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Lysosomal SLC46A3 modulates hepatic cytosolic copper homeostasis

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The environmental contaminant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) causes hepatic toxicity associated with prominent lipid accumulation in humans. Here, the authors report that the lysosomal copper transporter SLC46A3 is induced by TCDD and underlies the hepatic lipid accumulation in mice, potentially via effects on mitochondrial function. SLC46A3 was localized to the lysosome where it modulated intracellular copper levels. Forced expression of hepatic SLC46A3 resulted in decreased mitochondrial membrane potential and abnormal mitochondria morphology consistent with lower copper levels. SLC46A3 expression increased hepatic lipid accumulation similar to the known effects of TCDD exposure in mice and humans. The TCDD-induced hepatic triglyceride accumulation was significantly decreased in Slc46a3−/− mice and was more pronounced when these mice were fed a high-fat diet, as compared to wild-type mice. These data are consistent with a model where lysosomal SLC46A3 induction by TCDD leads to cytosolic copper deficiency resulting in mitochondrial dysfunction leading to lower lipid catabolism, thus linking copper status to mitochondrial function, lipid metabolism and TCDD-induced liver toxicity.
Nonalcoholic fatty liver disease (NAFLD) is one of the most common chronic liver diseases in developed countries. Fatty liver can be caused by diet, drugs, viruses, genetic factors, hormones, or environmental pollutants. Hepatic lipid accumulation can lead to nonalcoholic steatohepatitis, cirrhosis, liver failure, and hepatocellular carcinoma. Abnormal hepatic lipids can also be caused by chemically-induced toxicity by compounds like dioxins that are constituents of environmental pollution that threaten the ecosystem and exist as complex isomers of aromatic halogen compounds. The most poisonous of these dioxins is 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), a major contaminant in Agent Orange, a herbicide used in the Vietnam War between 1962 and 1971 to destroy forests for military purposes. TCDD is also produced during the burning of fossil fuels and is a byproduct of the manufacture of dioxins. It is a potent carcinogen and can induce tissue-specific toxicity with decreased immune function, increased hepatic drug metabolizing enzyme induction, teratogenesis, thymic degeneration, cirrhosis, endocrine disruption, infertility, liver toxicity associated with increased lipid accumulation, and cancer.

TCDD toxicity is due to its ability to activate the aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor that is activated by xenobiotics including dioxins and polycyclic aromatic hydrocarbons. The molecular mechanisms of many specific toxicities elicited by TCDD exposure are still not fully understood. In experimental animal models, these effects are due to activation of the AhR. The AhR nuclear translocator (ARNT) and ligand-bound AhR form a heterodimer that binds to elements in the promoter regions of target genes and activates transcription.

The function of solute carrier 46a3 (SLC56A3) is not known. SLC46A3 mRNA, similar to the AhR-regulated Cyp1a2 mRNA, is constitutively expressed in livers of wild-type mice and upon treatment with TCDD, was induced with similar kinetics to Cyp1a2 mRNA; induction of both mRNAs was not found in Ahr-null and Arnt liver-specific conditional-null mice. In view of the effects of TCDD on hepatic steatosis, it was of interest to determine whether SLC46A3 expression and its induction by AhR, affects hepatic lipid levels. While phylogenetically SLC46A3 is a solute carrier, its function and substrate(s) are unknown. Thus, the function of SLC46A3 and its role in liver lipid homeostasis was studied using Slc46a3−/− mice, cultured primary hepatocytes, and forced expression of recombinant SLC46A3 in cultured cells. Induction of lysosomal SLC46A3 by TCDD induces intracellular copper deficiency, which results in mitochondrial dysfunction resulting in lower lipid catabolism and hepatic lipid accumulation.

Results

Lysosomal SLC46A3 is regulated by AhR. Slc46a3 mRNA was induced by TCDD in the mouse liver while no induction was found in kidney or different regions of the small intestine and the colon, where constitutive Slc46a3 mRNA was detected, indicating liver-specific induction by the AhR (Fig. 1a). This is a similar pattern of induction found with another AhR target gene Cyp1a2, which shows liver-specific induction. Both Slc46a3 and Cyp1a2 mRNAs were induced in an AhR-dependent manner since no induction was found in Ahr−/− and Arnt−/− mice (Fig. 1a, b). As a negative control for TCDD, the non-dioxin-like polychlorinated biphenyl-153 (PCB-153) was analyzed by extraction of Gene Expression Omnibus (GEO) data sets (accession: GSE55084), and no induction of Slc46a3 mRNA was noted (Fig. 1c).

