Electronic Supplementary Material (ESI) for Chemical Communications.

Supplementary Information

Protein-recruiting synthetic molecules targeting the Golgi surface

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Fubito Nakatsu and Shinya Tsukiji*

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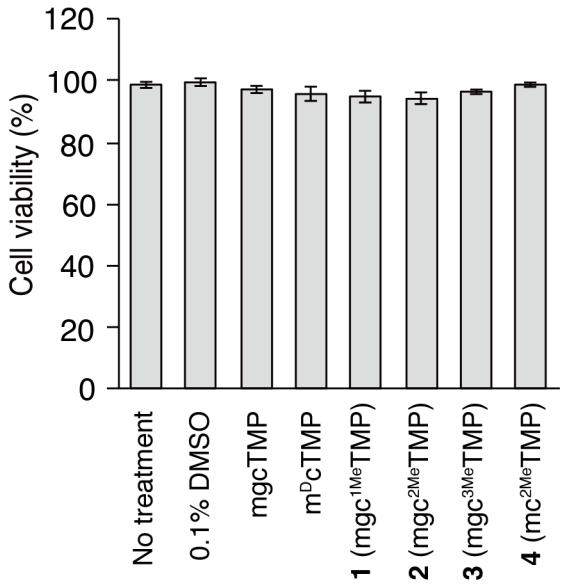

Fig. S1 Cytotoxicity assays. HeLa cells expressing eDHFR-EGFP were left untreated or incubated with DMSO (vehicle) or the indicated compound (10 μM) for 1 h. The cells were then assayed for viability by the propidium iodide exclusion assay. Data are presented as the mean ± SD of at least three independent experiments (for one experiment, n > 150 cells).

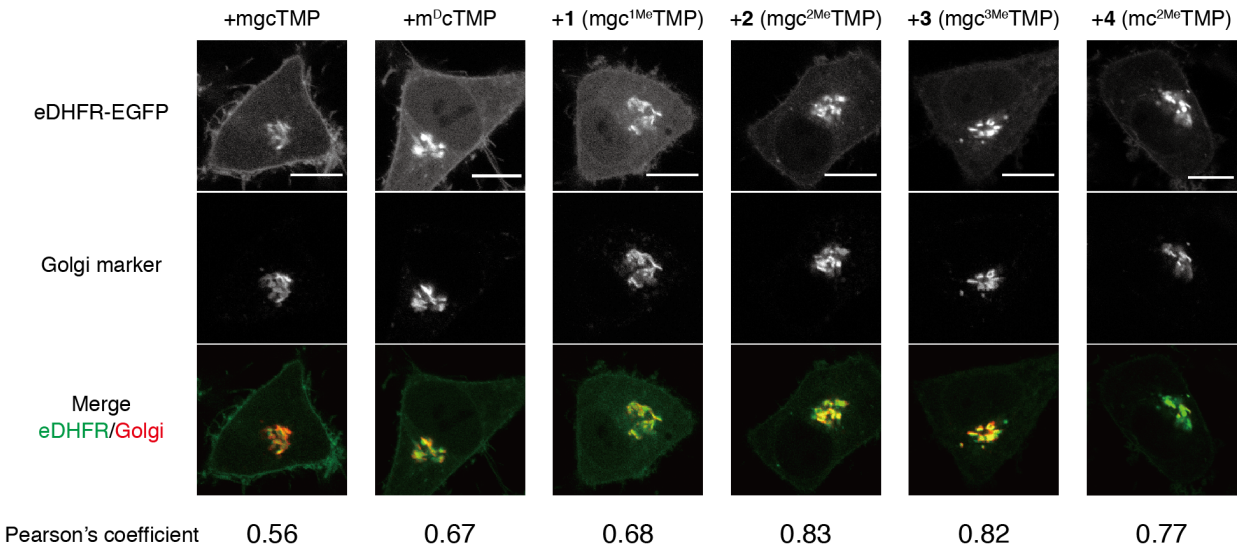

Fig. S2 Colocalization analysis of eDHFR-EGFP translocation sites. Confocal fluorescence images of HeLa cells coexpressing eDHFR-EGFP and mCherry-giantin (Golgi marker)[S1] were taken after treatment with the indicated compound (10 μM) for 10 min. Pearson's correlation coefficients were determined to evaluate the Golgi-targeting specificity of the SLs. Scale bars, 10 μm.

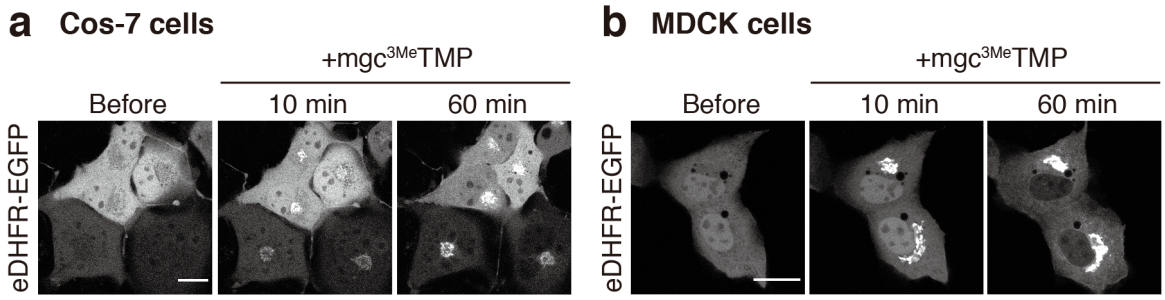

Fig. S3 mgc^{3Me}TMP-induced eDHFR-EGFP translocation in other cell lines. (**a**) Cos-7 cells. (**b**) MDCK cells. Confocal fluorescence images of cells expressing eDHFR-EGFP were taken before (left), and 10 min (center) and 60 min (right) after the addition of 10 μM mgc^{3Me}TMP. Scale bars, 10 μm.

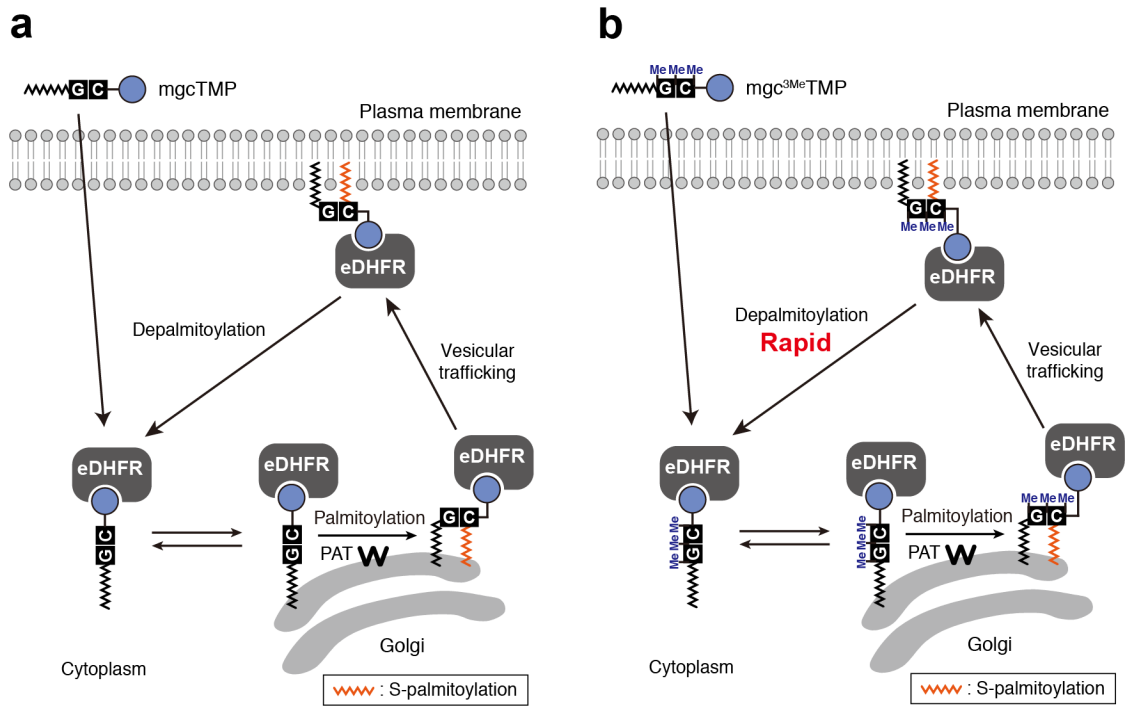

Fig. S4 Palmitoylation/depalmitoylation-dependent localization of mgcTMP and mgc^{3Me}TMP. (**a**) Schematic illustration of mgcTMP-induced eDHFR translocation. (**b**) Schematic illustration of the proposed mechanism for the Golgi-targeted eDHFR recruitment by mgc^{3Me}TMP. Enhanced Golgi-targeting specificity of mgc^{3Me}TMP over mgcTMP is considered to result from increased depalmitoylation kinetics of the myrGC^{3Me} motif.

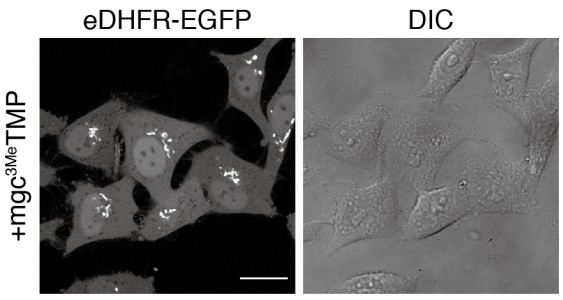

Fig. S5 SLIPT assay at 4 °C. Confocal fluorescence and differential interference contrast (DIC) images of HeLa cells expressing eDHFR-EGFP were taken after 10 min of incubation with 10 μM mgc^{3Me}TMP at 4 °C (on ice). Scale bar, 20 μm.

