The role of cation diffusion facilitator CDF-1 in lipid metabolism in Caenorhabditis elegans

Ying Hu,1,† Yanli Wang,2,† Xuanjun Wang,1 Xiaoyun Wu,1 Lin Fu,2 Xiayu Liu,2 Yu Wen,2 Jun Sheng,1,* and Jingjing Zhang1,2,*

1College of Food Science and Technology, Yunnan Agricultural University, Kunming 650201, China
2Center for Life Sciences, School of Life Sciences, Yunnan University, Kunming, Yunnan 650091, China

Abstract
Zinc is one of the most important trace elements as it plays a vital role in many biological processes. As well, aberrant zinc metabolism has been implicated in lipid-related metabolic diseases. Previously, we showed that zinc antagonizes iron to regulate sterol regulatory element-binding proteins (SREBP-SCD) pathway in lipid metabolism in the model organism Caenorhabditis elegans. In this study, we present the identification of another cation diffusion facilitator, CDF-1, which regulates lipid metabolism along with SUR-7 in response to zinc. Inactivation of SBP-1, the only homolog of SREBPs, leads to an increased zinc level but decreased lipid accumulation. However, either the cdf-1(n2527) or sur-7(tm6523) mutation could successfully restore the altered fatty acid profile, fat content, and zinc level of the sbp-1(ep79) mutant. Furthermore, we found that CDF-1/SUR-7 may functionally bypass SBP-1 to directly affect the conversion activity of SCD in the biosynthesis of unsaturated fatty acids and lipid accumulation. Collectively, these results consistently support the link between zinc homeostasis and lipid metabolism via the SREBP-SCD axis by the cation diffusion facilitators CDF-1 and SUR-7.

Keywords: zinc; sterol regulatory element-binding protein (SREBP); stearoyl-CoA desaturase (SCD); cation diffusion facilitator (CDF); lipid accumulation

Introduction
Zinc is one of essential trace elements for living organisms, and it plays numerous biological roles. For example, zinc determines both the structure and functionality of a variety of proteins, such as the metalloproteins. It can regulate both enzymatic activity and the stability of the proteins as either an activator or an inhibitor ion, and it modulates cellular signal transduction processes that are usually regulated by zinc transport or other interrelated proteins (Fukada et al. 2011; Chasapis et al. 2012; Escobedo-Monge et al. 2019). Thus, it is critical to maintain proper zinc homeostasis for living organisms.

Dysregulation of zinc homeostasis is related to many human diseases. In particular, deficiency of zinc has been shown to be closely associated with an altered lipid profile resulting in a series of lipid metabolism diseases, including obesity and comorbid conditions such as insulin resistance, type 2 diabetes, and inflammation (Costarelli et al. 2010; Blažewicz et al. 2013; Miao et al. 2015). Erythrocyte zinc levels are associated with body mass index and waist circumference (Blažewicz et al. 2013), and low zinc concentration was found in erythrocytes in obese women (Ennes Dourado Ferro et al. 2011). Moreover, there is a significant decrease in blood zinc levels in morbidly obese patients (de Luis et al. 2013). As well, zinc deficiency increases leptin production and exacerbates macrophage infiltration into adipose tissue in obese mice (Liu et al. 2013).

Zinc can be used as a nutrient to treat metabolic diseases. Zinc stimulates insulin secretion and increases the sensitivity to insulin. Therefore, zinc supplementation could improve insulin resistance, resulting in the improvement of blood pressure, glucose, and Low density lipoprotein (LDL) cholesterol serum level in obesity (Cruz et al. 2017; Olechnowicz et al. 2018). Obesity-related cardiac hypertrophy was exacerbated by zinc deficiency (ZD) in High fat diet/zinc deficiency (HFD/ZD) mice (de Luis et al. 2013), and this was attenuated by a zinc-supplemented diet (Wang et al. 2016). However, there may be deleterious effects with excessive zinc supplementation, since excessive zinc intake may cause an undesirable increase in HbA1c levels and high blood pressure (Miao et al. 2013). Again, the balance of zinc homeostasis in metabolism is critically important.

