Supplementary information

A Golgi-targeting fluorescent probe for labile Fe(II) to reveal abnormal cellular iron distribution induced by dysfunction of VPS35

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General procedure for synthesis and photophysical measurements: All chemicals used in this study were commercial products of the highest available purity and were further purified by the standard methods, if necessary. LysoRhoNox was prepared as previously reported (referred as HMRhoNox-M). ¹H-NMR spectra were obtained on a JEOLECA-500 spectrometer at 500 MHz and JEOL JNM AL-400 spectrometer at 400 MHz. ¹³C-NMR spectra were obtained on a JEOL ECA-500 spectrometer at 125 MHz and JEOL AL-400 spectrometer at 100 MHz. Chemical shifts of ¹H-NMR are referenced to tetramethylsilane (TMS). Chemical shifts of ¹³C-NMR are referenced to CDCl₃ (77.0) or CD₃OD (49.0). Chemical shifts and coupling constants were recorded in units of ppm and Hz, respectively. ESI-mass spectra were measured on a JEOL JMS-T100TD mass spectrometer. High-resolution mass spectra (HRMS) were measured on a JEOL JMS-T100TD by using polyethyleneglycol (PEG) as an internal standard. Reactions were monitored by silica gel TLC (Merck Silica gel 60 F₂₅₄) with visualization of components by UV light (254 nm) or with visual observation of the dye spots. Products were purified on a silica gel column chromatography (Taiko-shoji AP-300S).

1. Synthesis



Scheme S1. Synthesis of Gol-SiRhoNox

Gol-SiR

6-Carboxy-SiR¹ and (2-myristoylaminoethyl)carbamic acid *tert*-butyl ester² were prepared according to previous reports. (2-Myristoylaminoethyl)carbamic acid *tert*-butyl ester (30.5 mg, 82 µmol) was dissolved in trifluoroacetic acid (TFA, 1.0 mL) and CH₂Cl₂ (2.0 mL), and the resulting solution was stirred at room temperature for 1.5 h. The reaction mixture was evaporated and then azeotroped with toluene to obtain *N*-myristoylethylenediamine TFA salt quantitatively. This material was used to the next reaction without further purification. To a solution of 6-carboxy-SiR (36.3 mg, 69 µmol), Et₃N (29 µL, 206 µmol), and DMT-MM (28.5 mg, 103 µmol) in DMF (1.0 mL) was added a solution of *N*-myristoylehylenediamine in DMF (1.0 mL). The solution was stirred at room temperature for 21 h. CH₂Cl₂ (50 mL) was added to the solution, and the whole was washed with water (20 mL × 2) and brine (20 mL). The organic layer was dried over MgSO₄ and then evaporated. The residue was purified by silica gel column chromatography twice (*n*-hexane: EtOAc = 1:

1 and CHCl₃: MeOH = 40: 1) to give the target compound, Gol-SiR, as a white solid (8.4 mg, 16%).

TLC $R_f = 0.14$ (CHCl₃: MeOH = 40: 1)

¹H NMR (500 MHz, CDCl₃): δ 7.99 (d, J = 8.0 Hz, 1H), 7.90 (dd, J = 8.0, 1.1 Hz, 1H), 7.79 (s, 1H), 7.47 (t, J = 4.6 Hz, 1H), 6.90 (d, J = 3.4 Hz, 2H), 6.70 (d, J = 9.2 Hz, 2H), 6.47 (dd, J = 9.2, 2.9 Hz, 2H), 6.11 (t, J = 5.4 Hz, 1H), 3.54 (q, J = 5.2 Hz, 2H), 3.51–3.44 (m, 2H), 3.35 (q, J = 7.1 Hz, 8H), 2.16 (t, J = 7.7 Hz, 2H), 1.58 (q, J = 7.3 Hz, 2H), 1.34–1.18 (m, 22H), 1.15 (t, J = 7.2 Hz, 12H), 0.88 (t, J = 6.9 Hz, 3H), 0.64 (s, 3H), 0.59 (s, 3H).

¹³C NMR (125 MHz, CDCl₃): δ 175.3, 170.0, 166.8, 155.0, 146.5, 138.0, 130.0, 129.5, 128.4, 127.1, 125.8, 123.8, 115.8, 112.5, 92.5, 44.2, 42.0, 39.6, 36.2, 31.8, 29.7, 29.6, 29.5, 29.33, 29.25, 29.2, 25.7, 22.7, 14.1, 12.5, 0.3, -1.5.

HRMS (ESI+): *m/z* calculated for C₄₇H₆₈N₄NaO₄Si⁺ [M+Na]⁺: 803.4902, found 803.4921.

Gol-SiRhoNox

To a mixture of Gol-SiR (23.5 mg, 30 μ mol) in EtOAc (15 mL) was slowly added *m*-CPBA (8.9 mg, 36 μ mol) at 0 °C. The mixture was warmed to room temperature and then stirred for 5 min. Then, the reaction mixture was evaporated. The residue was purified with silica gel chromatography (CHCl₃: MeOH = 15: 1 to 10: 1) to give the title compound as a white solid (4.3 mg, 18%).