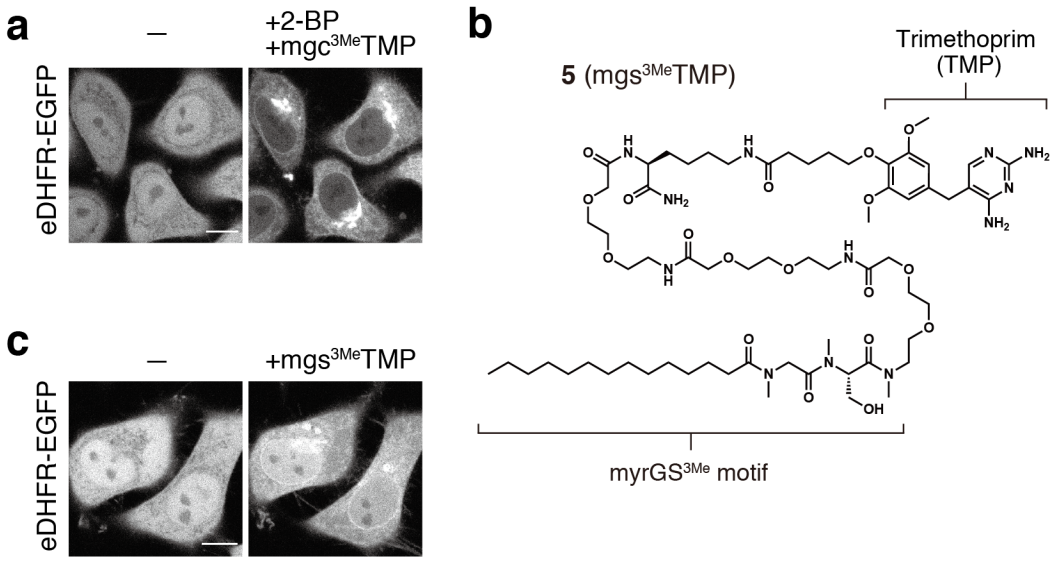

Fig. S6 The Golgi localization of mgc^{3Me}TMP is controlled by *S*-palmitoylation of the Cys residue. (**a**) Confocal fluorescence images of HeLa cells expressing eDHFR-EGFP were taken before (left) and 15 min (right) after incubation with 10 μM mgc^{3Me}TMP in the presence of 100 μM 2-BP. Scale bar, 10 μm. (**b**) Chemical structure of compound **5** (mgs^{3Me}TMP). (**c**) Confocal fluorescence images of HeLa cells expressing eDHFR-EGFP were taken before (left) and 5 min (right) after incubation with 10 μM mgs^{3Me}TMP. Scale bar, 10 μm.

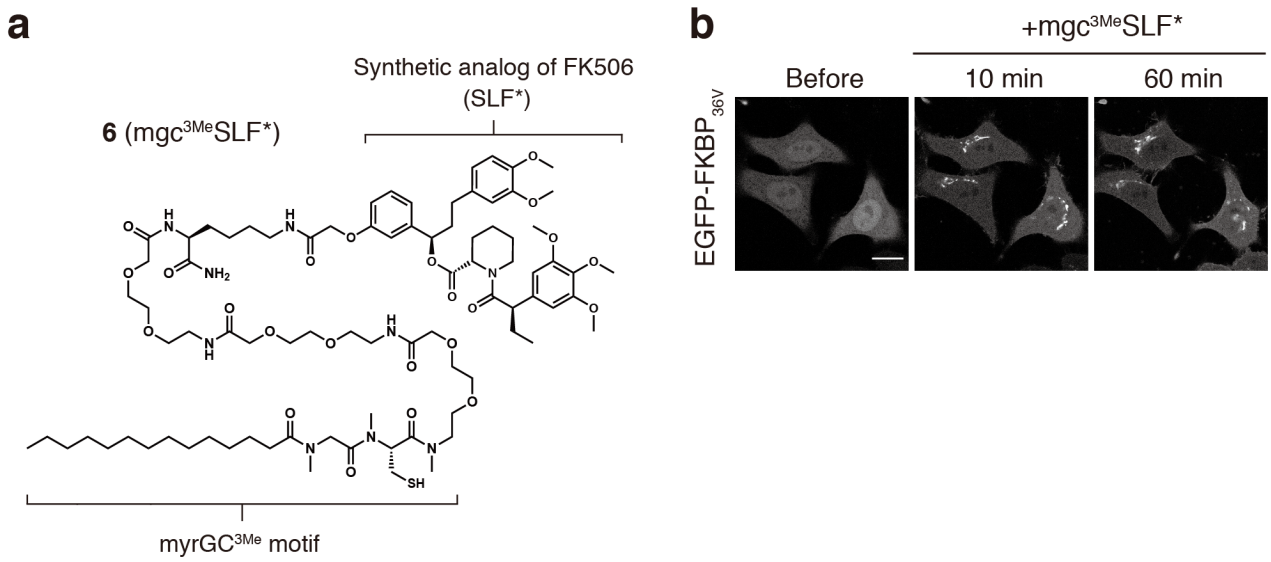

Fig. S7 EGFP-FKBP$_{36V}$ translocation using mgc^{3Me}SLF*. (**a**) Chemical structure of compound **6** (mgc^{3Me}SLF*). (**b**) Confocal fluorescence images of HeLa cells expressing EGFP-FKBP$_{36V}$ were taken before (left), and 10 min (center) and 60 min (right) after the addition of 10 μM mgc^{3Me}SLF*. Scale bar, 10 μm.

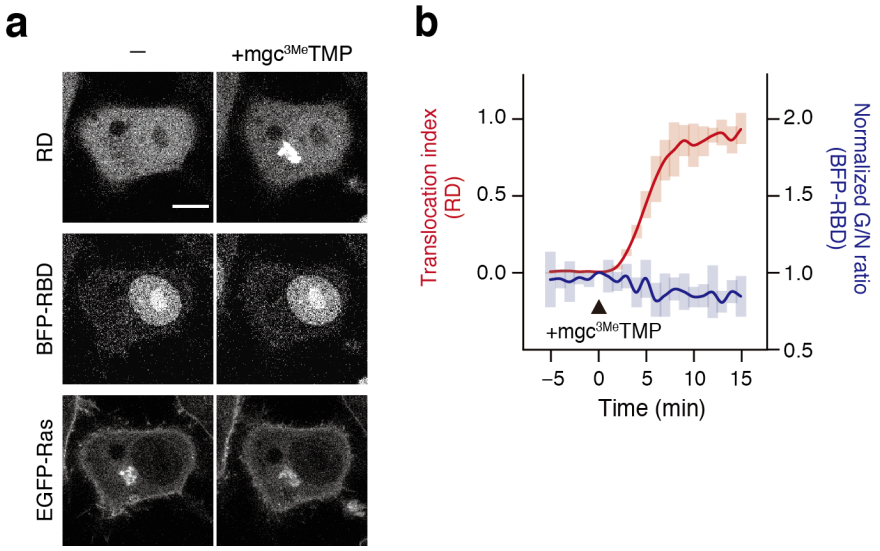

Fig. S8 Control experiment for synthetic Golgi Ras activation. (**a**) Confocal fluorescence images of HeLa cells coexpressing mCherry-eDHFR lacking the RasGEF domain (RD), EGFP-NRas, and BFP-RBD were taken before (left) and 15 min (right) after the addition of 2.5 μM mgc^{3Me}TMP. Scale bar, 10 μm. (**b**) Time course of RD translocation and Golgi Ras activation. Data were analyzed as described in the legend in **Fig. 3c** and are presented as the mean ± SD (n = 5 cells).