Maintenance of intracellular zinc homeostasis depends on the balance of zinc absorption, distribution, and excretion, which is mainly regulated by two protein families: metallothioneins and zinc transport proteins (Kimura and Kambe 2016). Zinc transport proteins are mainly responsible for mediating the compartmentalization of zinc into various organelles and vesicles for their storage, supplying zinc to various proteins that require it for functionality (Fukunaka and Fujitani 2018). Zinc transport proteins are divided into two major families: the zinc transporters or cation diffusion facilitator (ZnTs/CDFs) or solute-linked carrier family 30 (SLC30A), and the Zip (Zrt- and Irt-like proteins) family
or solute carrier family 39 A (SLC39A) (Liuzzi and Cousins 2004; Baltaci and Yuce 2018; Cotrim et al. 2019; Thokala et al. 2019). Several studies have shown that the expression of ZnT genes and disturbed zinc metabolism is associated with obesity and diabetes (Quraishi et al. 2005; Noh et al. 2014; Morais et al. 2019). The polymorphisms (SNPs) of ZnT8 have been connected with type 1 (Xu et al. 2011; Wenzlau and Hutton 2013) and type 2 diabetes (Rutter and Chimienti 2015; Drake et al. 2017; Virgili et al. 2018). ZnT5 is also involved in metabolic diseases (Cuadrado et al. 2018). However, the underlying mechanisms relating these ZnTs to metabolic diseases are largely unknown.

Our previous work showed that SUR-7, one of the zinc transport proteins in the model organism Caenorhabditis elegans (Yoder et al. 2004; Roh et al. 2013) synergistically affects zinc homeostasis and lipid metabolism via the sterol regulatory element-binding proteins and the steraryl-CoA desaturase (SREBP-SCD) axis (Zhang et al. 2017). In C. elegans, SBP-1 is the only homolog of sterol-regulatory element binding proteins (SREBPs). SREBPs are major regulators of lipid homeostasis, including fatty acids, triglycerides, and cholesterol in vertebrate cells (Goldstein et al. 2006; Shao and Espenshade 2012). Inactivation of SUR-7 restored the proper level of zinc and lowered fat accumulation and fatty acid profiles of sbp-1(ep79) via directly upregulating the activity of SCD (Zhang et al. 2017). Thus, this raised the question whether other zinc transport proteins were also involved in lipid metabolism. Therefore, we examined zinc transport related proteins in other zinc transport proteins were also involved in lipid metabolism via the sterol regulatory element-binding proteins and the stearyl-CoA desaturase (SREBP-SCD) axis (Zhang et al. 2017). In C. elegans, SBP-1 is the only homolog of sterol-regulatory element binding proteins (SREBPs). SREBPs are major regulators of lipid homeostasis, including fatty acids, triglycerides, and cholesterol in vertebrate cells (Goldstein et al. 2006; Shao and Espenshade 2012). Inactivation of SUR-7 restored the proper level of zinc and lowered fat accumulation and fatty acid profiles of sbp-1(ep79) via directly upregulating the activity of SCD (Zhang et al. 2017). Thus, this raised the question whether other zinc transport proteins were also involved in lipid metabolism. Therefore, we examined zinc transport related proteins in C. elegans and identified that the cdf-1(n2527) mutation acted as another suppressor of the sbp-1(ep79) mutant to restore its zinc levels and lipid content. This result provides further consistent evidence to link zinc homeostasis and lipid metabolism.

Materials and methods
Nematode strains, RNA interference, and culture conditions
C. elegans strains were maintained on NGM plates with Escherichia coli OP50 under standard culture conditions, unless otherwise specified. RNA interference (RNAi) was performed using the feeding method (Wu et al. 2018) and the bacterial strains feed were from the Ahringer C. elegans RNAi library. The wild-type strain was Bristol N2 (WT). All worm strains used in this study are listed in Supplementary Table S1.

Construct of sur-7RNAi clone
Construction of the sur-7RNAi used the L4440 empty vector (EV). The PCR primers used for the amplified EV and sur-7 gene are shown in Supplementary Table S2. These two linearized DNA fragments were recombined following the instructions of ClonExpress MultiS One Step Cloning Kit (Vazyme Biotech Co., Ltd) and then transformed into HT115 competent cells.

Construction of transgenic strains
Construction of the WT, kunEx203[caf-1p::caf-1::gfp, myo-2p::mcherry] and WT; kunEx187[sur-7p::sur-7::gfp, rol-6su1006] transgenic strains were done as follows. In general, DNA fragments of specific genes and their related promoters were isolated using PCR. The amplified DNA fragments were subsequently inserted into the transgenic plasmid pPD95.75 (Frøkjær-Jensen et al. 2008). An injection mixture with 50 ng/μL transgenic plasmid and 5 ng/μL pCFJ90(myo-2p::mcherry) or rol-6su1006) were injected into the gonads of young adult wild type N2 worms. The positive transgenic worms were selected based on fluorescence expression. The primers for the construction of transgenic worms are listed in Supplementary Table S2.