TLC $R_f = 0.31$ (CHCl₃: MeOH = 10: 1)

¹H NMR (500 MHz, CDCl₃): δ 8.23 (s, 1H), 8.19 (s, 1H), 8.01 (s, 2H), 7.98 (s, 1H), 7.45 (d, J = 8.0 Hz, 1H), 7.02 (d, J = 9.2 Hz, 1H), 6.92 (d, J = 2.9 Hz, 1H), 6.79 (d, J = 9.2 Hz, 1H), 6.58 (brs, 1H), 6.50 (dd, J = 9.2, 2.9 Hz, 1H), 3.78–3.58 (m, 4H), 3.54–3.52 (m, 2H), 3.52–3.48 (m, 2H), 3.36 (q, J = 7.1 Hz, 4H), 2.18 (t, J = 7.7 Hz, 2H), 1.57 (t, J = 6.6 Hz, 2H), 1.34–1.18 (m, 22H), 1.15 (d, J = 14.3 Hz, 6H), 1.09 (t, J = 6.9 Hz, 6H), 0.87 (t, J = 6.9 Hz, 3H), 0.71 (s, 3H), 0.65 (s, 3H)

¹³C NMR (125 MHz, CDCl₃): δ 175.6, 169.3, 166.4, 153.0, 146.9, 139.5, 139.0, 136.5, 129.0, 128.7, 128.4, 127.8, 127.4, 127.1, 126.3, 124.4, 122.1, 116.0, 112.5, 91.0, 66.8, 66.7, 44.3, 42.3, 39.5, 36.6, 31.9, 29.7, 29.61, 29.58, 29.46, 29.33, 29.29, 29.23, 25.7, 22.7, 14.1, 12.5, 8.4, 8.3, 0.3, -1.8.

HRMS (ESI+): *m/z* calculated for C₄₇H₆₈N₄NaO₅Si⁺: 819.4851, found: 819.4837.

2. Steady-state absorption and fluorescence spectroscopy

The UV-vis absorption spectra were recorded on an Agilent 8453 photodiode array spectrometer equipped with a UNISOKU thermo-static cell holder (USP-203). Fluorescence spectra were recorded using a JASCO FP6600 with a slit width of 5 nm and 6 nm for excitation and emission, respectively. To reduce fluctuation in the excitation intensity during measurement, the lamp was kept on for 30 min prior to the experiment. The path length was 1 cm with a cell volume of 3.0 mL.

Fluorescence responses of the probes to various metal ions were measured as follows. An aqueous solution of transition metal ion species (stock solutions: 10 mM for MnSO₄, CoSO₄, NiSO₄, FeSO₄, FeCl₃, CuSO₄, and

ZnSO₄; 100 mM for NaCl, KCl, MgCl₂, and CaCl₂) or [Cu(MeCN)₄]PF₄ (from 10 mM stock solution in MeCN) was added to give the final concentrations of 1 mM for Na⁺, K⁺, Mg²⁺, and Ca²⁺ and 20 μ M for other metal ion species. The mixtures were incubated for 1 h at room temperature, and then fluorescence spectra were measured after the addition of dioxane (30% v/v). Stability against reductants and reactive oxygen species, and effect of chelator were tested under the conditions as follows.

	$Na_2S_2O_3$: 100 µM from 100 mM stock solution in water
	Sodium ascorbate	: 1 mM from 100 mM stock solution in water
	Cysteine	: 1 mM from 100 mM stock solution in water
β -NADH (reduced): 100 μ M from 10 mM stock solution in water		
	Glutathione	: 1 mM from 100 mM stock solution in HEPES buffer (pH was adjusted to 7.4)
	NaNO ₂	: 100 µM from 100 mM stock solution in water
	O_2^{-}	: 100 μ M from saturated KO ₂ solution in DMSO (ca. 1 mM)
	H_2O_2	: 100 µM from 100 mM stock solution in water
	•OH	: 200 μ M H ₂ O ₂ and 20 μ M FeSO ₄
	NaOCl	: 100 µM from 100 mM stock solution in water
	NO	: 100 μ M NOC-5 from 10 mM stock solution in 0.1 M NaOHaq.
	$Fe^{2+} + Bpy$: 20 μM FeSO4 in the presence of 2,2'-bipyridyl (Bpy) from 100 mM stock solution is
		DMSO

Probe (2 μ M) was incubated under each condition in 50 mM HEPES buffer (pH 7.4) for 1 h, and then fluorescence spectra were measured after the addition of dioxane (30%v/v).

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For the fluorescence measurements in the presence of glutathione, Gol-SiRhoNox (2 μ M) was mixed with 20 μ M FeSO₄ in the presence or absence of 1 mM glutathione in HEPES buffer (50 mM, pH 7.4) at room temperature for 0, 15, 30, 45, and 60 min. then fluorescence spectra were measured after the addition of dioxane (30% v/v).

For the fluorescence measurements in the presence of amino acids solution, SiRhoNox-1,³ which is the Fe(II)sensor domain in Gol-SiRhoNox, was employed as a water-compatible analogue of Gol-SiRhoNox because fluorescence spectra was not measurable due to considerable precipitate upon addition of dioxiane to the amino acids mixture. SiRhoNox-1 (2 μ M form 1 mM stock solution in DMSO) was mixed with 20 μ M FeSO₄ in the presence or absence of essential amino acids and non-essential amino acids (from MEM essential amino acids solution (×50) and MEM non-essential amino acids solution (×100), FUJIFILM Wako Pure Chemical Corporation) in HEPES buffer (50 mM, pH 7.4), and the solutions were incubated at room temperature for 0, 15, 30, 45, and 60 min. Then fluorescence spectra were measured with excitation at 630 nm. The final amino acids contents are as follows.