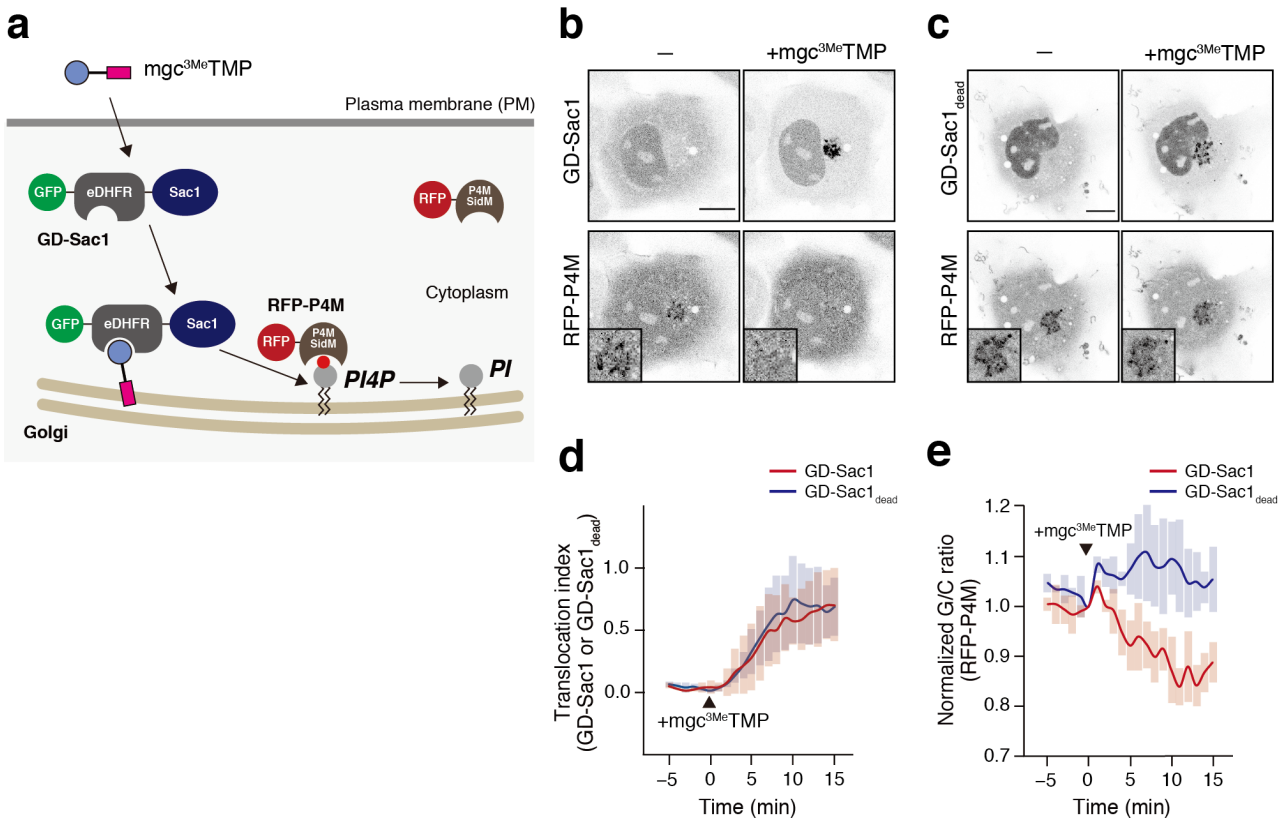

Fig. S9 Golgi PI4P depletion. (**a**) Schematic illustration of the experimental set-up for Golgi PI4P depletion. (**b**) Golgi recruitment of EGFP-eDHFR-Sac1 (GD-Sac1). Confocal fluorescence (black–white inverted) images of Cos-7 cells coexpressing GD-Sac1 and mCherry-P4M-SidM (RFP-P4M)[S2] were taken before (left) and 10 min (right) after the addition of 10 μM mgc^{3Me}TMP. (**c**) Golgi recruitment of EGFP-eDHFR-Sac1$_{dead}$ (GD-Sac1$_{dead}$) (control experiment for **b**). Confocal fluorescence images of Cos-7 cells coexpressing GD-Sac1$_{dead}$ and RFP-P4M were taken before (left) and 10 min (right) after the addition of 10 μM mgc^{3Me}TMP. (**d**) Time course of GD-Sac1/GD-Sac1$_{dead}$ translocation. To evaluate GD-Sac1 (red line) and GD-Sac1$_{dead}$ (blue line) recruitment, normalized ratios of the Golgi to the cytoplasmic fluorescence intensity of GD-Sac1/GD-Sac1$_{dead}$ were plotted as a function of time. (**e**) Time course of Golgi PI4P depletion. To evaluate Golgi PI4P depletion, normalized G/C ratios of RFP-P4M were plotted as a function of time. Data are presented as the mean ± SD (n = 3 cells). Scale bars, 15 μm.

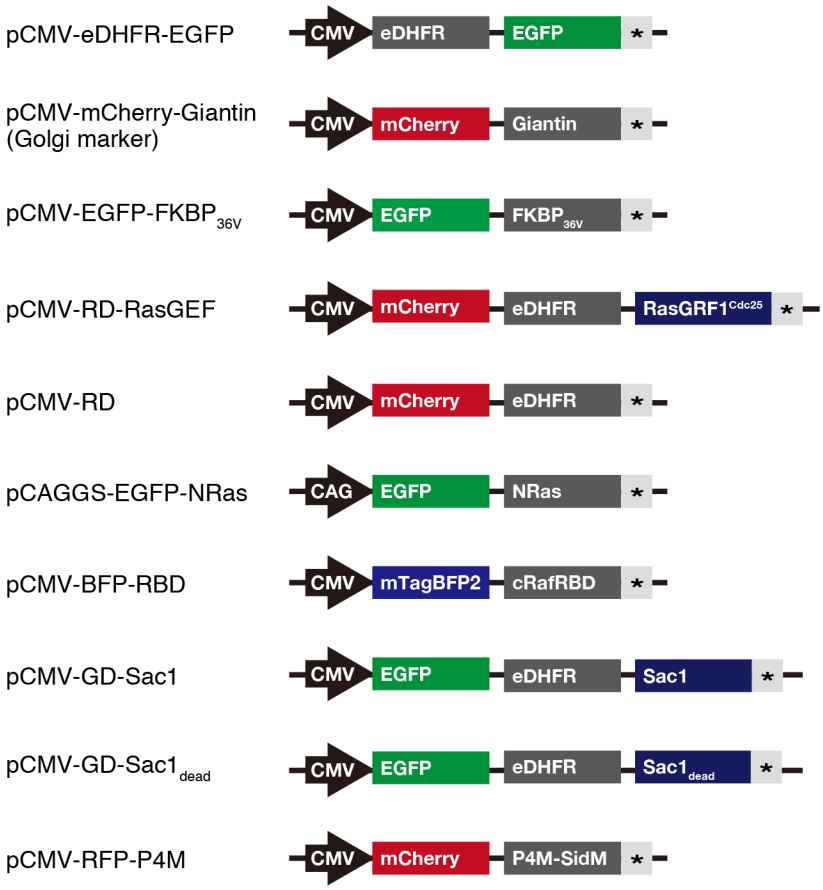

Fig. S10 Schematic illustration of the domain structures of constructs used in this study.

a

pCMV-eDHFR-EGFP

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pCMV-mCherry-Giantin

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pCMV-EGFP-FKBP$_{36V}$

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pCMV-RD

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pCAGGS-EGFP-NRas

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g

pCMV-BFP-RBD

>Amino acid sequence
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h

pCMV-GD-Sac1

>Amino acid sequence

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i

pCMV-GD-Sac1$_{dead}$

>Amino acid sequence

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j

pCMV-mCherry-P4M-SidM

>Amino acid sequence
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Fig. S11 DNA and amino acid sequences of constructs used in this study. (**a**) pCMV-eDHFR-EGFP: grey, eDHFR; green, EGFP. (**b**) pCMV-mCherry-Giantin: red, mCherry; grey, the Golgi targeting motif of human giantin (residues 3131–3259). (**c**) pCMV-EGFP-FKBP$_{36V}$: green, EGFP ; grey, FKBP$_{36V}$. (**d**) pCMV-RD-RasGEF: red, mCherry; grey, eDHFR; purple, the RasGEF domain of human RasGRF1 (residues 1018-1273). (**e**) pCMV-RD: red, mCherry; grey, eDHFR. (**f**) pCAGGS-EGFP-NRas: green, EGFP; grey, NRas. (**g**) pCMV-BFP-RBD: blue, mTagBFP2; grey, the Ras-binding domain of human cRaf (residues 51-131). (**h**) pCMV-GD-Sac1: green, EGFP; grey, eDHFR; purple, the catalytic domain of *S. cerevisiae* Sac1 (residues 2–517). (**i**) pCMV-GD-Sac1$_{dead}$: green, EGFP; grey, eDHFR; purple, Sac1$_{dead}$, the Sac1 domain containing an inactivating C392S mutation. (**j**) pCMV-mCherry-P4M-SidM: red, mCherry; grey, the P4M domain of *L. pneumophila* SidM (residues 546–647).

Note: In SLIPT-based signal control systems, the effector domain fused to the C-terminus of eDHFR needs to access its downstream target on the Golgi surface when it is anchored on the Golgi membrane by mgc^{3Me}TMP. Therefore, in the design of RD-RasGEF and GD-Sac1, relatively long flexible linkers (consisting of 28 and 76 amino acids, respectively) were used to connect eDHFR and the effector domain to ensure accessibility.

Supplementary Movies

Movie S1 mgc^{3Me}TMP (10 μM)-induced translocation of eDHFR-EGFP in HeLa cells. Scale bar, 20 μm.

Movie S2 Golgi Ras activation by mgc^{3Me}TMP-induced Golgi recruitment of RD-RasGEF (time-lapse movie of **Fig. 3b**). Scale bar, 10 μm.

Movie S3 Golgi PI4P depletion by mgc^{3Me}TMP-induced Golgi recruitment of GD-Sac1 (time-lapse movie of **Fig. S9b**). Scale bar, 15 μm.

Supplementary Methods: Chemical Synthesis

General materials and methods

All chemical reagents and solvents were purchased from commercial suppliers (Watanabe Chemical Industries, Tokyo Chemical Industry, FUJIFILM Wako Pure Chemical Corp., and Kanto Chemical) and used without further purification. Reverse-phase HPLC was performed on a Hitachi LaChrom Elite system with UV detection at 220 nm using a YMC-Pack ODS-A column (10 × 250 mm or 20 × 250 mm). ^{1}H NMR spectra were recorded on a Bruker AVANCE III HD400SJ (400 MHz) spectrometer. ^{1}H NMR chemical shifts were referenced to tetramethylsilane (0 ppm). High-resolution mass spectra were measured on a Thermo Scientific Extractive Plus Orbitrap mass spectrometer.