Nile Red staining of fixed worms and quantification of lipid droplet size
Nile Red staining of fixed worms was performed as previously described (Brooks et al. 2009; Liang et al. 2010). Young adult worms were collected and suspended in 1 mL of water on ice, then resuspended in 1 mL of buffer M9 with 50 μL of freshly prepared 10% paraformaldehyde solution. Worms were frozen in liquid nitrogen, subjected to two or three incomplete freeze/thaw cycles, and then washed with M9 buffer several times to remove the paraformaldehyde solution. Two microliters of 5 mg/mL Nile Red was added to the worm pellet (1 mL) and incubated for 30 min at 20°C, with occasional gentle agitation. Worms were washed two or three times with M9 buffer and mounted onto 2% agarose pads for microscopic observation and photography. For each animal, a projection image with proper intensity was acquired using an Olympus BX53 fluorescence microscope (Japan) (Shi et al. 2013). At least 15 animals were imaged, and lipid droplet (LD) size was quantified from approximately five worms for each worm strain or treatment.

Analysis of fatty acid compositions
Approximately 2000 young adults were harvested for fatty acid esterification and analysis. The harvested worms’ fatty acids were esterified with 1 mL of MeOH + 2.5% H2SO4 and heated for 1 hour at 70°C. Determination of the fatty acids was done using an Agilent 7890 series gas chromatographer equipped with a 15 m × 0.25 mm × 0.25 μm DB-WAX column (Agilent, Santa Clara, California, USA), with helium as the carrier gas at 1.4 mL/min, and a flame ionization detector. Four biological replicates were performed for gas chromatography (GC) analysis.

Analysis of triacylglycerols
About 4 × 10^5 synchronized young adults were harvested for total lipid extraction. Triacylglycerol (TAG) and phospholipids (PL) were separated by thin-layer chromatography, and the determination of fatty acids was by GC using an Agilent 7890A. The methyl ester of C15:0 was used as a standard for quantitative analysis.

Supplementation or sequestration of zinc
ZnSO4 supplementation and zinc reduction by N, N, N’,N’-tetrais(2- pyridylmethyl) ethylenediamine (TPEN) were performed as described previously (Zhang et al. 2017). In brief, synchronized L1 worms were placed and cultivated on NGM plates supplied with either ZnSO4 or TPEN with a final concentration of 50 μM and 5 μM, respectively, and young adult worms were harvested for further analysis.

Zinpyr-1 staining and visualization
Zinpyr-1 staining was performed as described previously (Roh et al. 2013). The fluorescence of Zinpyr-1 was visualized under an OLYMPUS BX53 fluorescence microscope (Olympus). Images were captured using identical settings and exposure times, unless specifically noted. The fluorescence intensity was quantified using Photoshop software (Blażewicz et al. 2013).

Visualization of GFP
L4 and young adult GFP positive worms were picked and plated on an agarose gel pad and visualized using a two-photon confocal microscope (Nikon A1MP+) or a fluorescent microscope (BX53;
Olympus). Images were captured using the same settings and exposure times for each worm, unless specifically indicated, and the GFP reporter expression was quantified using Photoshop software.

**Growth rate assay**

The gravid adult worms were treated with alkaline hypochlorite and the eggs were obtained. These were then seeded onto NGM plates and cultured for several days until adulthood. Forty-eight hours after seeding, the number of adults and the total number of worms were scored under a microscope every 12 hours. Each strain underwent three biological replications.

**Data analysis**

Data are presented as the mean ± SEM, except when specifically indicated. Statistical analysis was conducted using Student’s t-test. Figures were made using GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA) and Adobe Illustrator CS6.

**Data availability**

All data necessary for confirming the conclusions in this article are included in this article and in supplemental figures and tables. Worm strains or plasmids constructed by us are available upon request. Supplemental Material available at figshare: https://doi.org/10.25387/g3.14301467.

**Results**

The cation diffusion facilitator CDF-1 maintained zinc homeostasis and lipid metabolism

In eukaryotic cells, two major families of zinc transport proteins, cation diffusion facilitators (CDF/ZnT/SLC30) and zrt-, irt-like proteins (ZIP/SLC39), mediate zinc trafficking and homeostasis. In total, 14 CDFs and 14 ZIPs in *C. elegans* have been identified with the homologous protein sequences to the 10 CDF and 14 ZIP proteins in *Homo sapiens* (Kambe et al. 2015) (Supplementary Figure S1). We previously reported on zinc transport protein SUR-7, which coordinately affects zinc homeostasis and lipid metabolism (Zhang et al. 2017). To explore whether the other related transporters are also involved in zinc homeostasis and lipid metabolism, we tested 11 of the 28 zinc-related transporters, that had available mutants, to detect their labile zinc and pseudocellular zinc by zinpyr-1 fluorescence and their LDs by Nile Red staining (Brooks et al. 2013).