L-Alanine	100 µM
L-Arginine hydrochloride	599 µM
L-Asparagine	100 µM

L-Aspartic acid	100 µM
L-Cystine	100 µM
L-Glutamic acid	100 µM
L-Glysine	100 µM
L-Histidine hydrochloride	200 µM
L-Isoleucine	200 µM
L-Lysine hydrochloride	400 μΜ
L-Methionine	396 µM
L-Phenylalanine	600 µM
L-Proline	100 µM
L-Serine	100 µM
L-Threonine	100 µM
L-Tryptophan	600 µM
L-Tryosine	100 µM

3. Product analysis by HPLC-MS

To a solution of a probe (100 μ M) in 50 mM HEPES buffer (pH 7.4, 5%DMSO as co-solvent) was added a solution of FeSO₄ (final, 1 mM). The mixture was kept for 1 h under an ambient condition. The products were analyzed with LC-MS system (Chromaster[®]5110, Hitachi High-tech) equipped with a photodiode-array detector (Chromaster[®]5430, Hitachi High-tech) and a mass spectrometer (Chromaster[®]5610 MS Detector, Hitachi High-tech) and with Waters symmetry C₁₈ column (3.5 μ m, 4.6 × 75 mm) eluted with a gradient system consisting of H₂O (solvent A) and CH₃CN (solvent B) containing 0.05% formic acid; 50%B to 90%B over 20 min followed by 90%B for 10 min. The retention times were compared with those of the parent dyes in 50 mM HEPES buffer (pH 7.4, 5%DMSO as co-solvent). Assignments of the compounds were based on the observed *m/z* values at each peak.

4. Cell culture experiments

Human hepatocellular carcinoma (HepG2) cells were cultured in modified essential medium (MEM, Gibco) containing 10% fetal bovine serum (FBS, Gibco), 1% Antibiotic-Antimyocotic (ABAM, Gibco), and 2 mM glutamine at 37 °C in a 5% CO₂/95% air incubator. The human neuroblastoma cell line SH-SY5Y was cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 10% FBS at 37 °C in a 5% CO₂/95% air incubator. Two days before use, cells (1.0×10^5) were seeded on Advanced TC glass-bottomed dishes (CELLviewTM Cell Culture Dish, Greiner). For R55 treatment, SH-SY5Y cells were treated with 5 µM R55 (TPT-260, Cayman) at 48 h in DMEM with 10%FBS.

5. Confocal fluorescence imaging experiments

Confocal fluorescence images were acquired with Zeiss LSM700 laser confocal microscopy or Olympus IX83 microscope equipped with a 130 W mercury lump, an EMCCD camera (Hamamatsu Photonics, ImagEM), and a disk scan confocal unit (DSU). Images were obtained with appropriate filter sets for each dye as follows.

For all imaging experiments, Hank's Balanced Salt Solution (HBSS, Gibco) containing calcium chloride and magnesium chloride without phenol red was used. For Fe(II) uptake experiments, cells were treated with 100, 50, 10, 5, or 1 μ M ferrous ammonium sulfate hexahydrate (FAS, Fe(NH₄)₂(SO₄)₂•6H₂O, from 10 mM, 1 mM, or 100 μ M stock solution in water) in MEM without FBS at 37 °C for 30 min. After the treatment with FAS followed by washing the cells thoroughly with HBSS (×3), a solution of Gol-SiRhoNox (final: 5 μ M, from a 1:1 mixture of Gol-SiRhoNox (2 mM in DMSO) and Pluronic F-127 (20% w/v in DMSO)) in HBSS was added to the cells. After incubation for 30 min at 37 °C, the cells were washed with HBSS (×1), and then imaged with a Cy5 filter set (ex: 608–648 nm, em: 672–712 nm, dichroic: 660). For chelation experiments, 1 mM 2,2'-bipyridyl (Bpy, from 100 mM stock solution in DMSO) was added to the cells simultaneously with the probe. Fluorescence intensities in each image (230 cells on average) were measured (n = 4; 4 images for each condition) by using an automated evaluation process by ImageJ (background subtraction, thresholding, and then brightness measurement). Statistical analyses were performed with a Students' *t*-test.

For the photostability assay, HepG2 cells were stained with Gol-SiR (final: 5 μ M, from a 1:1 mixture of Gol-SiR (2 mM in DMSO) and Pluronic F-127 (20% w/v in DMSO) at 37 °C for 30 min in HBSS. The cells were washed with HBSS and then were irradiated with the same microscopic setting and the excitation light with the Fe(II) uptake experiments as above. The scanning and acquisition of images were repeated 30 times over 5 min, and the average fluorescence intensities were measured for each image by ImageJ. Total 3 fields of view at each time point were analyzed (n=3).

For inhibition tests of NAD(P)H-dependent enzymes, diphenyliodonium chloride⁴⁻⁶ (100 μ M DPI, from 10 mM stock solution in water) was added to the cells simultaneously with the probe. After incubation for 60 min, the cells were washed with HBSS and then imaged with the Cy5 filter set. Fluorescence intensities in each image (230 cells on average) were measured (n = 4; 4 images for each condition) by using ImageJ. Statistical analyses were performed with a Students' *t*-test.

