Supporting Information

Synthetic self-localizing ligands that control the spatial location of proteins in living cells

Manabu Ishida,[1] Hideaki Watanabe,[2] Kazumasa Takigawa,[2] Yasutaka Kurishita,[4] Choji Oki,[2] Akinobu Nakamura,[3] Itaru Hamachi[4,5] & Shinya Tsukiji[1]

[1]*Top Runner Incubation Center for Academia-Industry Fusion,* [2]*Department of Bioengineering,* [3]*Department of Electrical Engineering, Nagaoka University of Technology, 1603-1 Kamitomioka, Nagaoka, Niigata 940-2188, Japan*

[4]*Department of Synthetic Chemistry and Biological Chemistry, Graduate School of Engineering, Kyoto University, Katsura, Kyoto 615-8510, Japan*

[5]*Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Agency, 5 Sanbancho, Chiyoda-ku, Tokyo 102-0075, Japan*

Correspondence should be addressed to S.T. (tsukiji@vos.nagaokaut.ac.jp).

Supplementary Results

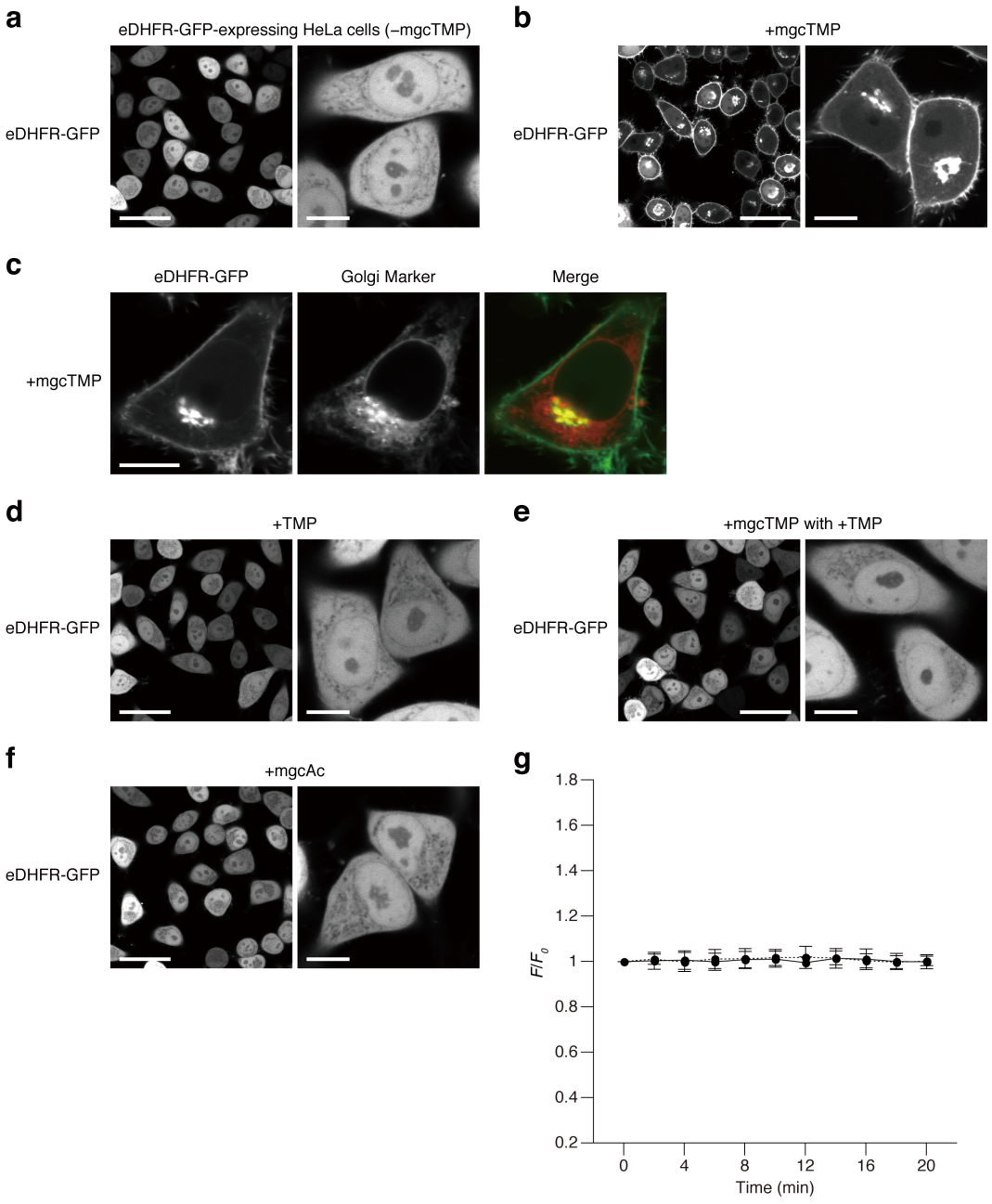


Figure S1. mgcTMP-induced eDHFR-GFP translocation. (**a**) Low- and high-magnification (L/H) confocal fluorescence images of HeLa cells expressing eDHFR-GFP (no treatment). (**b**) L/H confocal fluorescence images of eDHFR-GFP-expressing HeLa cells after 20 min incubation with 5 μM mgcTMP. (**c**)

eDHFR-GFP is partially recruited to the Golgi by mgcTMP. Intracellular fluorescence signals observed in eDHFR-GFP-expressing HeLa cells treated with mgcTMP co-localizes with the Golgi marker BODIPY TR-ceramide (as seen in yellow in the merged image). Left, GFP fluorescence; center, BODIPY TR fluorescence; right, merged image. Scale bar, 10 μm. (**d**) L/H confocal fluorescence images of eDHFR-GFP-expressing HeLa cells after 60 min incubation with 5 μM TMP. (**e**) L/H confocal fluorescence images of eDHFR-GFP-expressing HeLa cells after 20 min incubation with 5 μM mgcTMP in the presence of 50 μM TMP. (**f**) L/H confocal fluorescence images of eDHFR-GFP-expressing HeLa cells after 20 min incubation with 5 μM mgcAc. (**g**) Time course of eDHFR-GFP translocation induced by 5 μM mgcAc. Relative fluorescence signal intensities (F/F_0) in the iPM (solid line) and cytoplasm (dashed line) of cells were plotted as a function of time after the ligand addition. Data are represented as mean ± s.d. (n = 10 cells). Scale bars except **c**, 40 μm (left) and 10 μm (right).