Zinpyr-1 staining showed a differential intensity and location of fluorescence among the various worm mutants under treatment with or without ZnSO₄ (Figure 1, A and B, Supplementary Figure S2). Under normal conditions, like the *sur-7(tm6523)* mutant, the *cdf-1(n2527)*, *zipt-2.3(ok2094)*, and *zipt-15(ok2160)* mutants displayed reduced fluorescence (Figure 1, A and B, Supplementary Figure S2). This was mainly restricted in the area of the intestine lumen, suggesting a defect in zinc uptake. In contrast, the majority of mutants, especially the *tm-1(tm6669)* mutant, displayed higher zinpyr-1 fluorescence than the wild type N2 (Figure 1, A and B, Supplementary Figure S2). In the presence of ZnSO₄, most of the tested zinc-related transporters showed an increased level of Zinpyr-1 fluorescence to some extent (Figure 1, A and B, Supplementary Figure S2). Interestingly, among the 11 transporter mutants, only the *cdf-1(n2527)* mutant displayed a reduced LD size and lipid accumulation similar to the *sur-7(tm6523)* mutant in response to the ZnSO₄ treatment. However, the LD size and lipid accumulation of the other worms were not altered under normal conditions (Figure 1, C and D, Supplementary Figure S3). Furthermore, consistent with our previous report (Zhang et al. 2017), reduction of zinc by TPEN [N, N', N'-Tetrakis (2-pyridylmethyl) ethylenediamine], a zinc chelator, led to significantly increased LD size and lipid accumulation in all tested transporter mutants, like the wild type N2 (Figure 1, C and D, Supplementary Figure S3). Taken together, these results indicated that CDF-1 may function like SUR-7 in regulating zinc homeostasis and lipid metabolism.

**Inactivation of CDF-1 reversed lipid metabolism defects of the *sbp-1(ep79)* mutant**

Our previous study showed that SUR-7 functions via the SREBP and its target SCD to regulate lipid metabolism (Zhang et al. 2017). Inactivation of *sur-7* restores the LD size of the *sbp-1(ep79)* mutant by upregulating the activity of SCD. As mentioned above, the *cdf-1(n2527)* mutant displayed similar phenotypes like the *sur-7(tm6523)* mutation in response to zinc, therefore, we wondered whether CDF-1 also affected lipid metabolism via the SREBP-SCD axis.

SUR-7, CDF-1, and CDF-2 proteins belong to the CDF family, and they possess six conservative transmembrane domains (Supplementary Figures S1 and S4). The *cdf-1(n2527)* mutation contains an SNP in the third transmembrane domain, leading to a premature termination (Supplementary Figure S4A). The *cdf-2(tm788)* mutation contains an 804 bp deletion and 68 bp insertion, which causes a frame shift mutation (Supplementary Figure S4B). The *sur-7(tm6523)* mutation contains a 564 bp deletion and 4 bp insertion, which causes early termination of translation (Supplementary Figure S4C). To verify whether CDF-1 could be a suppressor of *sbp-1(ep79)*, we constructed the *sbp-1(ep79); cdf-1(n2527)* double mutant. *sbp-1(ep79); sur-7(tm6523)* was used as positive control, while *sbp-1(ep79); cdf-2(tm788)* was used as negative control. As we expected, the LD size and TAG content of both *sbp-1(ep79); cdf-1(n2527)* and *sbp-1(ep79); sur-7(tm6523)*, but not the *sbp-1(ep79); cdf-2(tm788)* mutant were significantly increased compared to the *sbp-1(ep79)* mutant (Figure 2, A–C). Similarly, the *sbp-1(ep79); cdf-1(n2527)* worms developed to adulthood about 24 hours earlier than the *sbp-1(ep79)* worms (Figure 2D). Taken together, these results demonstrated that the *cdf-1(n2527)* mutation was another suppressor of the *sbp-1(ep79)* mutant, in that it could restore the lipid accumulation and growth rate of the *sbp-1(ep79)* mutant.

**The suppression of *sbp-1(ep79)* by *cdf-1(n2527)* was related to zinc level**

As our previous study demonstrated, the *sbp-1(ep79)* mutant displays increased level of Zinpyr-1 fluorescence but reduced lipid accumulation, which can be reversed by *sur-7(tm6523)* (Zhang et al. 2017). Consistently, like the *sbp-1(ep79); sur-7(tm6523)* mutant, the zinc level indicated by Zinpyr-1 fluorescence in the *sbp-1(ep79); cdf-1(n2527)* mutant was significantly reduced compared with the *sbp-1(ep79)* worms, even under the treatment of ZnSO₄ (Figure 3, A and B), suggesting the upregulated zinc level of *sbp-1(ep79)* mutant depends on the activity of SUR-7 and CDF-1.