For co-staining experiments, Golgi apparatus of cells were visualized by green fluorescent protein (GFP) by using CellLight[®] Golgi-GFP BacMam 2.0* (Thermo Fischer Scientific inc.) or BODIPYTM FL C₅-Ceramide/bovine serum albumin (BSA) complex (1:1) (Thermo Fischer Scientific inc.), according to the manufacturer's protocol. After induction of GFP at Golgi apparatus of HepG2 cells, the cells were incubated with 100 μ M FAS at 37 °C for 30 min. Then, the cells were washed with HBSS (×3) and stained with 5 μ M Gol-SiRhoNox (by using the Pluronic method as above). After incubation of the cells at 37 °C for 30 min, the cells were washed with HBSS and then imaged using appropriate filter sets (an FITC filter set (ex: 465–500 nm, em: 516–556 nm, dichroic: 495 nm) for Golgi-GFP and the Cy5 filter set for Gol-SiRhoNox). Colocalization analysis was performed on each image by setting ROI (region of interests) of GFP-expressing

cells (20 cells in each image on average), and then Pearson's correlation values (R_{coloc}) were calculated for the FITC (Golgi-GFP) and Cy5 (Gol-SiRhoNox) channels by Coloc2 program of Fiji.⁷ R_{coloc} values were obtained for each field of view (20 cells in each image on average), and the averages of R_{coloc} values for each condition were calculated (n = 8: 8 images for each condition were analyzed). Statistical analyses were performed with a Students' *t*-test.

For staining with BODIPY FL C₅-Ceramide, the BSA complex of the dye was prepared (1:1, final 5 μ M in HBSS), according to the manufacturer's protocol. HepG2 cells pretreated with 100 μ M FAS and 5 μ M Gol-SiRhoNox as described above were stained with the BODIPY FL C₅-Ceramide/BSA complex (5 μ M) at 4 °C for 30 min. The medium was replaced by MEM (+10%FBS), and the cells were further incubated at 37 °C for 10 min. Then, the cells were washed with HBSS (×1) and imaged using the appropriate filter sets (the FITC filter set for BODIPY FL C₅ Ceramide and the Cy5 filter set for Gol-SiRhoNox). For staining with other organelle-staining dyes, HepG2 cells pretreated with Fe(II) (100 μ M, as FAS). After thorough washing with HBSS (×3), the cells were stained with a mixture of Gol-SiRhoNox (5 μ M) and each organelle-staining dye (MitoTracker[®] Green FM (0.5 μ M), LysoTracker[®] Green DND-26 (0.5 μ M), ER-TrackerTM Green (1 μ M) in HBSS for 30 min at 37 °C. The cells were washed with HBSS (×1) and then imaged by the appropriate filter sets (the FITC filter set for the organelle-staining dyes and the Cy5 filter set for Gol-SiRhoNox).

Colocalization analysis was performed by calculation of Pearson's correlation values (R_{coloc}) for the FITC channel (BODIPY FL C₅ Ceramide, MitoTracker[®] Green FM, LysoTracker[®] Green DND-26, ER-TrackerTM Green) and Cy5 (Gol-SiRhoNox) channel with Coloc2 program of Fiji.⁷ R_{coloc} values were obtained for each field of view (30 cells in each image on average), and the average of R_{coloc} values for each condition were calculated (n = 15: 15 images for each condition were analyzed).

For fluorescence microscopic analysis of local labile Fe(II) levels affected by knockdown of VPS35 and supplementation of R55 (5 μ M) in SH-SY5Y cells, the cells pretreated with R55 (5 μ M, 48 h, at 37 °C for 48 h) were washed with HBSS (×3) and stained with 5 μ M Gol-SiRhoNox (Pluronic-coincubation method as above) or 3 μ M LysoRhoNox (from 1 mM stock in DMSO) in HBSS at 37 °C for 1 h in HBSS. After incubation for 1 h, the cells were washed with HBSS and then imaged using a laser scanning confocal microscopy (Zeiss LSM700). The average fluorescence signal intensities in the GFP-positive cells (knockdown of VPS35 cells) were quantified for each image ("n" means the numbers of the fields of view analyzed for each condition) using ImageJ. Statistical analyses were performed with a Students' *t*-test.

For fluorescence imaging study of organelle-specific sensitivity against exogenous Fe(II), wild-type (mock), VPS35 knockdown, wild-type VPS35 overexpressing (control), or mutant VPS35 (D620N) overexpressing SH-SY5Y cells were treated without or with 100 μ M FAS for 30 min in DMEM (–FBS) and then washed with HBSS (×3). Then, the cells were stained with 5 μ M Gol-SiRhoNox or 3 μ M LysoRhoNox for 1 h in HBSS. The cells were washed with HBSS and then imaged by using the laser scanning confocal microscopy (Zeiss LSM700). The average fluorescence signal intensities in the GFP-positive cells (VPS35 mutant cells) were quantified for each image (n = 4: 4 images for each condition) using ImageJ. Statistical analyses were

performed with a Students' t-test.

For double-immunofluorescence staining, the treated cells were fixed with 4% paraformaldehyde for 10 min. Subsequently, these cells were permeabilized with 0.1% Triton X / PBS for 30 min at RT. The 2% goat serum was used for blocking reaction for 60 min. These cells were co-incubated with mouse monoclonal antibody against DMT1 (1:200, Sigma) and rabbit polyclonal antibody against TGN46 antibody (1:200, Novus bio.) or Lamp2 (1:200, GeneTex Inc.). Subsequently, we incubated cells with second antibodies (goat anti-mouse antibody Alexia 555, goat anti-mouse antibody Alexia 488 (Thermo Fisher Scientific, USA)) at RT for 30 min. In addition, cells were incubated with Hoechst 33342 (Molecular Probes) for 15 min at room temperature. Fluorescence was observed using a laser scanning confocal microscope (Zeiss LSM 700). Image analysis was performed with ImageJ. Statistical analyses were performed with a Students' *t*-test.