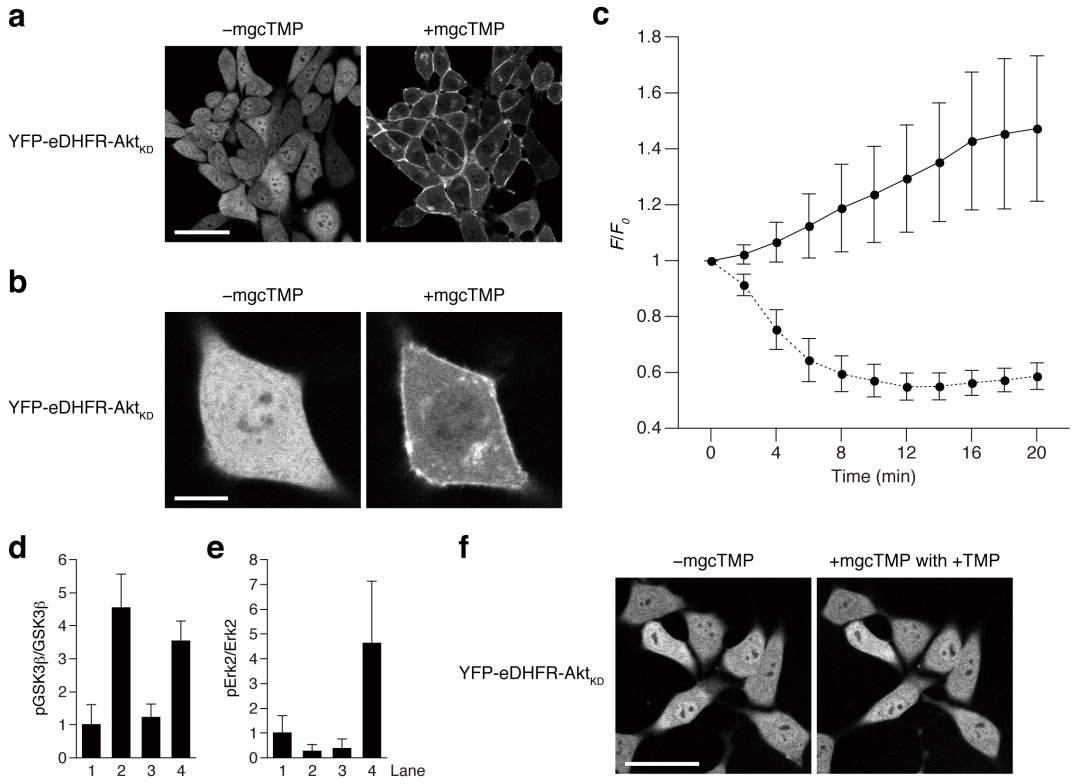


Figure S2. mgcTMP-induced YFP-eDHFR-Akt$_{KD}$ translocation and activation. (**a**,**b**) L/H confocal fluorescence images of NIH3T3 cells expressing YFP-eDHFR-Akt$_{KD}$ before and 20 min after addition of 5 μM mgcTMP. Scale bars, 40 μm (**a**) and 10 μm (**b**). (**c**) Time course of YFP-eDHFR-Akt$_{KD}$ translocation induced by 5 μM mgcTMP. Relative fluorescence signal intensities (F/F_0) in the iPM (solid line) and cytoplasm (dashed line) of cells were plotted as a function of time after the ligand addition. Data are represented as mean ± s.d. (n = 10 cells). (**d**,**e**) Quantification of GSK3β and Erk2 phosphorylation. See **Fig. 2c–g** for details. (**f**) Confocal fluorescence images of YFP-eDHFR-Akt$_{KD}$-expressing NIH3T3 cells before and 20 min after addition of 5 μM mgcTMP together with 50 μM TMP. Scale bar, 40 μm.