As we mentioned above and as reported in our previous work (Zhang et al. 2017), zinc negatively regulates lipid accumulation, and zinc reduction leads to increased lipid accumulation. Next, we asked whether the restoration of lipid accumulation of the *sbp-1(ep79)* mutant by *cdf-1(n2527)* was related to the zinc level. Nile Red staining of fixed worms showed that the LD sizes of both the *sbp-1(ep79); cdf-1(n2527)* and *sbp-1(ep79); sur-7(tm6523)* mutants were significantly reduced to the level of the *sbp-1(ep79)*
Figure 1 Zinc transporter mutants with zinc level and fat accumulation. (A) Fluorescence microscopy of WT, cdf-1(n2527), sur-7(tm6523), and cdf-2(tm788) treated with or without ZnSO₄, which stained with Zinpyr-1 and captured using identical settings and exposure times. Scale bar represents 50 µm for whole worms and 10 µm for enlarged worms. The concentration of ZnSO₄ is 50 µM. The red arrowheads indicate the lumen zinc and the white arrows show pseudocoelomic zinc. (B) Quantitation of the fluorescence intensity about pseudocoelomic zinc from (A). (C) Nile red staining of fixed worms treated with ZnSO₄ or TPEN. For representative animals, the anterior was on the left and the posterior was on the right; Bar, 5 µm. (D) Quantitation of lipid droplet size in the posterior region of intestines from five worms of each worm strain. The data are presented as mean ± SEM. For all statistical analysis, significant difference between WT N2 and a specific condition: ***P < 0.001; ****P < 0.0001 (unpaired t-test). Significant difference between the control and ZnSO₄/TPEN treatment of each strain: **P < 0.01; ***P < 0.001 (unpaired t-test).
Figure 2  cdf-1 and sur-7 restored lipid profiles of sbp-1. (A) The lipid droplets by Nile red staining in the worms of WT, sbp-1(ep79), cdf-1(n2527), sbp-1(ep79; cdf-1(n2527)), sur-7(tm6523), sbp-1(ep79; sur-7(tm6523), cdf-2(tm788), and sbp-1(ep79; cdf-2(tm788)). For representative animals, the anterior was on the left and the posterior was on the right; Bar, 5 μm. (B) Quantitation of lipid droplet diameters in the posterior region of intestines from five worms of each worm strain. The data are presented as mean ± SEM. (C) The percentage content of triacylglycerol (TAG %) in total lipids (TAG+PL, phospholipids) of each worm strain. The data are presented as mean ± SEM of four biological repeats. (D) The growth rate of worms. The data are presented as the mean ± SEM with three biological repeats, n: the number of scored worms for each strain. For all statistical analysis, significant difference between WT N2 and each mutant: *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. Significant difference between sbp-1(ep79) and double mutant: $P < 0.05; $$P < 0.01; $$$P < 0.001;$$$$P < 0.0001. Significant difference between WT N2 and each mutant: *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. Significant difference between sbp-1(ep79) and double mutant: $P < 0.05; $$P < 0.01; $$$P < 0.001;$$$$P < 0.0001.
Zinc regulated fat accumulation in *C. elegans*. (A) Zinc levels of WT, sbp-1(ep79), cdf-1(n2527), sbp-1(ep79, cdf-1(n2527)), sur-7(tm6523), sbp-1(ep79, sur-7(tm6523), cdf-2(tm788), and sbp-1(ep79; cdf-2(tm788)) treated with or without ZnSO₄ by stained with Zinpyr-1. Images were captured using identical settings and exposure times. Scale bar represents 50 μm for whole worms and 5 μm for enlarged worms. The concentration of ZnSO₄ is 50 μM. The red arrowheads indicate the lumen zinc accumulates of the intestine, and the white arrows show pseudocoelomic zinc. (B) Quantitation of the fluorescence intensity. (C) Nile red staining of fixed worms’ lipid droplets; Bar, 5 μm. (D) Quantitation of lipid droplet size and frequency distribution of LD diameter in the posterior region of intestines from five worms of each worm strain. All of quantitation of the data is presented as mean ± SEM. For all statistical analysis, significant difference between N2 (WT) and a specific condition: *P* < 0.05; **P** < 0.01; ***P*** < 0.001; ****P < 0.0001. Significant difference between within groups of the control and ZnSO₄ treatment: ‘*P* < 0.05; ‘**P** < 0.01; ‘***P*** < 0.001; ‘****P** < 0.0001 (unpaired t-test), which applies to all subsequent statistical analysis.
mutant when treated with ZnSO₄, similar to the cdf-1(n2527) or sur-7(tm6523) single mutant alone (Figure 3, C and D). However, the LD size in N2 and the sbp-1(ep79) mutant was not altered with the ZnSO₄ treatment (Figure 3, C and D). Taken altogether, these results further support a tight connection of lipid accumulation and zinc homeostasis regulated by SREBP-CDF-1/SUR-7.