Reagent abbreviations

DBU: 1,8-diazabicyclo[5.4.0]undec-7-ene
DIPEA: *N*,*N*-diisopropylethylamine
DMF: *N*,*N*-dimethylformamide
EDT: 1,2-ethanedithiol
Fmoc-Adox-OH: Fmoc-8-amino-3,6-dioxaoctanoic acid
HATU:*O*-(7-aza-1H-benzotriazol-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium hexafluorophosphate
HBTU: *O*-(benzotriazole-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium hexafluorophosphate
HOAt: 1-hydroxy-7-azabenzotriazole
HOBt: 1-hydroxybenzotriazole (monohydrate)
NMP: *N*-methyl-2-pyrrolidone
o-Ns-Cl: 2-nitrobenzenesulfonyl chloride
TFA: trifluoroacetic acid
TIPS: triisopropylsilane

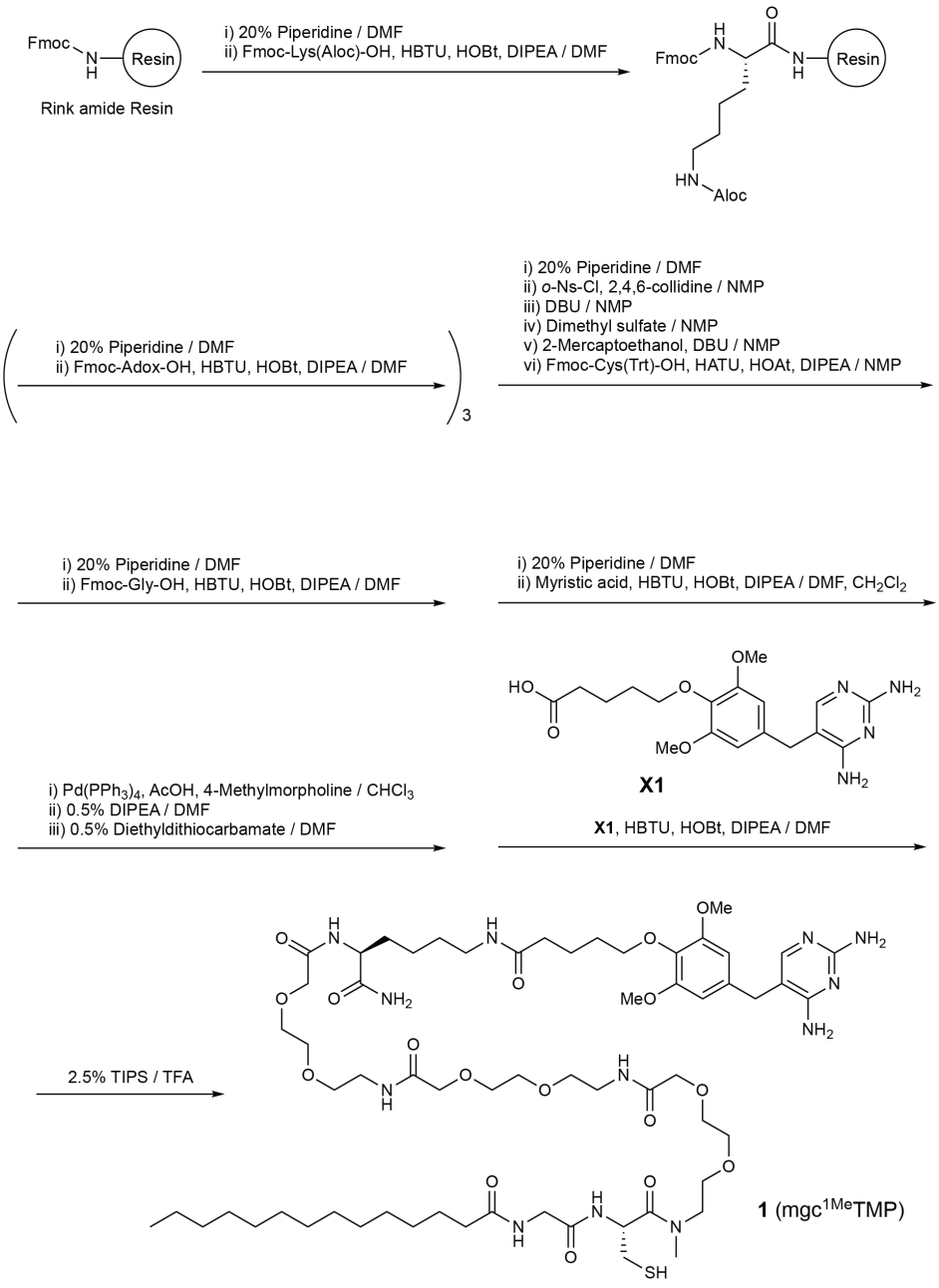
General methods for solid-phase synthesis

Compounds **1**–**6** were synthesized manually on Rink Amide resin or Sieber Amide resin by standard Fmoc-based solid-phase peptide synthesis protocols. Fmoc deprotection was performed with 20% piperidine in DMF at room temperature for 15 min. Amino acid coupling reactions were performed at room temperature with a mixture of Fmoc-protected

amino acid (3.1 eq.), HBTU (3.0 eq.), HOBt (3.0 eq.), and DIPEA (6.0 eq.) in DMF. All standard Fmoc deprotection and coupling reactions were monitored by the Kaiser test.[S3] Unless otherwise stated, all washing procedures were performed with DMF. Compounds **X1** (TMP-COOH)[S4] and **X2** (SLF*-COOH)[S5] were synthesized as described previously.

N-Methylation and subsequent amino acid coupling reactions were performed at room temperature as follows:[S6] Following Fmoc deprotection, the resin was treated with a mixture of *o*-Ns-Cl (4.0 eq.) and 2,4,6-collidine (10.0 eq.) in NMP for 15 min. After washing the resin with NMP, the resin was treated with DBU (3.0 eq.) in NMP for 3 min. After removing the DBU solution, the resin was treated with dimethyl sulfate (10.0 eq.) in NMP for 2 min twice. After washing the resin with NMP, the resin was treated with a mixture of 2-mercaptoethanol (10.0 eq.) and DBU (5.0 eq.) in NMP for 5 min twice, yielding the *N*-methylated *N*-terminus. Subsequent amino acid coupling reactions were performed with a mixture of Fmoc-protected amino acid (4.1 eq.), HATU (4.0 eq.), HOAt (4.0 eq.), and DIPEA (8.0 eq.) in NMP. Amino acid coupling reactions were monitored by the chloranil test.[S7]

Synthesis of Compound 1 (mgc^{1Me}TMP)



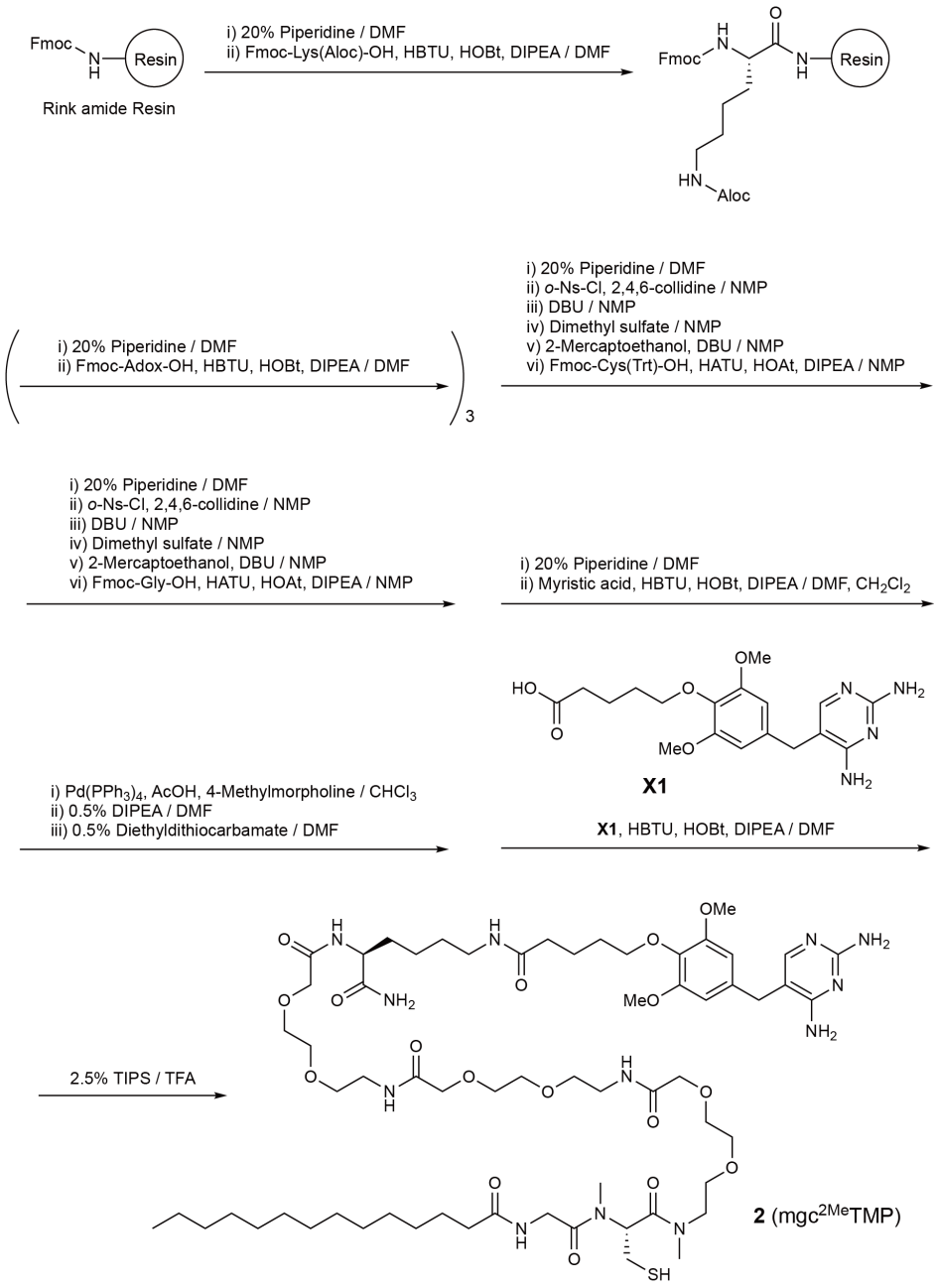
Scheme S1 Synthetic route of **1**

Compound **1** was synthesized manually on Rink amide resin (0.55 mmol/g) (36.3 mg, 20 μmol). First, Fmoc-Lys(Aloc)-OH and Fmoc-Adox-OH (×3) were coupled to the resin. The *N*-methylation of the Adox residue and subsequent Fmoc-Cys(Trt)-OH coupling were performed as described in "**General methods for solid-phase synthesis**". Fmoc-Gly-OH was then coupled to the resin. The *N*-terminus was myristoylated using a mixture

