misfolding or directly through interference with protein-protein interactions may explain the low protein stability obtained under treatment with cycloheximide (17) and the higher degradation rate through protein degradation systems, including the ubiquitin–proteasome and the lysosome pathways. Interestingly, however, S296L ZnT2 nonetheless showed some vesicular localization, thereby providing the first explanation for residual zinc accumulation, which was detected here by the BiFC-Zinquin assay despite the relatively weak interaction between S296L monomers. Although no experimental evidence was provided, it was recently proposed that mutant S296L ZnT2 had a dominant negative effect on WT ZnT2 activity (17). Here we showed that the strength of interaction between mutant S296L ZnT2 and WT ZnT2 monomers is moderate, as indicated by the relatively significant BiFC fluorescence signal. The moderate interaction between these monomers resulted in a higher Zinquin accumulation when compared with mutant S296L ZnT2 homodimers. However, it is not clear whether this interaction will result in a dominant negative effect on WT ZnT2 stability, because no significant alterations were detected between mutant ZnTs using BiFC analysis, as consistently observed using Western blot analysis (17). Hence, it is likely that moderate interactions between S296L ZnT2 and WT ZnT2 occur that increase S296L ZnT2 stability and hence minimize its degradation. Consistent with previous results (17), a very weak interaction was detected between inactive W152R ZnT2 and the more active S296L ZnT2. This interaction resulted in inactive homodimers, thereby providing a molecular explanation for the severe consequences of the double mutant W152R and S296L on zinc concentration in mother’s milk. Hence, one could predict that heterozygous S296L mothers or homozygous S296L mothers will produce milk with higher zinc concentration compared with mothers harboring both the S296L and W152R compound mutations, which produce very low milk zinc concentrations (>90% reduction) (17). We further show here that mutant W152R ZnT2 was found to be localized in the heart of the monomer-monomer interface, in a highly hydrophobic region. Substitution of this hydrophobic Trp-152 residue (−0.9 according to the Kyte and Doolittle hydropathy scale) with a positively charged residue, such as arginine, will necessarily disrupt the interaction between ZnT2 monomers, thereby leading to a profound disruption of homodimer formation. Additionally, it is possible that the W152R mutation impairs the stability of the dimer-membrane interaction, because Trp-152 is partially exposed to the membrane. These predictions explain the protein misfolding and the ER retention of W152R ZnT2, which may indirectly interfere with protein-protein interactions (17). It is also possible that Trp-152 may contribute directly to homodimerization due to its vicinity to the ZnT2 interface; hence, mutation in Trp-152 will possibly interfere with ZnT2 homodimerization, thereby resulting in ER retention as well (17, 21). The weak interactions between W152R ZnT2 monomers and its ER retention resulted in low Zinquin accumulation. In contrast to previous findings, which showed no interaction between mutant W152R ZnT2 and WT ZnT2, we show here that the interaction between W152R ZnT2 and WT ZnT2 was moderate and enabled moderate Zinquin accumulation, which was 1.7-fold lower than Zinquin accumulation of the half-amount of WT ZnT2, thereby providing the first evidence for some dominant negative effect of W152R ZnT2 on WT ZnT2 activity, which is somewhat less pronounced than the dominant negative effect of mutant G87R ZnT2 on WT ZnT2. Hence, it is possible that mothers who are heterozygous for the W152R mutation will produce milk that contains higher zinc concentrations than mothers who are heterozygous for the G87R mutation or who are homozygous for the W152R mutation.

In summary, we provided here the first direct visual evidence for the in situ dimerization of normal and mutant ZnTs using the sensitive BiFC technique, which enabled the direct visualization of specific protein-protein interactions in live cells. Combining this sensitive BiFC approach with a compatible fluorescent zinc probe like Zinquin in live cells revealed the in situ...
localization and function of these homo- and heterodimers. Using BiFC analysis, we shed light on the molecular dynamics of homodimerization of ZnT and its control cellular zinc homeostasis. Furthermore, this study paves the way for the direct analysis of zinc deficiency mutations interfering with zinc transporter oligomerization, subcellular localization, and zinc transport.

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