**CDF-1 regulated the activity of SCD**

In *C. elegans*, FAT-5, FAT-6, and FAT-7 are three SCDs that convert the saturated fatty acids palmitic acid (C16:0) and stearic acid (C18:0) to palmitoleic acid [C16:1(n-7)] and oleic acid [C18:1(n-9)] ([Brock et al. 2007; Shi et al. 2013; He et al. 2018]). Our previous study showed that reduction of lipid accumulation in the *sur-7(tm6523)* mutant was due to a decreased conversion activity of SCD ([Zhang et al. 2017]). To confirm that the SCD conversion activity in the mutants involved cdf-1(n2527), we detected their fatty acid profiles via GC under either TPEN or ZnSO₄ treatment. The levels of oleic acid C18:1(n-9) and the conversion activity of SCD [C18:1(n-9)/C18:0] were increased when treated with TPEN in all worm strains (Figure 4, A–C), while they were reduced in cdf-1(n2527) and *sur-7(tm6523)* mutants in the presence of ZnSO₄ (Figure 4, A–C). Meanwhile, the levels of saturated fatty acids (C16:0 and C18:0) in *sbp-1(ep79)*, cdf-1(n2527) and sbp-1(ep79); sur-7(tm6523) mutants were reduced compared with the sbp-1(ep79) mutant (Figure 4, A and D). Consistently, the SCD conversion activity was increased in both sbp-1(ep79); cdf-1(n2527) and sbp-1(ep79); sur-7(tm6523) mutants (Figure 4, C and F). Collectively, these results indicated that the cdf-1(n2527) mutant was similar to the *sur-7(tm6523)* mutant in response to dietary ZnSO₄ or TPEN treatment in regulating SCD activity.

**Zinc promotes SCD expression but reversely inhibits its conversion activity**

Since the n2527 mutation of cdf-1 suppressed the SCD activity under ZnSO₄ treatment, we therefore questioned whether it was due to the altered expression of SCDs. To test the expression of SCDs, we opted to use cdf-1RNAi and sur-7RNAi for experimental convenience. Meanwhile, we constructed two transgenic strains, WT; kunEx203[cdf-1p::cdf-1::gfp, myo-2p::mcherry] and WT; kunEx187[sur-7p::sur-7::gfp, rol-6(a1006)]. The CDF-1::GFP is mainly expressed in the intestine and SUR-7::GFP is mainly expressed in the muscle. The cdf-1RNAi specifically inhibited the fluorescence expression of CDF-1::GFP but not SUR-7::GFP. Likewise, the sur-7RNAi only inhibited the fluorescence expression of SUR-7::GFP but not CDF-1::GFP, suggesting the efficiency and specificity of each gene RNAi (Supplementary Figure S5). The fluorescence intensity of FAT-5::GFP, FAT-6::GFP, and FAT-7::GFP was not changed in the cdf-1RNAi and sur-7RNAi worms compared to the control (EV) (Figure 5). Interestingly, distinguishing from the SCD conversion activity (Figure 4C), the fluorescence intensity of FAT-5::GFP, FAT-6::GFP, and FAT-7::GFP was significantly increased in the presence of ZnSO₄ but was decreased under zinc reduction by TPEN treatment in control (EV), cdf-1RNAi, and sur-7RNAi worms (Figure 5), which were contradictory with the SCD conversion activity reduced under ZnSO₄ or increased TPEN treatment. Nevertheless, these results suggest that zinc promotes SCD expression but reversely inhibits its conversion activity.

**Discussion**

Zinc is an essential element for living organisms. Dysfunction of zinc homeostasis is associated with lipid metabolism–related diseases. Our previous work uncovered a cation diffusion facilitator, SUR-7, that is required for the function of SREBP-SCD in lipid synthesis and accumulation in *C. elegans*. In this study, we further identified another cation diffusion facilitator, CDF-1, from among other zinc-related transporters that functions similarly to SUR-7 in regulating lipid metabolism. We propose a model (Figure 6) to better illustrate the function of CDF-1/SUR-7 in lipid metabolism. Under normal conditions, CDF-1/SUR-7 may be involved in zinc uptake and transportation for the maintenance of zinc homeostasis as well as the downregulation of fatty acid biosynthesis and lipid accumulation. Inactivation of CDF-1 or SUR-7 impairs zinc uptake and transportation, which results in the reduction of free zinc levels that consequently upregulate the conversion activity of SCD, further promoting the biosynthesis of unsaturated fatty acids and increasing lipid accumulation (Figure 6).