of myristic acid (3.1 eq.), HBTU (3.0 eq.), HOBt (3.0 eq.), and DIPEA (6.0 eq.) in DMF/CH_2Cl_2 (1/1). After washing the resin with DMF, MeOH, and CH_2Cl_2, it was dried *in vacuo*. The Aloc group of the lysine residue was deprotected by treatment with $CHCl_3$ containing $Pd(PPh_3)_4$ (3.0 eq.), 5.0% acetic acid, and 2.5% 4-methylmorpholine under an Ar atmosphere. The resin was washed with DMF containing 0.5% DIPEA (×5), DMF containing 0.5% sodium diethyldithiocarbamate (×5), and DMF only (×5). Compound **X1** (TMP-COOH) was coupled to the side chain of the lysine residue with a mixture of **X1** (3.1 eq.), HBTU (3.0 eq.), HOBt (3.0 eq.), and DIPEA (6.0 eq.) in DMF. After washing the resin with DMF, MeOH, and CH_2Cl_2, it was dried *in vacuo*. Deprotection and cleavage from the resin were performed with TFA containing 2.5% TIPS. The crude product was purified by reversed-phase HPLC using a semi-preparative C18 column (a linear gradient of MeCN containing 0.1% TFA and 0.1% aqueous TFA) to afford **1** as a white solid [8.5 mg, 42% (as a mono-TFA salt)].

^{1}H NMR (400 MHz, CD_3OD): δ 7.21 (1H, s), 6.56 (2H, s), 5.12–4.96 (1H, m), 4.42 (1H, m), 4.03 (2H, s), 4.00 (4H, s), 3.91 (2H, t, J = 6.0 Hz), 3.86 (2H, s, s), 3.80 (6H, s), 3.66 (14H, m), 3.60 (6H, m), 3.46 (6H, m), 3.23–2.96 (5H, m), 2.88–2.72 (2H, m), 2.25 (4H, m), 1.80 (2H, m), 1.72 (2H, m), 1.61 (2H, m), 1.53 (2H, m), 1.40 (2H, m), 1.28 (22H, m), 0.89 (3H, t, J = 6.0 Hz).
HRMS (ESI): calculated for $[M+H]^+$, 1323.7592; found, 1323.7628.

Synthesis of Compound 2 (mgc^{2Me}TMP)



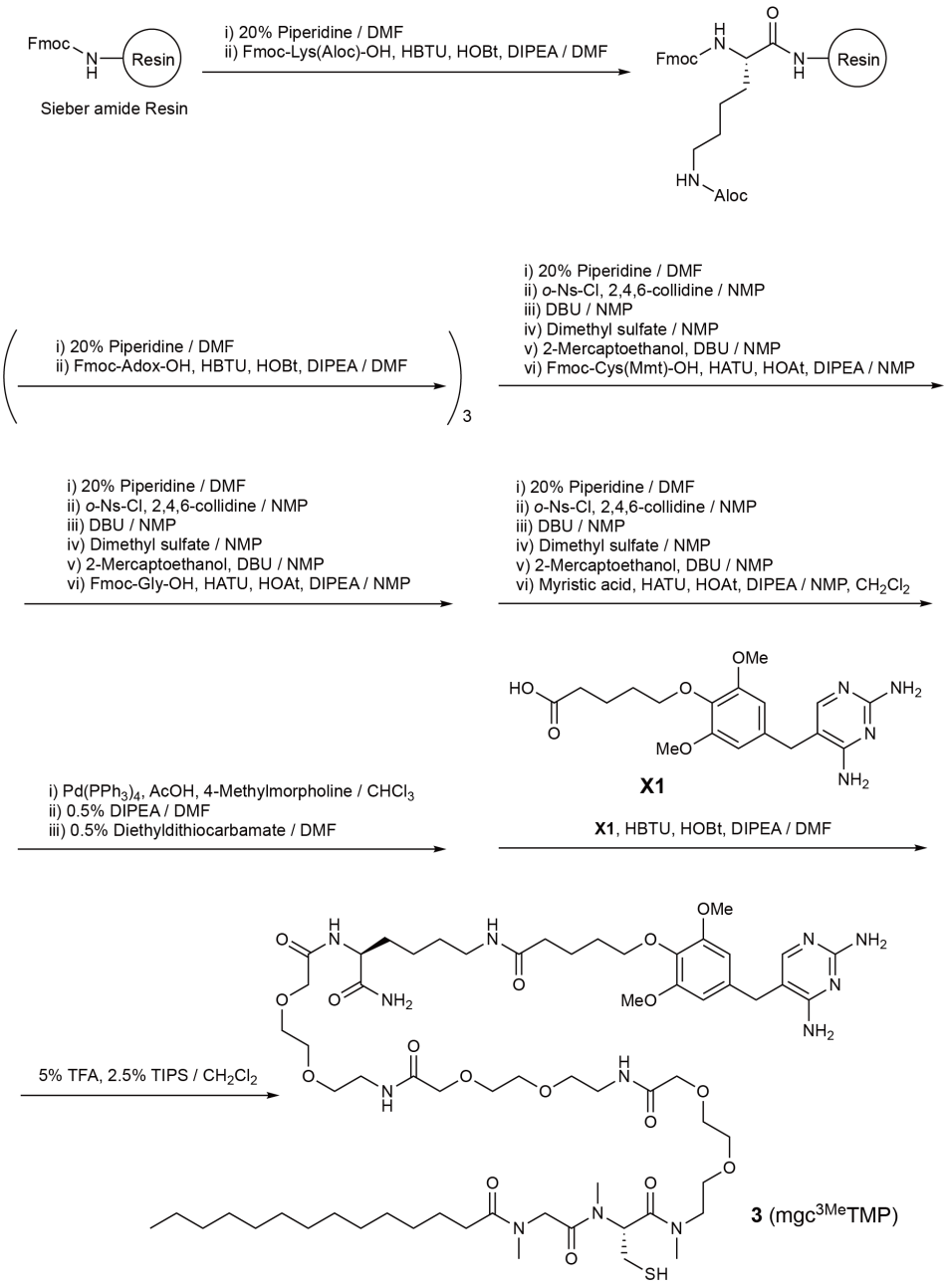
Scheme S2 Synthetic route of **2**

Compound **2** was synthesized manually on Rink amide resin (0.58 mmol/g) (34.5 mg, 20 μmol). First, Fmoc-Lys(Aloc)-OH and Fmoc-Adox-OH (×3) were coupled to the resin. The *N*-methylation and coupling reactions of Fmoc-Cys(Trt)-OH and Fmoc-Gly-OH were performed as described in "**General methods for solid-phase synthesis**". The *N*-terminus was myristoylated using a mixture of myristic acid (3.1 eq.), HBTU (3.0 eq.),

HOBt (3.0 eq.), and DIPEA (6.0 eq.) in DMF/CH_2Cl_2 (1/1). After washing the resin with DMF, MeOH, and CH_2Cl_2, it was dried *in vacuo*. The Aloc group of the lysine residue was deprotected by treatment with $CHCl_3$ containing $Pd(PPh_3)_4$ (3.0 eq.), 5.0% acetic acid, and 2.5% 4-methylmorpholine under an Ar atmosphere. The resin was washed with DMF containing 0.5% DIPEA (×5), DMF containing 0.5% sodium diethyldithiocarbamate (×5), and DMF only (×5). Compound **X1** (TMP-COOH) was coupled to the side chain of the lysine residue with a mixture of **X1** (3.1 eq.), HBTU (3.0 eq.), HOBt (3.0 eq.), and DIPEA (6.0 eq.) in DMF. After washing the resin with DMF, MeOH, and CH_2Cl_2, it was dried *in vacuo*. Deprotection and cleavage from the resin were performed with TFA containing 2.5% TIPS. The crude product was purified by reversed-phase HPLC using a semi-preparative C18 column (a linear gradient of MeCN containing 0.1% TFA and 0.1% aqueous TFA) to afford **2** as a white solid [6.4 mg, 22% (as a mono-TFA salt)].

^{1}H NMR (400 MHz, CD_3OD): δ 7.21 (1H, s), 6.56 (2H, s), 5.62–5.40 (1H, m), 4.41 (1H, m), 4.03 (2H, s), 4.00 (4H, s), 3.91 (2H, t, J = 6.0 Hz), 3.80 (6H, s), 3.66 (16H, m), 3.60 (6H, m), 3.46 (6H, m), 3.17 (2H, t, J = 6.6 Hz), 3.04–2.90 (6H, m), 2.68–2.56 (2H, m), 2.27 (4H, m), 1.80 (2H, m), 1.72 (2H, m), 1.62 (2H, m), 1.53 (2H, m), 1.40 (2H, m), 1.28 (22H, m), 0.90 (3H, t, J = 6.0 Hz).
HRMS (ESI): calculated for $[M+H]^+$, 1337.7749; found, 1337.7767.