6. Cytotoxicity assay by CCK-8 assay kit

Cytotoxicity assays were carried out according to the incubation conditions of the imaging experiments. The cytotoxicity was assayed by Cell Counting Kit-8, following the manufacturer's instruction (Wako Pure Chemical Industries Ltd.). Briefly, SH-SY5Y or HepG2 cells were seeded at 5.0×10^4 cells/mL in 96-well plates for 24 h. After seeding, the cells were treated with 100 μ M Fe(II) (FAS) at 37 °C for 30 min, then washed with HBSS (×3), and stained with 5 μ M Gol-SiRhoNox or 3 μ M LysoRhoNox in HBSS at 37 °C for 1 h. Bpy (1 mM) was added to the cells simultaneously with the probes. Subsequently, Cell Counting Kit-8 reagent was added into the wells, and the plate was incubated at 37 °C for 4 h. The optical density of formazan was detected at 450 nm by GloMax® (Promega, Madison, WI, USA) for calculating cell viability. The wavelength of 600 nm was used as reference.

7. Plasmid construction and preparation of viral vectors

The BLOCK-iTTM Pol II miR RNAi Expression Vector Kit with EmGFP (Invitrogen) was used for RNAi experiments. Artificial miRNA sequences targeting human VPS35 were designed using BLOCK-iTTM RNAi Designer (http://rnaidesigner.Invitrogen.com/rnaiexpress/). The designed oligonucleotides were annealed, followed by ligation into the pcDNA6.2-GW/EmGFP-miR vector (Invitrogen), which facilitates transfer into a suitable destination vector via Gateway recombination reactions. As the control, pcDNA6.2-GW/EmGFP-miR negative control plasmid (NC) (Invitrogen) was used. The sequence containing the miRNA coding region was transferred to the lentivirus vector via the Gateway cloning system (Invitrogen). Briefly, the miRNA coding region was subcloned into the entry plasmid pDONR221 (Invitrogen) using Gateway® BP ClonaseTM II Enzyme Mix (Invitrogen). The sequences in the entry plasmids were then transferred to the lentiviral expression vector, plenti CMV Puro Dest (w118-1) (a gift from Eric Campeau, Addgene plasmid #17452)⁸ using Gateway® LR ClonaseTM II Enzyme Mix (Invitrogen).

For the over-expressing experiment, human VPS35 and α -synuclein cDNA were purchased from TransOMIC Technologies, and subcloned into a pENTR entry vector (Invitrogen) with flag tag at C-terminal. The mutant

VPS35 (D620N) gene was generated by PrimeSTAR® Mutagenesis Basal Kit (Takara Bio Inc.) according to the manufacturer's protocol. The primer pairs were as follows: 5'-AATCAGCAATTCCAAAGCACAGCTAGC-3' and 5'-TTGGAATTGCTGATTTCATCTTCATAC-3' for plenti CMV Puro Dest-VPS35 (D620N). Subsequently, the VPS35 and α -synuclein cDNA transferred to plenti CMV Puro Dest and plenti CMV Blast Dest (706-1) (a gift from Eric Campeau, Addgene plasmid #17451)⁸ respectively.

8. Lentivirus infection

Lentiviral stocks were produced in 293FT cells according to the manufacturer's protocol (Invitrogen). In brief, virus-containing medium was collected 48 h post-transfection and filtered through a 0.45 µm filter. The miRNA expression was monitored by checking the simultaneous coexpression of the EmGFP reporter gene by fluorescence microscopy. The transduced cells were screened using western blotting assay to determine the levels of gene expression.

9. Western blotting

For VPS35 detection, the treated cells were lysed by adding lysis buffer (150 mM NaCl, 10 mM Tris-HCl, 1% NP-40, 1 mM EDTA, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 1% phosphatase inhibitor, 0.1 mM PMSF) and centrifuged under the conditions of 14,000g at 4 °C for 30 min. The supernatant was collected as protein samples. For α -synuclein detection, the treated cells were lysed by adding RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, 0.5% Na-deoxycholate, 0.1% SDS, 1% NP-40, 1 mM EDTA, 10 µg/mL aprotinin, 10 µg/mL leupeptin, 1% phosphatase inhibitor, 0.1 mM PMSF) with brief sonication on ice. The concentration of protein was determined using the Pierce BCA protein assay kit (Thermo Fisher Scientific). Samples underwent SDS-PAGE in order to separate proteins on the basis of molecular weight. SDS-PAGE was performed under constant voltage at 200V at room temperature for 60 min. The separated proteins in polyacrylamide gel were transferred to a PVDF membrane in transfer buffer (0.3% Tris, 1.44% glycine, 20% methanol) under constant voltage at 100V at 4 °C for 60 min. The transferred membrane was incubated in 5% BSA (Wako) at room temperature for 60 min. After a blocking reaction, the membrane was incubated with primary antibodies: the mouse monoclonal antibodies, β-actin (1:2,000, Santa Cruz Biotechnology), Flag (1:1,000, Sigma); rabbit polyclonal antibody: VPS35 (1:1,000, Sigma) dissolved in 5% BSA at 4 °C overnight. After the primary antibody reaction, the membrane was incubated with the secondary antibody: goat anti-rabbit antibody conjugated with HRP (1:2000, Santa Cruz Biotechnology), and goat anti-mouse antibody conjugated with HRP (1:2000, Santa Cruz Biotechnology) dissolved in 3% BSA for 30 min. The membrane was incubated in ECL Prime (GE Healthcare, Buckinghamshire, UK) to generate the chemiluminescence from HRP antibodies. The chemiluminescence was detected using the LAS3000 Mini (Fujifilm). The band density was measured by ImageJ software.