Putative DRE sites were found in a published ChIP-seq data set (GEO, accession: GSE97634) (Fig. 1d, e). A potential remote DRE site −17,135 bp of upstream of Slc46a3 was examined for trans-activation by AhR and TCDD using luciferase reporter gene assays, revealing that 3x-DRE-luciferase activity was significantly increased by 10 nM TCDD in Hepa1c1c7 cells and was abolished by mutation of the DRE site (Fig. 1f) and CH-223191, an AhR antagonist (Fig. 1g). Likewise, CH-223191 suppressed TCDD-induced Slc46a3 mRNA levels (Fig. 1h). These results suggest that AhR may be functioning through a DRE located in a remote long-range enhancer controlling AhR activation of the Slc46a3 gene.

Time and dose-dependent expression of Slc46a3 mRNA induction by TCDD. In mouse liver, Slc46a3 mRNA was developmentally gradually increased up to 8 weeks of age (Fig. 1i). In addition, Slc46a3 mRNA was elevated seven fold by 10 μg/kg TCDD in a time-dependent manner over 24 h (Fig. 1j). No significant loss of Slc46a3 mRNA levels was noted after 3 days and expression persisted for 7 days (Fig. 1k). This might be due to the high affinity of TCDD to AhR and its persistent activation, or due to a very stable Slc46a3 mRNA that, once induced, does not rapidly decay.

Generation and phenotype of Slc46a3−/− mice. To explore a role for Slc46a3 in the liver, Slc46a3−/− mice were generated (Fig. 2a–c). The mice were born at the expected frequency, showed no developmental defects, and no gross abnormal phenotype, as compared to matched wild-type controls. A striking color change was noted in the livers of Slc46a3−/− mice compared to wild-type mice. Notably, the livers of Slc46a3−/− mice were darker, and, after perfusion with saline, showed a more grayish color (Fig. 1d). However, H&E staining revealed no difference in both groups in the classic hepatic inflammation features in the livers of mice administered TCDD (Fig. 2e).

Localization of SLC46A3. SLC46A3 protein was localized in the lysosomal membrane (Fig. 2f) as revealed by lysosomes incorporated with eGFP-SLC46A3 that co-isolated with LAMP1 (Fig. 2g). The eGFP-SLC46A3 aggregated during denaturing by boiling as revealed by western blot analysis which indicates the marked hydrophobicity of SLC46A3, as expected from this class of membrane-bound proteins (Fig. 2h).

Copper is a possible substrate of lysosomal SLC46A3. Based on the color change in the liver of Slc46a3−/− mice, hepatic metal contents were measured in WT and Slc46a3−/− mice (Supplementary Fig. 1a). Among the physiologically relevant metal ions, copper levels were notably increased by around 30% in perfused livers of Slc46a3−/− mice, with no differences in iron contents (Fig. 3a and Supplementary Fig. 1b). This observation might be due to lysosomal copper accumulation. However, liver copper-related mRNA levels were not changed after 7 days of 10 μg/kg TCDD (Fig. 4f). For lysosomal copper analysis, hepatic lysosomes were isolated using magnetic beads after hydrodynamic injection with eGFP-LAMP1 and Dsred or Dsred-SLC46A3. The lysosomal fraction was isolated only with the N-terminal eGFP tagged LAMP1 (Supplementary Fig. 2). Copper levels were significantly increased by Dsred-SLC46A3 from the isolated lysosome (Fig. 3b). Furthermore, the 13,000 g pellet from the lystate of Hepa1c1c7 cells had elevated FLAG-SLC46A3 protein levels (Fig. 3c), and copper levels were significantly increased in the presence of copper sulfate (Fig. 3d). Based on copper analysis using liver and cell lines, SLC46A3 may transport copper into the lysosome. Thus, a quenching effect was tested in lysosomes using Phen Green FL dye in the presence of recombinant SLC46A3 expression in Hepa1c1c7 cells. A quenching effect was found in lysosomes embedded with mCherry-SLC46A3 (Fig. 3e) as revealed by decreased levels of Phen Green fluorescence in the liver.
lysosome by mCherry-SLC46A3 (Fig. 3f). This might be due to certain metals such as copper, transferred into the lysosome.