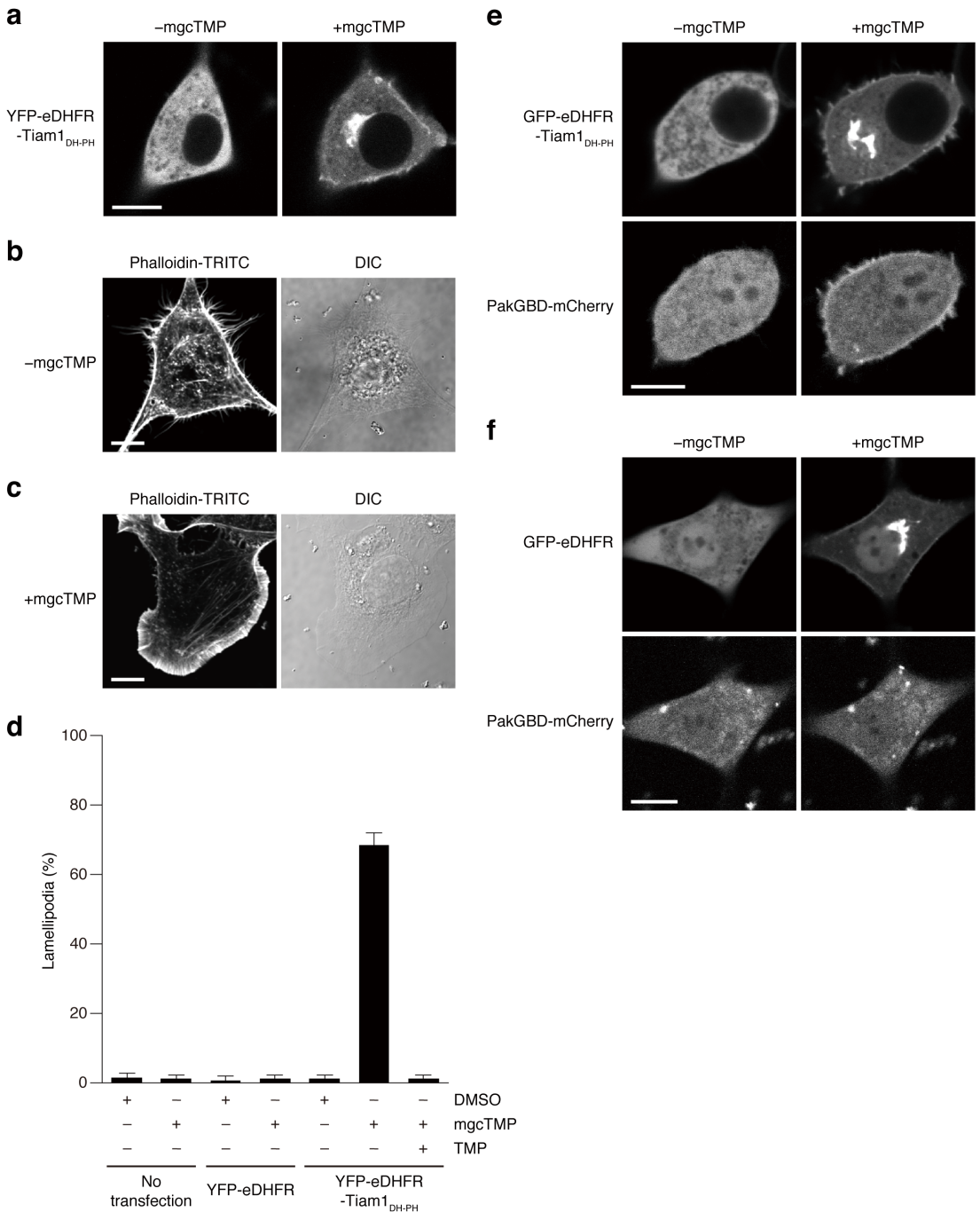


Figure S3. mgcTMP-induced YFP-eDHFR-Tiam1$_{DH-PH}$ translocation, endogenous Rac activation, and lamellipodia formation. (**a**,**b**) Confocal fluorescence images of NIH3T3 cells expressing YFP-eDHFR-Tiam1$_{DH-PH}$ before and 20 min after addition of 5 μM mgcTMP. (**b**,**c**) Representative confocal images of YFP-eDHFR-Tiam1$_{DH-PH}$-expessing (serum-starved) NIH3T3 cells stained with phalloidin-TRITC before (**b**) and 1 h after incubation with 5 μM mgcTMP (**c**). Left,

TRITC fluorescence; right, DIC image. (**d**) Quantification of lamellipodia formation. Non-transfected and YFP-eDHFR-expressing NIH3T3 cells were used as controls. After serum-starvation, cells were incubated for 1 h with 0.1% DMSO (vehicle) or 5 μM mgcTMP in the absence or presence of 50 μM TMP. The lamellipodia % shown was determined by dividing "the number of transfected cells with lamellipodia" by "the number of transfected cells". For no transfection control, cells were randomly chosen and counted. Data are represented as mean ± s.d. of three independent experiments (n > 40 cells for each experiment). (**e**) Monitoring of Rac activation using the biosensor PakGBD-mCherry.[S1] For this experiment, a GFP-tagged variant (GFP-eDHFR-Tiam1$_{DH-PH}$) was used. Confocal fluorescence images of NIH3T3 cells co-expressing GFP-eDHFR-Tiam1$_{DH-PH}$ and PakGBD-mCherry were obtained before and 20 min after addition of 5 μM mgcTMP. Top, GFP fluorescence; bottom, mCherry fluorescence. Translocation of PakGBD-mCherry occurred upon mgcTMP-induced GFP-eDHFR-Tiam1$_{DH-PH}$ translocation, indicating that GFP-eDHFR-Tiam1$_{DH-PH}$ recruitment activated endogenous Rac. (**f**) Negative control for **e**. iPM translocation of a GFP-eDHFR only construct does not induce PakGBD-mCherry translocation. Scale bars for all panels, 10 μm.