10. Quantification of total intracellular iron by atomic absorption spectrometry

HepG2 cells (5.0×10^6 cells) or SH-SY5Y cells (4.0×10^6 cells) were seeded on a plastic dish (10 cm) 3 days prior to use. The medium was washed with cold phosphate-buffered saline (PBS, 6 mL × 3). The cells were removed from the dishes by using a scraper, and then the suspension was centrifuged (1,000 rpm, 5 min). The supernatant was carefully removed, and the cells were re-suspended into conc. HNO₃ (100μ L). The suspension was heated at 90 °C for 4 h to dissolve the cell bodies. The lysate was diluted to 2 mL with distilled water. Concentrations of iron in the samples were measured by furnace atomic absorption spectroscopy with a Shimadzu AA-7000 atomic absorption spectrometer. The obtained values (ng/mL) were normalized with the cell numbers (per 10^6 cells).

Total 4 dishes were prepared for each experiment as described above. 3 dishes were used for iron quantification, and the rest was used to calculate the number of cells. To determine the cell numbers, the cells were collected by trypsinization at the same time point with the other 3 dishes, and the number of the cells was counted by a hematocytometer or an automated cell counter (Countess II[®] FL, Thermo Fisher Scientific).

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<Supplementary figures>



Figure S1. (a) Absorption spectral change of Gol-SiR (5 μ M) upon various concentrations of 50 mM HEPES (pH = 7.4)/dioxane (0–100%). (b) Plot of absorption at 655 nm against concentration of dioxane. (c) Fluorescence spectral change of Gol-SiR (2 μ M) upon various concentrations of dioxane. Excitation was provided at 630 nm. (d) Plot of absorption at 655 nm of Gol-SiR (red) and Gol-SiRhoNox (blue) in aqueous solutions (circles) and 30%dioxane aqueous solution (triangles) at each pH (200 mM phosphate solution). (e) Plot of fluorescence intensities at 670 nm in aqueous solutions (circles) and 30%dioxane aqueous solution). Probe concentrations for the aqueous solution and the 30% dioxane aqueous solutions are 5 μ M and 3.5 μ M, respectively. Excitation was provided at 630 nm. (f) Fluorescence intensities of Gol-SiRhoNox (2 μ M) after incubation with various concentrations of Fe(II) (provided as ferrous sulfate hexahydrate) at room temperature for 1 h. The same experiments were repeated thrice. Statistical analysis was performed with Student's *t*-test. **p* < 0.05, ***p* < 0.005 (*n* = 3). Error bars indicate ± standard error of the mean (SEM).



Figure S2. (a) Fluorescence response of Gol-SiRhoNox (2 μ M) against various biological reductants, reactive oxygen species. Bars represent relative fluorescence intensities at 665 nm after incubation with the chemicals at room temperature for 1 h in 50 mM HEPES buffer (pH 7.4). Excitation was provided at 640 nm. Dioxane (final 30%v/v) was added prior to fluorescence measurements. (b) Fluorescence response of Gol-SiRhoNox (2 μ M) to 20 μ M Fe(II) in the presence of absence of glutathione (GSH, 1 mM) or β -nicotinamide adenine dinucleotide reduced form (β -NADH, 100 μ M) in 50 mM HEPES buffer (pH 7.4). The relative fluorescence intensities at 665 nm after incubation for 0 (white), 15 (light gray), 30 (gray), 45 (dark gray), and 60 min (black) at room temperature are plotted. Dioxane (final 30%v/v) was added prior to fluorescence response of SiRhoNox-1 (2 μ M) to 20 μ M Fe(II) in the absence or presence of the mixture of essential and non-essential amino acids in 50 mM HEPES buffer (pH 7.4). The relative fluorescence intensities at 660 nm after incubation for 0 (white), 15 (light gray), 30 (gray), 45 (light gray), 30 (gray), 45 (dark gray), and 60 min (black) at room temperature of the mixture of essential and non-essential amino acids in 50 mM HEPES buffer (pH 7.4). The relative fluorescence intensities at 660 nm after incubation for 0 (white), 15 (light gray), 30 (gray), 45 (dark gray), 30 (gray), 45 (dark gray), and 60 min (black) at room temperature are plotted. Excitation was provided at 630 nm.



Figure S3. HPLC-MS monitoring of the reaction of Gol-SiRhoNox (100 μ M) and Fe(II) (1 mM). (a) Gol-SiRhoNox, (b) the mixture of Gol-SiRhoNox and Fe(II), and (c) Gol-SiR. Data collection by monitoring absorption at 256 nm and mass spectra was started at 5 min after eluting highly polar components such as iron salt and HEPES with the initial solvents, and the gradients started then. The numbers on the peaks indicate retention times. Lower indicate the mass spectra observed at each time-point of the peaks.