Zinc transport proteins play crucial roles in maintaining zinc homeostasis. Dysfunction of ZnTs is associated with obesity and diabetes ([Quraishi et al. 2005; Noh et al. 2014; Morais et al. 2019]). In *C. elegans*, a total of 28 potential zinc transport proteins have been identified based on human protein sequence homologues ([Kambe et al. 2015]). Among these zinc transport proteins, we found that CDF-1 and SUR-7 function redundantly to regulate zinc homeostasis and lipid metabolism. Inactivation of either CDF-1 or SUR-7 reduced Zinpyr-1 fluorescence in wild-type N2 and also in the *sbp-1(ep79)* mutant. More importantly, both gene mutations could recover the LD size in the *sbp-1(ep79)* mutant, and both displayed a similar response with a reduction of LD size under ZnSO₄ treatment. Therefore, our current work in *C. elegans* also supports a negative relationship between zinc level and lipid accumulation.

In this study, some transporter mutants showed higher levels of zinc than wild type, and some showed lower levels of zinc than wild type, but not all of them could rescue sbp-1. This could be due to different, specific expression locations of these ZnTs, and because the routes of zinc transport in various cell types may be different as well ([Kambe et al. 2015]). Another reason can be based on a different structure-function of these ZnTs ([Cotrim et al. 2019]). Moreover, the expression of different ZnTs at different stages of disease may be altered. For example, the expression level of specific ZnTs identified in the whole blood of patients with systemic inflammatory response syndrome at admission and on day 7 of ICU was different ([Florea et al. 2018]). By previous reports, some transporter mutants are very sensitive to zinc. The other ZnTs could not transport excessive zinc from the pseudocoelome ([Yoder et al. 2004; Davis et al. 2009]), resulting in high levels of zinc in the pseudocoelome. Therefore, it is possible that not all of these zinc transport mutants are sensitive to zinc and thus affect fat synthesis. At this time, only two mutants, cdf-1 and sur-7, have been found to be extremely sensitive to zinc, affecting fat synthesis through zinc level (Figure 1). While the zinc content is lower in zipt-2.3 and zipt-15 mutants under normal conditions (Supplementary Figure S2), which is consistent with the cdf-1 and sur-7 mutants, however the zinc content in the pseudocoelome is significantly increased when zinc is supplemented. The response of the zipt-2.3 and zipt-15 mutants to an excess of zinc is clearly different from the cdf-1 and sur-7 mutants. This may be one of the reasons why the zinc content of zipt-2.3 and zipt-15 mutants is reduced under normal conditions but could not restore the phenotype(s) of sbp-1(ep79). In addition, different subunits of the same protein can have different locations and functions. Through expression from different transcriptional start sites, *C. elegans* TTM-1 encodes two proteins TTM-1A and TTM-1B. TTM-1A is localized to vesicles and may also promote zinc
Figure 4: **cdf-1** and **sur-7** mediated the activity of SCD by zinc. (A), (B), (D), and (E) The fatty acid profiles of N2 (WT), **sbp-1(ep79; cdf-1(n2527))**, **sur-7(tm6523)** and **sbp-1(ep79; sur-7(tm6523))** were quantified using GC analysis. (A) and (B) Percentage of C18:0 and C18:1(n-9) in total fatty acids. (C) The conversion activity of SCD presented by the ratio of C18:1(n-9)/C18:0. (D) and (E) Percentage of C16:0 and C16:1(n-7) in total fatty acids. (F) The conversion activity of SCD presented by the ratio of C16:1(n-7)/C16:0. The data are presented as mean ± SEM of more than three biologic repeats.
Figure 5 The expression of FAT-6::GFP, FAT-7::GFP, and FAT-5::GFP under empty vector or a specific condition. (A), (C), and (E) The fluorescence intensity of FAT-6::GFP, FAT-7::GFP, and FAT-5::GFP. Bar, 50 μm. From left to right were EV, cdf-1RNAi, and sur-7RNAi, from top to bottom were control, ZnSO₄ supplementation, and TPEN supplementation. (B), (D), and (F) The quantization of the GFP fluorescence intensity corresponding to figure (A), (C), (E), respectively.
excretion and/or sequestration, TTM-1B functions in excretion; it is localized to the apical surface of the plasma membrane of intestinal cells and transports cytoplasmic zinc into the intestinal lumen (Roh et al. 2013). Therefore, it is understandable that other ZnTs could not restore the phenotypes in a SBP-1 mutant.