Synthesis of Compound 3 (mgc^{3Me}TMP)



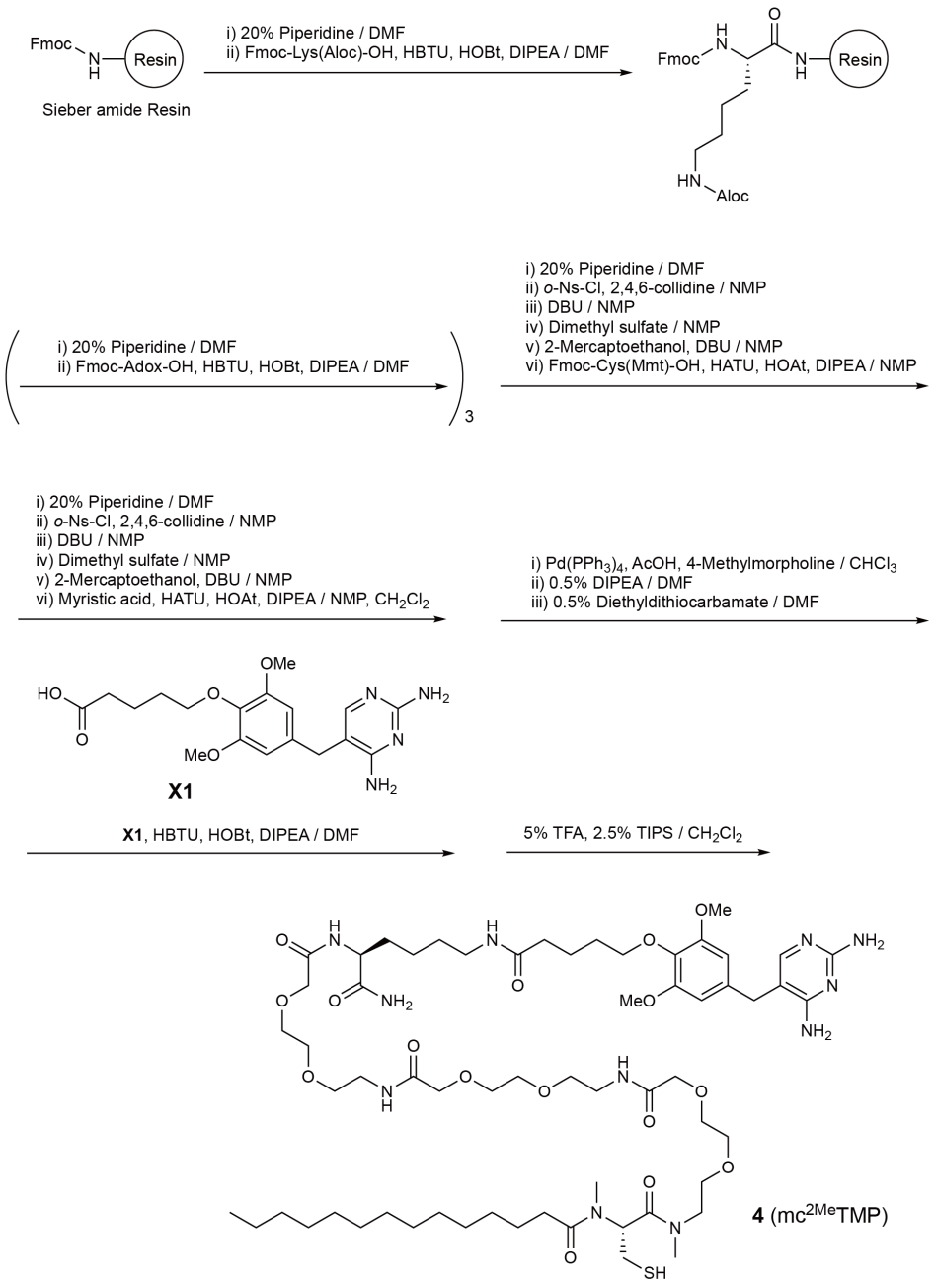
Scheme S3 Synthetic route of **3**

Compound **3** was synthesized manually on Sieber amide resin (0.79 mmol/g) (38.0 mg, 30 μmol). First, Fmoc-Lys(Aloc)-OH and Fmoc-Adox-OH (×3) were coupled to the resin. The *N*-methylation and coupling reactions of Fmoc-Cys(Mmt)-OH and Fmoc-Gly-OH were performed as described in "**General methods for solid-phase synthesis**". The *N*-terminus was myristoylated using a mixture of myristic acid (3.1 eq.), HATU (3.0 eq.),

HOAt (3.0 eq.), and DIPEA (6.0 eq.) in NMP/CH_2Cl_2. After washing the resin with NMP, MeOH, and CH_2Cl_2, it was dried *in vacuo*. The Aloc group of the lysine residue was deprotected by treatment with $CHCl_3$ containing $Pd(PPh_3)_4$ (3.0 eq.), 5.0% acetic acid, and 2.5% 4-methylmorpholine under an Ar atmosphere. The resin was washed with DMF containing 0.5% DIPEA (×5), DMF containing 0.5% sodium diethyldithiocarbamate (×5), and DMF only (×5). Compound **X1** (TMP-COOH) was coupled to the side chain of the lysine residue with a mixture of **X1** (3.1 eq.), HBTU (3.0 eq.), HOBt (3.0 eq.), and DIPEA (6.0 eq.) in DMF. After washing the resin with DMF, MeOH, and CH_2Cl_2, it was dried *in vacuo*. Deprotection and cleavage from the resin were performed with CH_2Cl_2 containing 5% TFA and 2.5% TIPS. The crude product was purified by reversed-phase HPLC using a semi-preparative C18 column (a linear gradient of MeCN containing 0.1% TFA and 0.1% aqueous TFA) to afford **3** as a white solid [4.9 mg, 11% (as a mono-TFA salt)].

^{1}H NMR (400 MHz, CD_3OD): δ 7.21 (1H, s), 6.56 (2H, s), 5.66–5.44 (1H, m), 4.41 (1H, m), 4.03 (2H, s), 3.99 (4H, s), 3.91 (2H, t, J = 6.0 Hz), 3.80 (6H, s), 3.66 (16H, m), 3.60 (6H, m), 3.45 (6H, m), 3.17 (2H, t, J = 6.6 Hz), 3.10–2.89 (9H, m), 2.76–2.60 (2H, m), 2.44 (2H, m), 2.25 (2H, m), 1.80 (2H, m), 1.70 (2H, m), 1.61 (2H, m), 1.53 (2H, m), 1.38 (2H, m), 1.28 (22H, m), 0.90 (3H, t, J = 6.0 Hz).
HRMS (ESI): calculated for $[M+H]^+$, 1373.7724; found, 1373.7708.

Synthesis of Compound 4 (mc^{2Me}TMP)



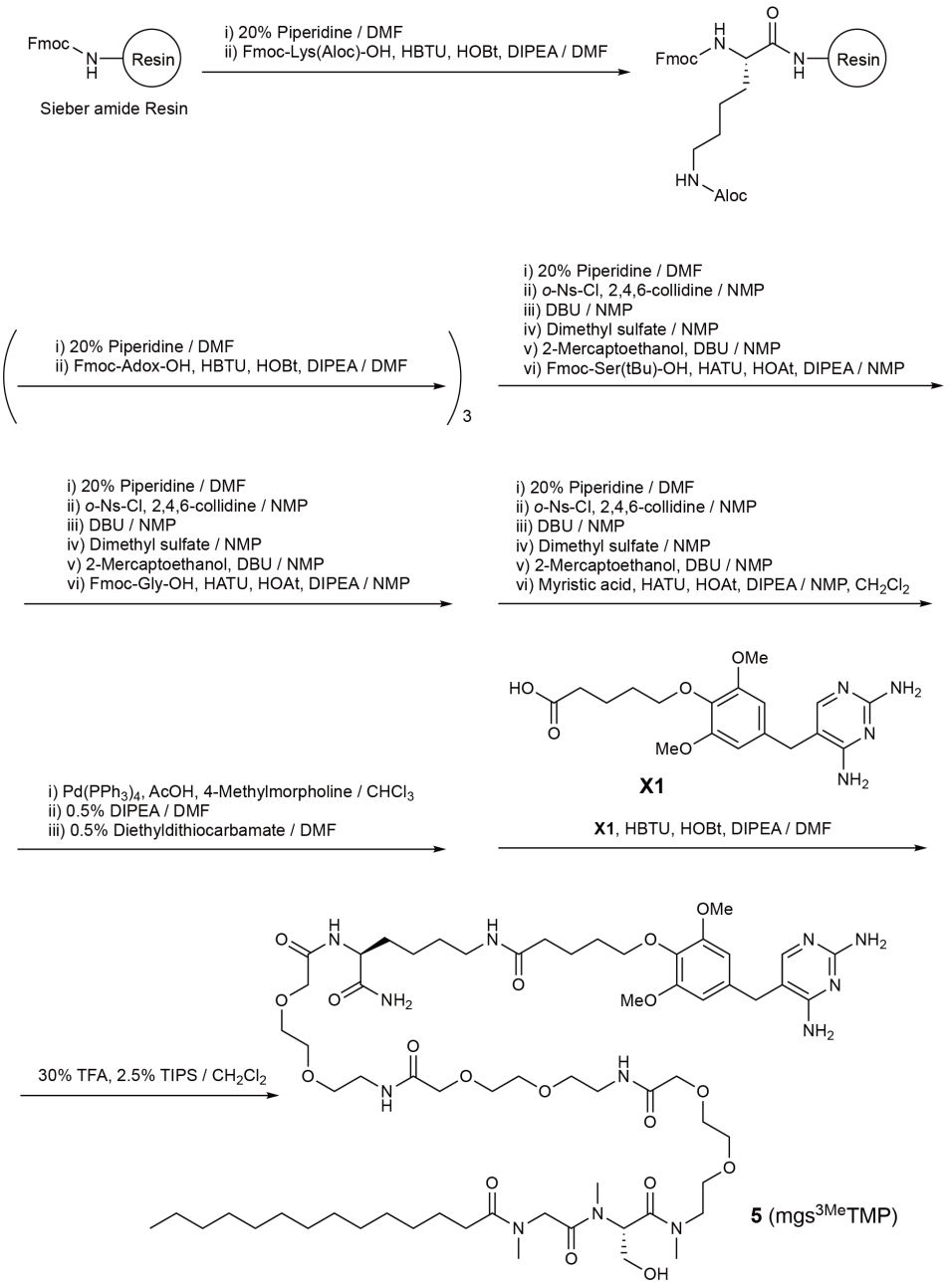
Scheme S4 Synthetic route of Compound **4**

