Supporting Information

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SI Materials and Methods

Cell Culture and Lentiviral Infection. SH-SY5Y cells were cultured in RPMI media supplemented with 15% FCS, 2 mM L-glutamate, and 50 μg/mL PenStrep. Lentiviral infection of SH-SY5Y cells was used to make stable cell lines of inducible Ndfip1-Flag (see Fig. 2*A*) and shRNAi Ndfip1 knockdown cells (Open Biosystems clone V2LHS_99041). To generate lentiviral particles, we transfected HEK293T cells with packaging constructs pCMV ðR8.2 and VSVg and the relevant lentiviral plasmid using Effectene reagent according to the manufactures protocol (Qiagen). For inducible Ndfip1-Flag, two separate viruses were made, one containing the Ndfip1-Flag construct and the other containing the GEV16 Super construct, infection with both viruses created the inducible Ndfip1-Flag cells. The virus containing supernatants were harvested at 100,000 $\times$ *g*. Target cells were infected with virus supernatant for 24 h. Successful infection was selected for with puromycin (2 mg/mL, Sigma) or hygromycin B (100 mg/mL, Sigma). From this selection stable cell lines for inducible Ndfip1-Flag and shRNAi Ndfip1 were produced.

Metal Toxicity and Apoptosis Quantification by Flow Cytometry. Assays to determine Ndfip1's regulation in response to metals were conducted by treating cells with varying concentrations of $CoCl_2$ or $FeCl_2$ for 18 h before analysis by western blot. Rabbit polyclonal anti-N terminus Ndfip1 antibody (polyclonal Ndfip1) was used as the primary antibody to detect Ndfip1 levels. Apoptosis was assessed quantitatively by flow cytometry using double labeling with propidium iodide and annexin-V-FLUOS (Roche) according to the manufactures protocol. Briefly 2×10^5 cells were grown in 6-well plates, Ndfip1 was induced by the addition of 100 nM 4-hydroxy tamoxifen 15 h before addition of $CoCl_2$ or $FeCl_2$. Control cells contained no 4-hydroxy tamoxifen. Cells were incubated for 18 h after addition of metals. All cells (adherent and in suspension) were collected by trypsinization and centrifugation before being washed with binding buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, and 2.5 mM $CaCl_2$), and labeled with annexin-V-FLUOS, 10 μg/mL PI for 15 min at RT. At least 40,000 cells from each sample were analyzed by FACScaliber flow cytometer (Becton Dickinson). Positive staining of the plasma membrane with annexin V in the absence of concomitant staining of nuclei with PI indicates apoptosis at an early stage (quadrant 2 in Fig. 2*B*). Double-positive staining with both annexin V and PI suggests apoptosis at later stages (quadrant 3 in Fig. 2*B*). Control cells of wild type SH-SY5Y treated with 4-hydroxy tamoxifen showed no protection in the metal toxicity assay. For Ebselen experiments, wild-type SH-SY5Y cells were treated with Ebselen (1 μM) for 30 min before the addition of $FeCl_2$ for 18 h. Cell death was measured by flow cytometry as outlined above.

Iron Uptake and Staining of SH-SY5Y Cells. Inducible SH-SY5Y cells were treated with 150 and 300 μM $FeCl_2$ for 18 h. Cells were washed three times in TBS and 0.12% ammonium sulfide was added for 5 min. Cells were washed and then fixed in 4% PFA for 5 min. Elemental Fe was then stained using a silver enhancer kit as described in the manufacturer's protocol (Sigma-Aldrich).