Copper ions may be sequestered in the lysosome via SLC46A3. To probe the lysosomal copper contents, eGFP-SLC46A3-expressing Hepa1c1c7 cells were incubated with the labile copper sensing probe CF4 (1 µM) and its non-responsive control Ctrl-CF4 (1 µM) for 10 min and a red filtered fluorescence signal detected under fluorescence microscopy. The CF4 signal was strongly detected in the eGFP-SLC46A3-embedded lysosomes compared to Ctrl-CF4 (Fig. 3g). By high-magnification images of eGFP-SLC46A and CF4, their sensing location was confirmed using a BZ-X analyzer. EGFP-SLC46A3 protein was localized in the lysosomal membrane and CF4-Cu was encapsulated inside of the lysosome (Fig. 3h, i). In addition, the lysosome size was significantly increased by CuSO4 in a dose-dependent manner (Fig. 3j).

Oil (n = 6 per group), PCB-153 (n = 3 per group), and TCDD (n = 3 per group). NS not significant. 

**Fig. 1** Regulation of Slc46a3. 

a, Slc46a3 mRNA expression in different mouse tissues after oil (n = 3–4 per group) or TCDD (10 µg/kg for 24 h) (n = 3–4 per group) administration. 

b, Hepatic Slc46a3 mRNA expression after oil (n = 4 per group) or TCDD (10 µg/kg for 24 h) (n = 5 per group) administration in Amt−/− mice. Two-way ANOVA with Bonferroni’s multiple comparisons tests, ***p < 0.0002, ****p < 0.0001. 

c, Male mouse liver transcription profiling by Affymetrix microarray (GEO: GSE55084) shows Slc46a3 mRNA after treatment with PCB-153 (IP injection, 80 mg/kg for 48 h) and TCDD (IP injection, 10 µg/kg for 48 h). Oil (n = 6 per group), PCB-153 (n = 3 per group), and TCDD (n = 3 per group). 

f, Counting of putative dioxin-responsive enhancer (DRE)/xenobiotic response element (XRE) binding sites of Slc46a3 promoter were extracted using ChIP-seq data (GEO accession: GSE97634) and possible enhancer region (−17,135 bp from the 5′-flanking region of Slc46a3) was analyzed using original file sets using Integrated Genome Browser (Bioviz). 

g, Expressional analysis of hepatic Slc46a3 mRNA as a function of dose and time of oil (n = 5 per group). 

h, Inhibitory effect of the AhR inhibitor, CH-223191, on the putative 3×DRE luciferase activity in Hepa1c1c7 cells. After transfection with the 3×DRE luciferase constructs (n = 3 biologically independent experiments) overnight and treated with indicated doses of TCDD for 6 h. Transfection was normalized with the Renilla luciferase signal. One-way ANOVA with Tukey’s multiple comparisons test, ***p < 0.0001. 

i, Expression of hepatic Slc46a3, Cyp1a2, and AhR mRNAs as a function of mouse age (n = more than 6 per group). One-way ANOVA with Tukey’s multiple comparisons test, ****p < 0.0001. 

j, Expression of hepatic Slc46a3, Cyp1a2, and AhR mRNAs as a function of time after TCDD treatment (n = 4 per group). One-way ANOVA with Tukey’s multiple comparisons test, ****p < 0.0001.
TCDD-treated (10 µg/kg for 7 days) livers (Fig. 4a) and eGFP-SLC46A3-expressing Hepa1c1c7 cells (Fig. 4b). Furthermore, the effect was enhanced in the presence of BCS, a Cu (I) chelator in the media, in the presence of eGFP-SLC46A3 expressing cells (Fig. 4b). However, ferritin-heavy chain, a marker for the labile cytosolic iron pool, was not changed by eGFP-SLC46A3 expression level changes in Hepa1c1c7 cells (Fig. 4c). In addition, mitochondrial SOD activity was increased as reported previously (Fig. 4d).10. Inversely, the hepatic CCS level was dramatically decreased by disruption of the Slc46a3 gene in mice (Fig. 4e). Thus, copper may be a substrate for SLC46A3 in the hepatic lysosome.