Figure S4. (a) Total iron contents of HepG2 cells (per 10^6 cells) after incubation without or with Fe(II) (100 μ M, as FAS, at 37 °C for 30min in MEM without FBS) by atomic absorption spectrometry. Statistical analyses were performed with Student's *t*-test. ** p < 0.001 (n = 3). Error bars indicate ± standard error of the mean (SEM) (n=3) (b) Cytotoxicity assay (CCK-8 assay) of HepG2 cells treated with Gol-SiRhoNox (5 μ M) in the presence of Fe(II) (100 μ M) and 2,2'-bipyridyl (Bpy) (1 mM) as in the microscopic analysis (Fig S7). The treatments with the probe, Fe(II), and Bpy did not induce significant cytotoxitity (n = 6). Error bars indicate ±SEM. (c) Representative images of HepG2 cells stained with Gol-SiR (5 μ M). The cells were stained at 37 °C for 30 min, washed by HBSS, and then imaged. Scanning was repeated 30 times over 5 min. Scale bars indicate 25 μ m. (d) Quantification of fluorescence signal intensities from Gol-SiR. 3 fields of view were imaged analyzed for each time point. Error bars indicate ±SEM (n = 3). No significant change was observed.



Figure S5. Confocal fluorescence images to verify intracellular localization of Gol-SiRhoNox. Co-staining of Fe(II)-treated HepG2 cells with Gol-SiRhoNox 5 μ M with (column 1) BODIPY FL C₅-ceramide/BSA complex (1:1, 5 μ M), (column 2) MitoTracker[®] Green FM (0.5 μ M), (column 3) LysoTracker[®] Green DND-26 (0.5 μ M), or (coumun 4) ER-TrackerTM Green (1 μ M). (a) Representative images obtained using a Cy5 filter set (for Gol-SiRhoNox) and (b) that obtained using an FITC filter set (for BODIPY FL C₅-ceramide/BSA complex, MitoTracker[®] Green FM, LysoTracker® Green DND-26, and ER-TrackerTM Green). (c) Merged images of (a) and (b). Fe(II) (100 μ M) was supplemented as ferrous ammonium sulfate hexahydrate (FAS). Scale bars indicate 20 μ m.

Pearson's correlation values are listed as follows. (n = 15: 15 images for each condition were analyzed)

Golgi: $R_{coloc} = 0.73 \pm 0.06$ Mitochondria: $R_{coloc} = 0.31 \pm 0.09$ Lysosome: $R_{coloc} = 0.35 \pm 0.06$ ER: $R_{coloc} = 0.54 \pm 0.08$



Figure S6. Representative fluorescence microscopic images of HepG2 cells pretreated with (a) 0 μ M, (b) 1 μ M, (c) 5 μ M, (d) 10 μ M, and (e) 50 μ M of Fe(II) (supplemented as FAS). The cells were stained with Gol-SiRhoNox (5 μ M) after washing with HBSS (×3). (f) Quantification of fluorescence intensities of the cells treated under each condition. Statistical analyses were performed with Student's *t*-test. **p < 0.01 (n = 6: 6 images for each condition were analyzed). Error bars indicate ± SEM. Scale bars indicate 50 μ m.



Figure S7. (a and b) Representative fluorescence microscopic images of HepG2 cells treated with Gol-SiRhoNox (5 μ M) in the absence (a) and in the presence (b) of 1 mM 2,2'-bipyridyl (Bpy) at 37 °C for 30 min in HBSS to detect endogenous labile Fe(II) at Golgi. (c and d) Differential interference contrast (DIC) images for the same field of view for a and b, respectively. (e) Quantification of fluorescence intensities of the Bpy-untreated and Bpy-treated cells. Statistical analysis was performed with Student's *t*-test. **p < 0.01 (n = 4: 4 images for each condition were analyzed). (f and g) Representative fluorescence microscopic images of the cells treated with Gol-SiRhoNox (5 μ M) in the absence (f) and in the presence (g) of 100 μ M diphenyliodonium chloride (DPI) for 60 min in HBSS to investigate the effect of endogenous NAD(P)H-dependent reductases on fluorescence signal change. (H and I) DIC images for the same field of view for f and g, respectively. (j) Quantification of fluorescence intensities of the DPI-untreated and DPI-treated cells. Statistical analyses were performed with Student's *t*-test. (n = 6: 6 images for each condition were analyzed). Error bars indicate ± SEM. Scale bars indicate 50 μ m.



Figure S8. Representative fluorescence microscopic images of live SH-SY5Y cells loaded with Gol-SiRhoNox for detection of exogenously supplemented Fe(II). (a and b) Representative images of the cells treated without (a) and with (b) 100 μ M Fe(II) (supplemented as Fe(NH₄)₂(SO₄)₂•6H₂O) at 37 °C for 30 min in MEM without FBS prior to incubation with the probe in HBSS for 30 min at 37 °C. (c) A representative image of the cells treated with Fe(II) prior to incubation with the probe in the presence of 1 mM 2,2'-bipyridyl (Bpy) in HBSS (HBSS) at 37 °C for 30 min. (d–e) Differential interference contrast (DIC) images for the same slices of (a–c), respectively. (g) Quantification of fluorescence intensities of the cells treated with each condition. Statistical analyses were performed with Student's *t*-test. **p < 0.05 (n = 5: 5 images for each condition were analyzed). Error bars indicate ± SEM. Scale bars indicate 50 µm.