Compound **4** was synthesized manually on Sieber amide resin (0.79 mmol/g) (25.3 mg, 20 μmol). First, Fmoc-Lys(Aloc)-OH and Fmoc-Adox-OH (×3) were coupled to the resin. The *N*-methylation and coupling reactions of Fmoc-Cys(Mmt)-OH were performed as described in "**General methods for solid-phase synthesis**". The *N*-terminus was myristoylated using a mixture of myristic acid (3.1 eq.), HATU (3.0 eq.), HOAt (3.0 eq.),

and DIPEA (6.0 eq.) in NMP/CH_2Cl_2. After washing the resin with NMP, MeOH, and CH_2Cl_2, it was dried *in vacuo*. The Aloc group of the lysine residue was deprotected by treatment with $CHCl_3$ containing $Pd(PPh_3)_4$ (3.0 eq.), 5.0% acetic acid, and 2.5% 4-methylmorpholine under an Ar atmosphere. The resin was washed with DMF containing 0.5% DIPEA (×5), DMF containing 0.5% sodium diethyldithiocarbamate (×5), and DMF only (×5). Compound **X1** (TMP-COOH) was coupled to the side chain of the lysine residue with a mixture of **X1** (3.1 eq.), HBTU (3.0 eq.), HOBt (3.0 eq.), and DIPEA (6.0 eq.) in DMF. After washing the resin with DMF, MeOH, and CH_2Cl_2, it was dried *in vacuo*. Deprotection and cleavage from the resin were performed with CH_2Cl_2 containing 5% TFA and 2.5% TIPS. The crude product was purified by reversed-phase HPLC using a semi-preparative C18 column (a linear gradient of MeCN containing 0.1% TFA and 0.1% aqueous TFA) to afford **4** as a white solid [3.2 mg, 8% (as a mono-TFA salt)].

^{1}H NMR (400 MHz, CD_3OD): δ 7.22 (1H, s), 6.56 (2H, s), 5.66–5.44 (1H, m), 4.41 (1H, m), 4.03 (2H, s), 3.99 (4H, s), 3.93 (2H, t, J = 6.0 Hz), 3.80 (6H, s), 3.66 (16H, m), 3.60 (6H, m), 3.46 (6H, m), 3.36–2.90 (6H, m), 2.68–2.54 (2H, m), 2.42 (2H, m), 2.25 (2H, m), 1.80 (2H, m), 1.72 (2H, m), 1.61 (2H, m), 1.53 (2H, m), 1.38 (2H, m), 1.28 (22H, m), 0.90 (3H, t, J = 6.0 Hz).
HRMS (ESI): calculated for $[M+2H]^{2+}$, 640.8803; found, 640.8787.

Synthesis of Compound 5 (mgs^{3Me}TMP)



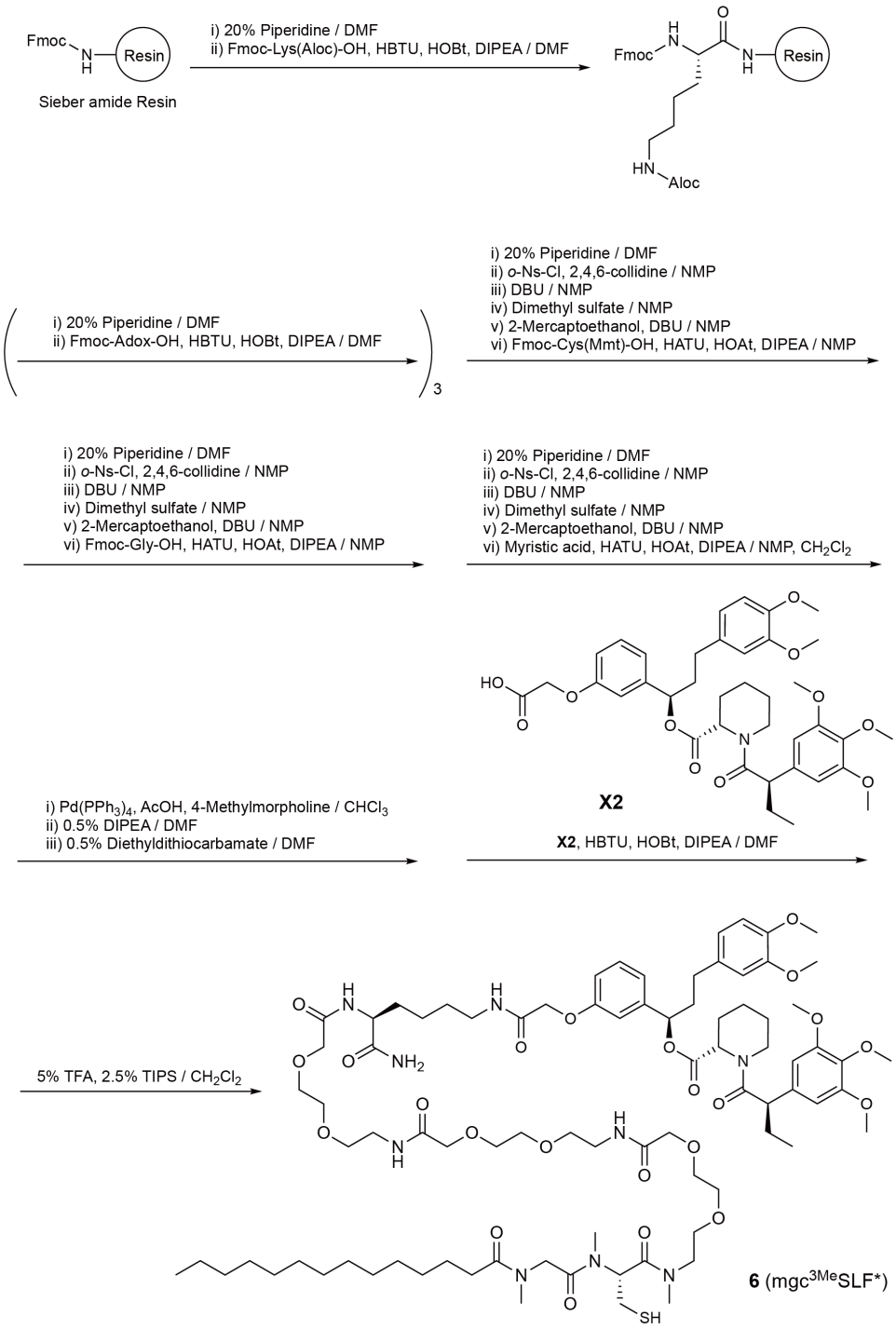
Scheme S5 Synthetic route of **5**

Compound **5** was synthesized manually on Sieber amide resin (0.79 mmol/g) (38.0 mg, 30 μmol). First, Fmoc-Lys(Aloc)-OH and Fmoc-Adox-OH (×3) were coupled to the resin. The *N*-methylation and coupling reactions of Fmoc-Ser(tBu)-OH and Fmoc-Gly-OH were performed as described in "**General methods for solid-phase synthesis**". The *N*-terminus was myristoylated using a mixture of myristic acid (3.1 eq.), HATU (3.0 eq.),

HOAt (3.0 eq.), and DIPEA (6.0 eq.) in NMP/CH_2Cl_2. After washing the resin with NMP, MeOH, and CH_2Cl_2, it was dried *in vacuo*. The Aloc group of the lysine residue was deprotected by treatment with $CHCl_3$ containing $Pd(PPh_3)_4$ (3.0 eq.), 5.0% acetic acid, and 2.5% 4-methylmorpholine under an Ar atmosphere. The resin was washed with DMF containing 0.5% DIPEA (×5), DMF containing 0.5% sodium diethyldithiocarbamate (×5), and DMF only (×5). Compound **X1** (TMP-COOH) was coupled to the side chain of the lysine residue with a mixture of **X1** (3.1 eq.), HBTU (3.0 eq.), HOBt (3.0 eq.), and DIPEA (6.0 eq.) in DMF. After washing the resin with DMF, MeOH, and CH_2Cl_2, it was dried *in vacuo*. Deprotection and cleavage from the resin were performed with CH_2Cl_2 containing 30% TFA and 2.5% TIPS. The crude product was purified by reversed-phase HPLC using a semi-preparative C18 column (a linear gradient of MeCN containing 0.1% TFA and 0.1% aqueous TFA) to afford **5** as a white solid [3.3 mg, 8% (as a mono-TFA salt)].

^{1}H NMR (400 MHz, CD_3OD): δ 7.22 (1H, s), 6.56 (2H, s), 5.58–5.39 (1H, m), 4.41 (1H, m), 4.03 (2H, s), 3.99 (4H, s), 3.91 (2H, t, J = 6.2 Hz), 3.80 (6H, s), 3.66 (16H, m), 3.60 (6H, m), 3.45 (6H, m), 3.17 (2H, t, J = 6.0 Hz), 3.10–2.90 (9H, m), 2.80–2.70 (2H, m), 2.43 (2H, m), 2.25 (2H, m), 1.80 (2H, m), 1.70 (2H, m), 1.61 (2H, m), 1.53 (2H, m), 1.38 (2H, m), 1.29 (22H, m), 0.90 (3H, t, J = 6.0 Hz).
HRMS (ESI): calculated for $[M+H]^+$, 1335.8134; found, 1335.8073.