SLC46A3 alters mitochondrial potential and morphology. Because copper ion is a cofactor of the complex IV of the respiratory chain, the mitochondrial morphology and mitochondrial potential were analyzed in the presence or absence of SLC46A3. In primary hepatocytes of Slc46a3−/− mice, mitochondria distal from the nucleus, were thick and elongated compared to small round organelles found in WT mouse liver (Fig. 5a). The mitochondrial area of Slc46a3−/− mice was significantly larger than in WT hepatocytes (Fig. 5b). The altered mitochondrial morphology was further confirmed by fluorescence microscopy after MitoTracker treatment in mCherry- or mCherry-SLC46A3-expressing primary hepatocytes. In particular, in mcherry-SLC46A3-expressing hepatocytes, mitochondria were transformed to thin and long thread-like structures associated with weak mitoTracker signals (Fig. 5c). In this experiment, the mCherry signal was not visible because the MitoTracker intensity overwhelms the mCherry signal. In addition, MitoTracker intensity was notably decreased in eGFP-SLC46A3-expressing Hepa1c1c7 cells (Fig. 5d). SLC46A3 induction could inhibit mitochondrial potential in tetramethylrhodamine ethyl ester (TMRE)-treated Hepa1c1c7 cells (Fig. 5e). Furthermore, the initial oxygen consumption rate (OCR) was significantly reduced in eGFP-SLC46A3-expressing Hepa1c1c7 cells (Fig. 5f). As mitochondrial potential was changed by SLC46A3 overexpression, hepatic ATP levels were measured. ATP was significantly decreased by overexpression of eGFP-SLC46A3 in Hepa1c1c7 cells (Fig. 5g). In addition, mitochondrial images were taken using transmission electron microscopy (TEM) to determine whether overexpression of SLC46A3 could change mitochondrial morphology. The mitochondrial morphology in livers of Slc46a3−/− mice were longer compared to those in WT livers (Fig. 5h). However, a truncated or donut-shaped mitochondrial form was found in livers expressing eGFP-SLC46A3 (Fig. 5i).

Slc46a3−/− mice are resistant to TCDD-induced hepatic triglyceride accumulation. To investigate the possible function of hepatic SLC46A3 after TCDD (200 µg/kg for 7 days) exposure, ALT and ALP were measured in serum. However, a difference between WT and Slc46a3−/− mice was not observed. Interestingly, TCDD did not alter the hepatic triglyceride (TG) levels in Slc46a3−/− mice.
compared to WT mice. In addition, differences in levels of hepatic free fatty acids (non-esterified fatty acid, NEFA) were not observed between both groups in the presence or absence of TCDD (Fig. 6a). Oil red O staining revealed that lipid droplet accumulation in livers of TCDD-treated Slc46a3−/− mice were significantly reduced compared to WT mice (Fig. 6b). Since Slc46a3−/− mice were resistant to TCDD-induced TG accumulation in the liver, a high-fat diet (HFD) model was applied to determine whether Slc46a3−/− mice were resistant to hepatic TG accumulation. Notably, after 3 weeks of HFD feeding, body weight changes in Slc46a3−/− mice were significantly reduced compared to WT mice (Fig. 6c). Glucose tolerance test (GTT) showed no significant difference between WT and Slc46a3−/− mice (Supplementary Fig. 3). In addition, liver mass and epidydimal fat mass were also lower in Slc46a3−/− than in WT livers (Fig. 6d,e). Hepatic TG levels were lower in Slc46a3−/− mice than in WT mice (Fig. 6f), while differences in serum TG and NEFA levels in the two groups were not observed (Fig. 6g). These results suggest that SLC46A3 influences hepatic TG generation from NEFA in the liver. Due to the change of hepatic TG levels in Slc46a3−/− mice, lipid metabolites were analyzed in livers from WT and Slc46a3−/− mice. Two peaks from 16.88 min were significantly changed (Supplementary Fig. 4a). PLS-DA (Supplementary Fig. 4b) and S-plots (Supplementary Fig. 4c) using UPLC-MS data by SIMCA-P+12 software, as well as GC-MS analysis with the FAME-derivatization method,
SLC46A3 was confirmed by Nile Red staining in primary hepatocytes (Fig. 7f). In addition, lipid droplets were increased by tetraethylenepentamine (TEPT), a copper chelator, in Hepa1c1c7 cells (Supplementary Fig. 5). Furthermore, SLC46A3 could alter the phosphorylation of some energy homeostasis-related proteins such as AMPK and ACC. Ad-SLC46A3 increased phosphorylation of AMPKα and decreased phosphorylation of ACC in a titer-dependent manner in primary hepatocytes (Fig. 7g). Thus, SLC46A3 induction may cause unbalanced energy homeostasis.