Figure S9. Cytotoxicity assay of SH-SY5Y cells against Gol-SiRhoNox (5 μ M) in the presence of Fe(II) (100 μ M) and 2,2'-bipyridyl (Bpy) (1 mM). The cell viability assay was performed by CCK-8 assay. Cell viability of SH-SY5Y cells treated with Fe(II), Gol-SiRhoNox, and Bpy as those in the microscopic analysis (Fig S7) were assayed by CCK assay kit according to the manufacturer's protocol. The treatments with the probe, Fe(II), and Bpy did not induce significant cytotoxitity (n = 6).



Figure S10. Immuno-fluorescence staining of DMT1, TGN48 (Golgi marker), and Lamp2 in wild-type SH-SY5Y cells (WT), VPS35 KD cells, and VPS35 KD cells treated with R55 (5 μ M). (a) Immuno-staining of the cells with antibodies of DMT1 and TGN46. (b) Colocalization efficiency between DMT1 and TGN46 (n = 8). (c) Immuno-staining of the cells with antibodies of DMT1 and TGN46. (d) Colocalization efficiency between DMT1 and LAMP2 (n = 9: 9 images for each condition were analyzed). Statistical analyses were performed with Student's *t*-test. ***p*<0.005. Error bars indicate ± SEM. Scale bars indicate 10 μ m.



Figure S11. Fluorescence imaging study of organelle-specific sensitivity against exogenous Fe(II) ion. For the images, local labile Fe(II) levels at Golgi (Gol-SiRhoNox) and lysosome (LysoRhoNox) are indicated by

magenta and yellow, respectively. GFP (green) is an indicator of the efficiency of siRNA transfection (knockdown of VPS35). Blue color indicate nuclear stained by Hoechst 33342. (a–d) Images of wild-type (a and c) and VPS35 KD (b and d) SH-SY5Y cells without (a and b) and with (c and d) supplementation of 100 μ M Fe(II) ion (Fe(NH₄)₂(SO₄)₂•6H₂O was used as an iron source) stained with Gol-SiRhoNox. (e) Quantification data in (a–d). (f–i) Images of wild-type (f and h) and VPS35 KD (g and i) SH-SY5Y cells without (f and g) and with (h and i) supplementation of 100 μ M Fe(II) ion (Fe(NH₄)₂(SO₄)₂•6H₂O was used as an iron source) stained with Col-SiRhoNox. (e) Quantification data in (a–d). (f–i) Images of wild-type (f and h) and VPS35 KD (g and i) SH-SY5Y cells without (f and g) and with (h and i) supplementation of 100 μ M Fe(II) ion (Fe(NH₄)₂(SO₄)₂•6H₂O was used as an iron source) stained with LysoRhoNox. (j) Quantification data in (f–i). Statistical analyses were performed with Student's *t*-test. **p*<0.05, ***p*<0.01 (n = 4: 4 images for each condition were analyzed). Error bars indicate ± SEM. Scale bars indicate 10 μ m.



Figure S12. Fluorescence imaging study of organelle-specific sensitivity against exogenous Fe(II) ion in SH-SY5Y cells overexpressing mutant VPS35 (D620N). . For the images, local labile Fe(II) levels at Golgi (Gol-SiRhoNox) and lysosome (LysoRhoNox) are indicated by magenta and yellow, respectively. Blue color indicate nuclear stained by Hoechst 33342. (a–d) Images of wild-type (a and c) and VPS35(D620N) (b and d) SH-SY5Y cells without (a and b) and with (c and d) supplementation of 100 μ M Fe(II) ion (Fe(NH₄)₂(SO₄)₂•6H₂O was used as an iron source) stained with Gol-SiRhoNox. (e) Quantification data in (a–d). (f–i) Images of wild-type (a and c) and VPS35(D620N) (b and d) supplementation of 100 μ M Fe(II) ion (ferrous ammonium sulfate hexahydrate was used as an iron source) stained with LysoRhoNox. (j) Quantification data in (f–i). Statistical analyses were performed with Student's *t*-test. **p*<0.01 (n = 4: 4 images for each condition were analyzed). Error bars indicate ± SEM. Scale bars indicate 10 μ m.



Figure S13. Fluorescence imaging study of organelle-specific fluorescent compounds.. For the images, GFP (green) is an indicator of the efficiency of siRNA transfection (knockdown of VPS35). Blue color indicate nuclear stained by Hoechst 33342. (a–d) Images of wild-type (a and c) and VPS35 KD (b and d) SH-SY5Y cells stained with Gol-SiR (a and b, magenta) and LysoTrackerTM Deep-Red (c and d, yellow). (E)

Quantification data in (a and b). (F) Quantification data in (c and d). Statistical analyses were performed with Student's *t*-test (n = 30: 30 images for each condition were analyzed). Error bars indicate \pm SEM. Scale bars indicate 10 µm.



Figure S14. Quantification of total iron contents in VPS35 wild-type and VPS35 KD cells by atomic absorption. No significant change was observed. Statistical analysis was performed with Student's *t*-test (n = 3).



Figure S15. (a) Western blot analysis of α -synuclein expression level in VPS35 KD cells. SH-SY5Y cells overexpressing α -synuclein with flag tag at C-terminal were treated with microRNA to down-regulate VPS35 protein in the absence or presence of R55 (5 μ M, 48 h). (b) Quantification of (a). Statistical analysis was performed with Student's *t*-test. **p*<0.05 (n = 3). Error bars indicate ± SEM.

¹H- and ¹³C-NMR spectra of the newly synthesized compounds Gol-SiR (CDCl₃)



Gol-SiRhoNox (CDCl₃)