Synthesis of Compound 6 (mgc^{3Me}SLF*)



Scheme S6 Synthetic route of **6**

Compound **6** was synthesized manually on Sieber amide resin (0.79 mmol/g) (38.0 mg, 30 μmol). First, Fmoc-Lys(Aloc)-OH and Fmoc-Adox-OH (×3) were coupled to the resin. The *N*-methylation and coupling reactions of Fmoc-Cys(Mmt)-OH and Fmoc-Gly-OH

were performed as described in "**General methods for solid-phase synthesis**". The *N*-terminus was myristoylated using a mixture of myristic acid (3.1 eq.), HATU (3.0 eq.), HOAt (3.0 eq.), and DIPEA (6.0 eq.) in NMP/CH_2Cl_2. After washing the resin with NMP, MeOH, and CH_2Cl_2, it was dried *in vacuo*. The Aloc group of the lysine residue was deprotected by treatment with $CHCl_3$ containing $Pd(PPh_3)_4$ (3.0 eq.), 5.0% acetic acid, and 2.5% 4-methylmorpholine under an Ar atmosphere. The resin was washed with DMF containing 0.5% DIPEA (×5), DMF containing 0.5% sodium diethyldithiocarbamate (×5), and DMF only (×5). Compound **X2** (SLF*-COOH) was coupled to the side chain of the lysine residue with a mixture of **X2** (3.1 eq.), HBTU (3.0 eq.), HOBt (3.0 eq.), and DIPEA (6.0 eq.) in DMF. After washing the resin with DMF, MeOH, and CH_2Cl_2, it was dried *in vacuo*. Deprotection and cleavage from the resin were performed with CH_2Cl_2 containing 5% TFA and 2.5% TIPS. The crude product was purified by reversed-phase HPLC using a semi-preparative C18 column (a linear gradient of MeCN containing 0.1% TFA and 0.1% aqueous TFA) to afford **6** as a pale yellow solid [8.5 mg, 22% (as a mono-TFA salt)].

^{1}H NMR (400 MHz, CD_3OD): δ7.19 (1H, m), 6.88–6.84 (2H, m), 6.81 (1H, m), 6.73 (1H, m), 6.68 (1H, m), 6.59(2H, s), 6.54(1H, m), 5.58 (1H, m), 5.50 (1H, m), 5.39 (1H, m), 4.52 (2H, m), 4.42 (1H, m), 4.03 (2H, s), 3.99 (4H, s), 3.88 (m, 2H), 3.82 (6H, m), 3.78 (3H, s), 3.69 (6H, s), 3.66 (14H, m), 3.59 (6H, m), 3.45 (6H, m), 3.26 (2H, m), 3.10–2.89 (9H, m), 2.73 (2H, m), 2.70 (1H, m), 2.43 (2H, m), 2.25 (2H, m), 2.03 (2H, m), 1.73 (2H, m), 1.61 (4H, m), 1.59 (6H, m), 1.37 (2H, m), 1.28 (22H, m), 0.89 (6H, t).
HRMS (ESI): calculated for $[M+H]^+$, 1668.9308; found, 1668.9263.

Supplementary Methods: Molecular and Cell Biology Experiments

Plasmid construction

All of the constructs used in this study and their cDNA and amino acid sequences are listed in **Fig. S8** and **Fig. S9**. pCAGGS-EGFP-NRas and pCMV-RFP-P4M (Addgene plasmid #51471: mCherry-P4M-SidM)[S2] were kind gifts from Dr. Michiyuki Matsuda (Kyoto University) and Dr. Tamas Balla (NIH), respectively. The plasmids encoding Sac1 [Addgene plasmid #37999: Pseudojanin (PJ)][S8] and $Sac1_{dead}$ (Addgene plasmid #38002: PJ-DEAD)[S8], which were used for constructing pCMV-GD-Sac1 and pCMV-GD-$Sac1_{dead}$, respectively, were gifts from Dr. Robin Irvine (University of Cambridge). We used pEGFP-N1(Clontech), pEGFP-C1 (Clontech), and pmCherry-C1 (Clontech) as vectors for constructing other expression plasmids. All expression plasmids were generated using standard cloning procedures. All PCR-amplified sequences were verified by DNA sequencing.

Cell culture and transfection

HeLa and Cos-7 cells were obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University. MDCK cells were a gift from Dr. Yasuo Mori (Kyoto University). Cells were cultured in DMEM (Wako) supplemented with 10% heat-inactivated FBS (Biowest), penicillin (100 U/mL), and streptomycin (100 μg/mL) [DMEM(+)] at 37 °C under a humidified 5% CO_2 atmosphere. For transient expression experiments, cells were transfected using 293fectin (Invitrogen) in accordance with the manufacturer's protocol.

Live-cell imaging

Confocal fluorescence imaging was performed with either (i) a LSM880 confocal laser-scanning microscope (Zeiss) equipped with a Plan-Apochromat 63×/1.40 NA oil objective (Zeiss), a Definite Focus.2 module (Zeiss), and an incubation chamber (Incubator PM 2000 RBT, Pecon), or (ii) an IX83/FV3000 confocal laser-scanning microscope (Olympus) equipped with a PlanApo N 60×/1.42 NA oil objective (Olympus), a Z drift compensator system (IX3-ZDC2, Olympus), and a stage top incubator (Tokai Hit). Lasers used for excitation were as follows: 405 nm for mTagBFP2 (ii), 488 nm for EGFP (i/ii), and 543 nm (i) or 561 nm (ii) for mCherry. Live-cell imaging was performed

at 37 °C. Fluorescence images were analyzed using the Fiji distribution of ImageJ.[S9]

SLIPT assays

To conduct SLIPT assays, HeLa cells stably expressing eDHFR-EGFP[S10] were plated at 2×10^5 cells in 35 mm glass-bottomed dishes (Iwaki Glass) and cultured for 24 h at 37 °C. The medium was changed to serum-free DMEM supplemented with penicillin (100 U/mL) and streptomycin (100 μg/mL) [DMEM(–)], and the cells were observed by time-lapse imaging before and after the addition of the indicated compound (10 μM) dissolved in DMSO (final DMSO concentration <0.1% v/v).

For palmitoylation inhibition experiments, 2×10^5 HeLa cells stably expressing eDHFR-EGFP were plated on 35 mm glass-bottomed dishes and cultured for 24 h at 37 °C in 5% CO_2. The cells were incubated with 2-BP (100 μM) in DMEM(+) for 3 h, and the medium was changed to DMEM(–). The cells were then imaged before and after treatment with mgc^{3Me}TMP (10 μM).

To conduct SLIPT assays in Cos-7 and MDCK cells, cells (1×10^5) were plated on 35 mm glass-bottomed dishes and cultured for 24 h at 37 °C in 5% CO_2. The cells were transfected with pCMV-eDHFR-EGFP using 293fectin. After incubation for 24 h, the medium was changed to DMEM(–), and the cells were observed by time-lapse imaging before and after the addition of mgc^{3Me}TMP (10 μM).

For FKBP$_{36V}$-EGFP translocation, HeLa cells (1×10^5) were plated on 35 mm glass-bottomed dishes and cultured for 24 h at 37 °C in 5% CO_2. The cells were transfected with pCMV-FKBP$_{36V}$-EGFP using 293fectin. After incubation for 24 h, the medium was changed to DMEM(–), and the cells were observed by time-lapse imaging before and after the addition of mgc^{3Me}TMP (10 μM).

Cytotoxicity assays

The cytotoxicity of SLs was evaluated by the propidium iodide (PI) exclusion test.[S11] HeLa cells (2×10^5) stably expressing eDHFR-EGFP were plated on 35 mm glass-bottomed dishes and cultured for 24 h at 37 °C in 5% CO_2. The medium was changed to DMEM(–), and the cells were left untreated or treated with DMSO (0.1%) or the indicated compound (10 μM) for 1 h. The cells were then incubated with PI (3 μg/mL) and observed by confocal fluorescence imaging. Cell viability was estimated by

determining the percentage of non-PI-stained (live) cells relative to the total cells counted (n > 150 cells).

Colocalization analysis

For colocalization analysis, 2×10^5 HeLa cells stably expressing eDHFR-EGFP were plated on 35 mm glass-bottomed dishes and cultured for 24 h at 37 °C in 5% CO_2. The cells were transfected with pCMV-mCherry-Giantin using 293fectin. After incubation for 24 h, the medium was changed to DMEM(–), and the cells were imaged 10 min after treatment with the indicated compound (10 μM).

Synthetic activation of Golgi membrane Ras

HeLa cells (1×10^5) were plated on 35 mm glass-bottomed dishes and cultured for 24 h at 37 °C in 5% CO_2. The cells were cotransfected with pCMV-RD-RasGEF (or pCMV-RD), pCAGGS-EGFP-NRas, and pCMV-BFP-RBD using 293fectin. After incubation for 24 h, the medium was changed to DMEM(–), and the cells were observed by time-lapse imaging before and after the addition of mgc^{3Me}TMP (2.5 μM).

Golgi PI4P depletion

HeLa cells (1×10^5) were plated on 35 mm glass-bottomed dishes and cultured for 24 h at 37 °C in 5% CO_2. The cells were cotransfected with pCMV-GD-Sac1 (or pCMV-GD-Sac1$_{dead}$) and pCMV-RFP-P4M using 293fectin. After incubation for 24 h, the medium was changed to DMEM(–), and the cells were observed by time-lapse imaging before and after the addition of mg^{3Me}TMP (10 μM).

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