Discussion
Here, the function of SLC46A3 was analyzed in mouse liver. Because SLC46A3 is specifically induced by TCDD in liver via AhR signaling, it might play an important role in liver pathophysiology, notably in the toxic response to TCDD. To investigate the function of SLC46A3, Slc46a3−/− mice were generated. The critical gross pathological feature of Slc46a3−/− mice was gray rather than yellow livers as compared to WT mice. Metal ions were measured in the liver to investigate whether the color change was the result of altered levels of intracellular metal ions. Interestingly, copper and iron were increased in the liver. For iron, no significant difference was noted after liver perfusion as well as in the 13,000 g fraction of the eGFP-SLC46A3-expressing liver

Slc46a3 induction triggers hepatic triglyceride accumulation. Since the phenotype of the Slc46a3−/− mice revealed that SLC46A3 affected hepatic TG accumulation, a direct role for SLC46A3 in lipid accumulation was investigated. Forced expression of SLC46A3 in the liver by both adenosine and hydrodynamic shear injection significantly increased hepatic lipids (Fig. 7a) and TGs (Fig. 7b). Since adipophilin (ADFP) is expressed in lipid droplets in concert with TG accumulation (Fig. 7a) and TGs (Fig. 7b). Since adipophilin (ADFP) is expressed in lipid droplets in concert with TG accumulation, ADFP mRNA levels were measured after Ad-SLC46A3 administration for 7 days. Hepatic Adfp mRNA was significantly increased by Ad-SLC46A3 administration (Fig. 7c). In addition, mCherry-SLC46A3 increased the size of eGFP-ADFP-expressing lipid droplets in primary hepatocytes (Fig. 7d, e). The increase of lipid droplet size by SLC46A3 was confirmed by Nile Red staining in primary hepatocytes (Fig. 7f). The increase of lipid droplet size by SLC46A3 in concert with TG accumulation, ADFP mRNA levels were measured after Ad-SLC46A3 administration for 7 days. Hepatic Adfp mRNA was significantly increased by Ad-SLC46A3 administration (Fig. 7c).

Fig. 4 TCDD and eGFP-SLC46A3 increases the copper chaperone for superoxide dismutase. a Western blotting data show the level of copper-related proteins in the liver after oil (n = 5 per group) or TCDD (10 μg/kg for 7 days) treatment (n = 5 per group). Densitometrical analysis is shown in the right panel. b Western blotting data show the level of copper chaperone for superoxide dismutase (CCS) protein level after treatment with BCS (Cu (I) chelator in the media) for 3 days in the presence of eGFP-SLC46A3 in Hepa1c1c7 cells. Densitometrical analysis is shown in the right panel. NB nonboiled sample. c The level of heavy chain of ferritin, an indicator of labile cytoplasmic iron levels, was measured after induction of eGFP-SLC46A3 in Hepa1c1c7 cells. Western blotting data show the level of copper chaperone for superoxide dismutase (CCS) in the livers from wild-type (WT) mice after oil (n = 4 per group) and Slc46a3−/− mice (n = 4 per group). Densitometrical analysis is shown in the right panel. Expression of mRNAs encoding metal transporters in the mice liver after oil (n = 4 per group) or TCDD (10 μg/kg for 24 h) (n = 5 per group) administration. Each data point represents the mean ± SD and adjusted p value, presented in the panels, was determined by unpaired two-tailed Student’s t test using indicated sample sizes and groups.

SLC46A3 induction may cause unbalanced energy homeostasis.
This may be due to an increase in iron in the blood. In this study, the relationship between copper ion and SLC46A3 in the liver was investigated. Because SLC46A3 was localized in the lysosome, the isolated lysosome fraction was subjected to metal analysis using inductively coupled plasma mass spectrometry (ICP-MS). These results suggested that copper could be a possible substrate of SLC46A3.

Lysosomes are catabolic organelles in eukaryotic cells that digest various components from the cytoplasm. Previous reports suggested that copper can be transported to hepatocytes through vesicle pathways and can be excreted into the bile by lysosomes11,12. Lysosomes were enlarged by SLC46A3 expression, however, the mechanism is not known. SLC46A3 may influence the recruitment of functional proteins for lysosomal activation such as LAMP1. Misregulated or mutant lysosomal membrane protein-coding genes can cause human diseases. For example, a mutation in MCOLN1 encoding mucolipin-1, a lysosomal cation channel for ions such as Na\(^+\), K\(^+\), Ca\(^{2+}\), Fe\(^{2+}\), causes mucolipidosis type IV13. As SLC46A3 protein could modulate lysosomal copper, unbalanced intracellular copper levels in lysosomes can lead to altered metabolic pathways as well as other metal-related transporting systems in the liver. Increased copper levels as a result of TCDD administration or SLC46A3 overexpression/deletion could influence the expression of copper-related genes or other metal concentrations in the cells. Iron-related gene mRNAs (Tf, Tfrc, Tth1, and Ftl1), zinc-related gene mRNAs (Zip5, Zip14, Znt9, and Znt7), and copper-related gene mRNAs (Ctr1, Atp7a, Atp7b, Ccs, and Cp) were measured and only Ftl1 mRNA was slightly increased by TCDD in the liver. This suggests that copper deficiency may trigger the increase of iron level as a compensatory mechanism. However, hepatic mRNA levels of zinc or copper-related proteins were unchanged after TCDD administration.