Western Blots and Immunoprecipitation Assays. Human cortical neuron cultures and SH-SY5Y cells were lysed in RIPA buffer containing complete protease inhibitors (Roche Applied Science). For ubiquitin assays N-ethyl maleimide (NEM) was added to the lysis buffer to prevent loss of ubiquitin tags. Lysates were incubated with appropriate antibody; polyclonal Ndfip1 (1:2,000), purified rabbit polyclonal Nedd4–2 (1:2,000), rabbit polyclonal DMT1 (1:2,000), mouse monoclonal Itch (1:2,000; BD Transduction Laboratories), for 1 h followed by 30 min incubation with Protein A agarose (Zymed). Beads were washed five times with RIPA buffer and separated on a 10% SDS/PAGE, before transfer to nitrocellulose membranes. Western blots were then probed with the appropriate primary antibody [polyclonal Ndfip1, monoclonal Flag M2 (Sigma), polyclonal DMT1, monoclonal multiubiquitin clone FK2 (MBL), and polyclonal Nedd4–2, monoclonal Itch] followed by anti-rabbit or mouse HRP. Blots were detected using Amersham ECL reagent (GE Healthcare). All blots are representative of at least three different experiments.

Immunohistochemistry. Immunohistochemistry was performed on 18-week-old human embryonic brain tissue fixed in 4% paraformaldehyde for 24 h. Tissue was sectioned into 14 μm slices and blocked in 10% normal horse serum in 0.1 M PB with 0.2% Triton X-100, and then incubated overnight in primary antibodies. Primary antibodies used were rat monoclonal Ndfip1 (1:200) and mouse monoclonal human DMT1 (1:500; Abnova). Secondary antibodies were Alexa Fluor 594-conjugated goat anti-rat IgG (1:500; Invitrogen) and Alex 488-conjugated goat antimouse IgG (1:500; Invitrogen). Images were taken on an inverted Zeiss Axiovert 200-LSM 5-Pa confocal microscope.

Histology and Brain Fe Measurements. Brains were dissected out of Ndfip1$^{+/-}$ and Ndfip1$^{-/-}$ mice at P3. Tissue was sectioned into 1-mm slices and cultured for 18 h in the presence of 200 μM $FeCl_2$. Sections were placed in 20% sucrose for 2 h then fresh frozen in OCT. Tissue was then cut into 20-μm sections before fixing in 4% PFA for 20 min. Iron accumulation was stained using Perls Prussian blue and counter stained with 2% neutral red. Ndfip1$^{+/+}$ and Ndfip1$^{-/-}$ animals at 3 weeks of age were fed a low Fe diet (15 mg/kg) or high Fe diet (20 g/kg) for 3 weeks (standard rodent diet contains 180 mg/kg Fe). Brain tissue was measured by weight and ICP mass spectroscopy was used to analyze the levels of Fe within the cortex of both Ndfip1$^{+/+}$ and Ndfip1$^{-/-}$ mice fed on the low or high Fe diet. For the analysis of DMT1 protein levels lysates were prepared from brain tissue from three animals per genotype for each of the diets. Western blots were performed and probed with anti-DMT1 rabbit antibody (Abnova, 4EC).

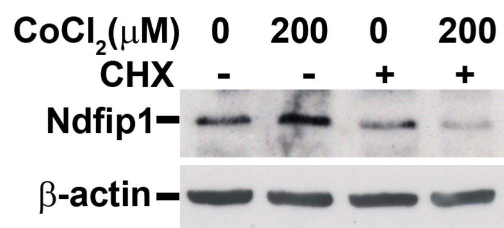

Fig. S1. The increase in Ndfip1 protein in metal treated cells is due to transcriptional upregulation. SH-SY5Y cells treated with $CoCl_2$ upregulate Ndfip1 (two left lanes). However, treatment with cycloheximide (CHX) for 6 h prevents the increased expression of Ndfip1 in response to $CoCl_2$ (two right lanes).

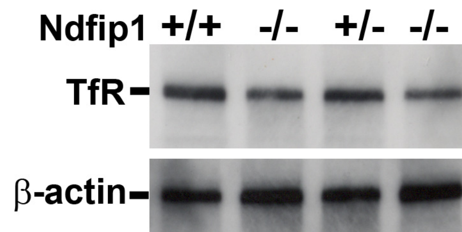

Fig. S2. Transferrin receptor (TfR) protein levels are lower in Ndfip1$^{-/-}$ mice brains. Transferrin receptor is a major pathway for Fe entry into cells. Ndfip1$^{-/-}$ mice brains (P7) were probed with TfR antibody (Invitrogen) and were found to have lower levels of TfR when compared with heterozygous or wild-type littermates (two separate litters shown).