While CDF-1 and SUR-7 depletion have a similar effect on the zinc and lipid levels of sbp-1(ep79) worms, Zipyr-1 stained cdf-1(n2527) and sur-7(tm6523) worms look different. cdf-1 worms show stronger luminal Zinpyr-1 staining than sur-7 worms (Figures 1A and 3A). The sur-7 mutant was more tolerant to high Zn2+ concentrations than cdf-1 mutant (Yoder et al. 2004). These differences between cdf-1 and sur-7 may be related to their different subcellular localization and reflect functional differences. Moreover, in C. elegans, SUR-7, located in the ER membrane (Supplementary Figure S5C) (Yoder et al. 2004), may transport zinc from the cytoplasm into the ER. CDF-1 promotes zinc excretion into the pseudocoelomic space (Supplementary Figure S5A) (Bruinsma et al. 2002; Davis et al. 2009). CDF-2 transports excess zinc into lysosome-related vesicles (Davis et al. 2009). TTM-1A is distributed in a punctate pattern in intestinal and hypodermal cells and promotes zinc excretion and/or sequestration, while TTM-1B transports zinc into the intestinal lumen (Roh et al. 2013). We analyzed mRNA levels of these genes using quantitative real-time PCR (qPCR) in the cdf-1 or sur-7 mutant (Supplementary Figure S6, A–C). The expression of tm-1a increased in the sur-7 mutant and the level of tm-1b was higher in the cdf-1 mutant (Supplementary Figure S6, A and B). This result suggested that the function of TTM-1B in cdf-1 worms was enhanced which accelerated zinc to the intestinal lumen, while the function of TTM-1A in sur-7 worms was enhanced which promoted zinc to the punctate organelles. Therefore, we suggest that the capacity of zinc is different between the organelles or organs in C. elegans, which leads to the different sensitivity of mutants to zinc and the different response of mutants in lipid metabolism.

SCD is a main target of SREBP. SCD converts saturated fatty acids (C16:0 and C18:0) to Monounsaturated fatty acids (MUFAs) [C16:1(n-7) and C18:1(n-9)] for the biosynthesis of TAGs, PLs, and cholesterol esters. The SCD catalysis is dependent on its di-iron center. We previously showed that zinc reduction led to iron overload, consequently activating the conversion activity of SCD and fat accumulation (Zhang et al. 2017). Although the expression of FAT-5::GFP, FAT-6::GFP, and FAT-7::GFP, the three SCDs in C. elegans, was upregulated by ZnSO4 treatment while being down-regulated by TPEN, the levels of oleic acid C18:1(n-9) and the conversion activity of SCD [C18:1(n-9)/C18:0] were increased under TPEN treatment. However, these were reduced in the cdf-1(n2527) and sur-7(tm6523) mutants by ZnSO4 treatment. Thus, these results demonstrate a negative regulation of SCD expression and conversion activity by zinc, further confirming our hypothesis that zinc acts by bypassing SREBP to directly determine the conversion activity of SCD in lipid biosynthesis and accumulation. On the other hand, our results also showed that the expression of FAT-5::GFP, FAT-6::GFP, and FAT-7::GFP was upregulated upon ZnSO4 feeding when CDF-1 and SUR-7 were depleted, but the conversion activity of SCD was decreased.

Upon the loss of function of SCDs, the phenotypes of the worms were very similar to sbp-1(ep79) worms, such as small LDs, which appeared very meager. So, does the loss of SCDs function also increase the zinc level like the loss of SBP-1 function? Is it possible to restore the phenotypes of SCDs loss of function with SUR-7 or CDF-1 depletion as well? Our results showed it partially did. fat-6RNAi worms had an increased level of zinc, and under either SUR-7 or CDF-1 depletion, the high zinc level in fat-6RNAi worms was rescued (Supplementary Figure S7, A and B). However, although cdf-1 or sur-7 mutants could restore the zinc level of fat-6RNAi worms, they could not restore the fat-6RNAi lipid droplets back to wild type. They still look meager (Supplementary Figures S7, C and D). This observation raises the question of whether the increased expression of SCDs might be due to feedback regulation by decreased levels of C18:1(n-9). Therefore, we speculate that targeting of SCD may potentially provide a possible treatment for zinc-related lipid metabolic diseases. This specific mechanism requires further study, however.

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