Copper is an essential transition metal that acts as a cofactor in many enzymes and plays an important role in cellular metabolism and bioenergy. Importantly, copper ion is a cofactor for
mitochondrial respiratory complex IV and cytochrome c oxidase, and thus intracellular copper deficiency may cause mitochondrial dysfunction. Features of impaired copper metabolism lead to genetic disorders such as Wilson’s and Menke’s disease. Moreover, copper regulates the lipolysis of triglycerides via cAMP signaling. Therefore, in the absence of SLC46A3, the levels of copper in the cells could be increased and mitochondrial function would be increased as well. Conversely, induction of SLC46A3 could lead to copper deficiency, since copper could migrate to the lysosome for excretion. Indeed, previous results show that hepatic copper deficiency appears at the early stages of HFD feeding. According to a previous report, ATP7B, which results in Wilson’s disease, removes excess copper into the bile via lysosomal exocytosis. Thus, lysosomal SLC46A3 trafficking is critical to understand copper deficiency in hepatocytes. As a conventional method for detecting copper accumulation in the liver, rhodamine staining was used and copper staining was noted in Slc46a3−/− mice. Because copper was increased by about 30% in the liver of Slc46a3−/− mice, copper levels were measured in isolated lysosomes using ICP-MS analysis. Copper accumulation was also measured in SLC46A3-embedded lysosomes by using the copper sensing probe CF4. Based on this study, the characteristics of mitochondria differed according to the levels of SLC46A3. As expected, intracellular copper deficiency resulted in inhibition of mitochondrial potential when SLC46A3 was overexpressed. This result is supported by a previous report that mitochondrial potential is significantly reduced by TCDD in rat hepatocytes. In addition, in a previous study, the morphology of some mitochondria was changed by copper deficiency to a donut shape in human hepatocytes as well as the thymus and spleen in mice. In addition, others reported that mitochondria were changed to a donut shape by carbonyl cyanide m-chlorophenyl hydrazine (CCCP), a mitochondrial oxidative phosphorylation uncoupler, without fusion or fission in MEF cells. This morphological feature was similar to the present result in a fusion- or fission-independent...
Fig. 7 SLC46A3 overexpression triggers TG accumulation in the liver. a Oil Red O staining after adenovirus-based (upper, n = 5 per group) and hydrodynamic injection-based (lower, n = 3 per group) expression of Slc46a3 mRNA in the liver. b Hepatic Slc46a3 mRNA expression (left) and TG level (right) after administration of Ad-eGFP-Cre (n = 3 mice per group) or Ad-Slc46a3 (n = 5–6 mice per group). c Hepatic Adfp mRNA expression after Ad-Slc46a3 and control Ad-Cre administration (n = 3 per group). d Representative fluorescence images in the eGFP-adipophilin/mcherry-SLC46A3-expressing primary hepatocytes. The representative images were obtained from three biologically independent experiments. Scale bar, 10 μm; N nucleus (blue color). e Relative volume of lipid droplets in the eGFP (n = 11 counts per cell) or eGFP-SLC46A3 (n = 32 counts per cell) expressing primary hepatocytes from (d). f Nile Red staining after adenoviral induction of SLC46A3 in primary hepatocytes with Ad-Slc46a3. The representative fluorescence images were obtained from three biologically independent experiments. Scale bar, 10 μm. g Western blotting of phosphorylated AMPKα and ACC using the mice liver after viral induction of Ad-Cre-GFP or Ad-Cre-Slc46a3. The representative western blot images were obtained from three biologically independent experiments. Each data point represents the mean ± SD and the adjusted p value, presented in the panels, was determined by unpaired two-tailed Student’s t test using indicated sample sizes and groups.
intrapеритонеально с одним дозой 10 или 200 μg/kg of TCD5 для 3 or 7 days. To investigate a role for SLC46A3 in hepatic TG accumulation, mice were fed a 60% HFD that was obtained from Research Diets (New Brunswick, NJ). Six weeks later, the mice were killed.

Cell culture. Hepa1c1c7 cells were purchased from the ATCC and cultured in DMEM supplemented with 10% heat-inactivated FBS and 50 U/ml of penicillin/streptomycin mixture (Invitrogen) at 37°C in a humidified atmosphere of 5% CO2/95% air. Cells were grown to 60-80% confluence, followed by trypsinization with 0.05% trypsin containing 0.02% EDTA. For ectopic expression of proteins in hepatocytes, recombinant adenosine or hydrodynamic shear plasmid DNA was delivered to the liver using tail vein injection. Primary hepatocytes were cultured overnight and treated with 20 nM MitoTracker or 0.5 μg/ml Nile Red for 15–30 m.

Plasmids. For mammalian expression, coding regions for mouse Slc46a3, Adfp, and Lamp1 cDNAs were subcloned into pEGFP, pDsred, and pmCherry plasmids (Clontech, Mountain View, CA, USA) for tagging eGFP, Dsred, and mCherry, respectively, at the N-terminal of the SLC46A3 protein. For the FLAG-SLC46A3-expressing plasmid (pCMV-Flag-Slc46a3), the EGFP cDNA in the pEGFP plasmid was replaced to make the final Flag-Slc46a3 fusion gene by modification.

In vivo and in vitro DNA delivery. For in vivo plasmid DNA delivery, hydrodynamic shear tail vein injection was carried out using 10-week-old C57BL/6j male mice. Mixtures with pEGFP-Slc46a3 (20 μg) or pmCherry-Slc46a3 (20 μg) or pEGFP-Adfp (10 μg) and 2.2 ml of TransIT-QR delivery solution were injected within 3 to 5 s through the tail vein. One day later, the mice were killed or primary hepatocytes isolated for culturing as previously described. Using small pieces of fresh liver, the expression of each fluorescence fusion protein was monitored under fluorescence microscopy. Adenoviral delivery of SLC46A3 using 10-week-old C57BL/6j male mice was carried out by injecting 200 μl of saline solution containing 2 × 1010 pfu/mouse through the tail vein. A week later, the mice were killed. For in vitro transfection, Hepa1c1c7 cells were transfected with different plasmids using JetPEI reagent for 16 h before the cells were harvested.

Quantitation of mRNA. Total RNA was isolated from the liver using Trizol (Invitrogen, Carlsbad, CA). After synthesis of complementary DNA (cDNA) using a SuperScript II reverse transcriptase kit (Invitrogen, Carlsbad, CA), qPCR was carried out using an Applied Biosystems Prism 7900HT Sequence Detection System (Foster City, CA) as described previously. Expression levels of mRNA were normalized to 18 S RNA or Gapdh mRNA as the internal standards. Primers for the qPCR are listed in Supplementary Table 1.

Western blotting. Hepa1c1c7 cells were lysed with M-PER buffer (Thermo Scientific) and the lysate was boiled for 5 min. Primary antibodies against Slc46a3 and Lamp1 were probed, followed by secondary antibodies and chemiluminescent substrate (Pierce) by an image analyzer as described previously. Protein concentration was measured using the bicinchoninic acid (BCA) reagent. Protein concentration was measured using the bicinchoninic acid (BCA) reagent. Protein concentration was measured using the bicinchoninic acid (BCA) reagent. Protein concentration was measured using the bicinchoninic acid (BCA) reagent. Protein concentration was measured using the bicinchoninic acid (BCA) reagent.

Mitochondria analysis. For mitochondrial OCR measurements, Hepa1c1c7 cells were cultured in specialized 24-well plates and transfected with 100 nM of pEGFP or pEGFP-Slc46a3 plasmid for 24 h and mitochondrial OCR was measured using the Seahorse XF24 analyzer as described previously. OCR was measured from the basal conditions and later cells were treated with the mitochondrial inhibitor oligomycin, a mitochondrial uncoupling compound cyanide-ferricyanide (FCCP), and the respiratory chain inhibitor antimycin A.

Biochemical analysis. Serum levels of alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were measured using ALT (Catachem, Bridgeport) and ALP kits (Catachem, Bridgeport). Serum or hepatic level of non-esterified fatty acids (NEFA) and hepatic TG level were quantified using TG kit (Wako Chemicals USA Inc.) and NEFA kit (Wako Chemicals USA Inc.) according to the manufacturer’s instructions.

Copper transport assay. To analyze the copper levels in the lysosomal fraction, hepatic lysosomal fraction was isolated using the magnetic bead method. In detail, 8-week-old FVB male mice were transfected with pEGFP-Lamp1 (10 μg) and pDsred-Slc46a3 (10 μg) or pDsred (10 μg) using the hydrodynamic injection method for 24 h. Liver tissue (300 mg) was homogenized with a tight dounce homogenizer in the presence of 0.1 M Tris-HCl buffer containing protease inhibitor cocktail followed by centrifugation at 10000 g for 5 min twice. The supernatants were incubated with biotinylated anti-GFP antibody (20 μg/ml) for 20 m at 4°C using a rotor shaker and the lysosomes-eGFP-Lamp1 complex was isolated with a streptavidin-magnetic bead (Pierce). The isolated lysosomal fractions were subjected to western blotting or metal analysis.

In vitro metal transport assays, Hepa1c1c7 cells were transfected with different amounts of pcMV-Flag-Slc46a3 plasmid using JetPEI transfection reagent for 24 h. The cells were then treated with copper sulfate (CuSO4·5H2O, 10 μg/ml) in serum-free DMEM media for 1 h. After extra incubation of cells in fresh serum-free DMEM for 30 m, the cells were lysed with M-PER buffer, and subjected to sequential fractionation by differential centrifugation at 800 for 5 m, 13000×g for 10 min, or 543000×g for 30 min at 4°C. Each pellet or supernatant were subjected to western blotting and metal analysis.

Reporter gene assays. To verify the role of AhR on Slc46a3 regulation, the putative DRE site (~17 kb) of Slc46a3 was inserted in pGL4.10 with additional minimal TATA box sequence using Ascl/PacI sites. pGL4.10-3×DREmin was described as wild-type and pGL4.10-3×mutDREmin was indicated as mutant as an experimental control. The inserted sequences for pGL4.10-3×mutDREmin and pGL4.10-3×mutDREmin are GASGATCGTGCCATCATCTGCCTGGAGATAGTCGCTTGTTTGGAGA TGACCTGCTTCTGCTGAGAACAATGAGGTTAATAAGAAGTACTGAC GCGGCT and TGAGAGATAAAACACATCTGCTGAGACATCGAGAATATAAAGAAATC GACCTGCTTCTGAGATAAAACACATCTGCTGAGACATCGAGAATATAAAGAAATC GACCTGCTTCTGAGATAAAACACATCTGCTGAGACATCGAGAATATAAAGAAATC.
Transmission electron microscopy (TEM). TEM images of livers were serviced by the Center for Cancer Research (CCR) of NCI at Frederick.

Northern blot analysis: Total DNA from the WT and Slc46a3−/− mice was performed as described previously38 using specific probes (Supplementary Table 1).

Northern blot analysis: Total RNA from different tissue samples was prepared by using Trizol reagent (Invitrogen, Carlsbad, CA). Northern blot analyses were performed as previously described38. Slc46a3 cDNA (901 bp) was used as a Northern probe.

ATP measurement. Hepa1c1c7 cells were transfected with pEGFP (8 µg) or pEGFP-Slec46a3 (8 µg) plasmids in 100 mm dishes overnight and cell lysates and ATP was measured according to the manufacturer’s instructions (DoGenBio, Seoul, Korea).

Statistical analysis. Experimental values are expressed as mean ± SD. Statistical analysis was performed by one-way ANOVA for multiple comparisons using Tukey’s multiple comparisons test, two-way ANOVA for comparisons of multiple factors using Bonferroni’s method, or two-tailed Student’s t test for unpaired data. P < 0.05 was considered statistically significant. GraphPad PRISM v.8.4.3 was used for statistical analysis.

Data availability

All relevant data are available from the corresponding authors upon request. Source data are provided with this paper.

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Author contributions

J.H.K. performed animal experiments and cell experiments as well as molecular biology. T.M. analyzed the Slc46a3−/− phenotype and lipid using the liver and serum samples. C.
F.F. and K.H. helped with the computational analysis. J.L. helped with metal analysis. H.Y. analyzed TG. T.N. followed the generation of Slc46a3−/− mice and evaluated the phenotype of Slc46a3−/− mice. S.Y. discovered that Slc46a3 was an AhR target gene and generated the Slc46a3−/− mice. S.J. synthesized the copper sensing probe. J.H.K., D.K., and K.W.K. performed metabolomics analysis. S.J. and C.J.C. contributed to the copper detection experiments. J.H.K. and F.J.G. wrote the manuscript and supervised the study with contributions from all authors.

**Competing interests**
The authors declare no competing interests.

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