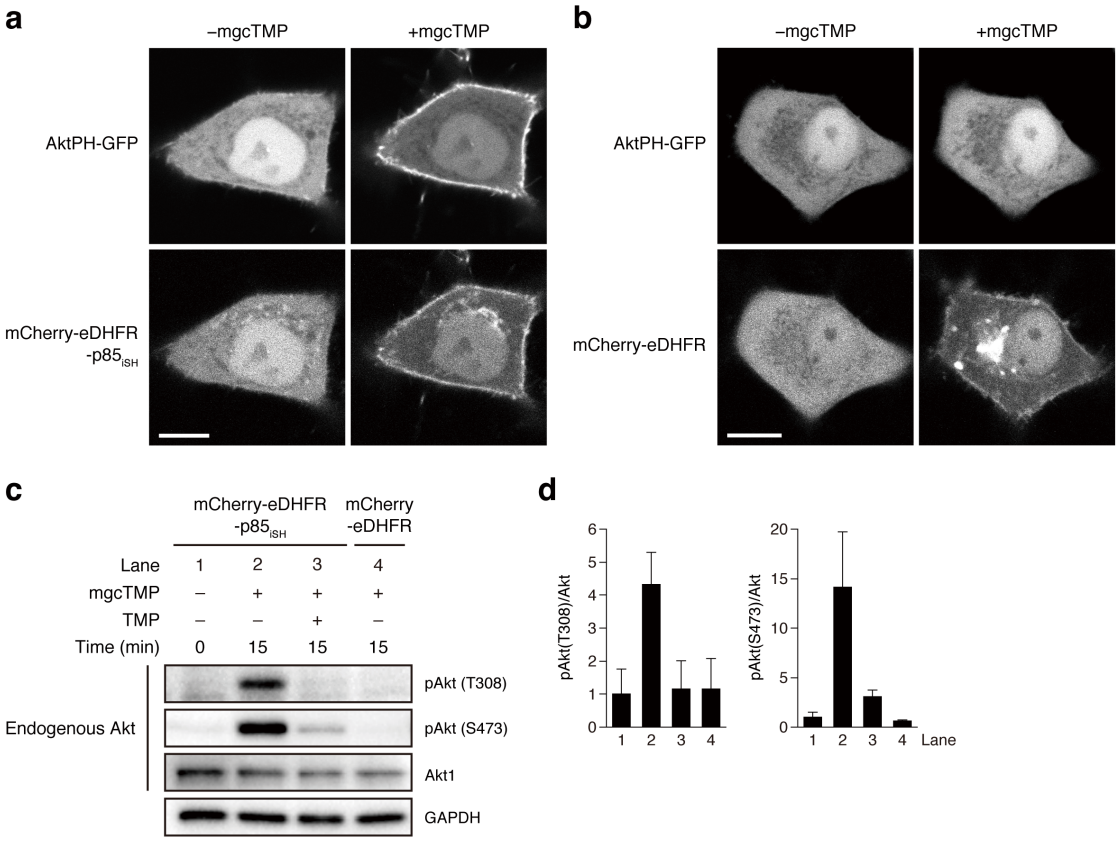


Figure S4. mgcTMP-induced mCherry-eDHFR-p85$_{iSH}$ translocation, PIP$_3$ formation, and endogenous Akt activation. (**a**) Detection of PIP$_3$ formation using the biosensor AktPH-GFP.[S2] Confocal fluorescence images of (serum-starved) HeLa cells co-expressing mCherry-eDHFR-p85$_{iSH}$ and AktPH-GFP were obtained before and 15 min after addition of 5 μM mgcTMP. Top, GFP fluorescence; bottom, mCherry fluorescence. (**b**) Negative control for **a**. Confocal fluorescence images of (serum-starved) HeLa cells co-expressing mCherry-eDHFR and AktPH-GFP were obtained before and 15 min after addition of 5 μM mgcTMP. Scale bars, 10 μm. (**c**) Immunoblot analysis of endogenous Akt activation. HeLa cells expressing mCherry-eDHFR-p85$_{iSH}$ (lane 1–3) or mCherry-eDHFR (lane 4) were serum-starved and incubated for 15 min under the following conditions: lane 1, none; lane 2, 5 μM mgcTMP; lane 3, 5 μM mgcTMP and 50 μM TMP; lane 4, 5 μM mgcTMP. Cell lysates were immunoblotted with the indicated antibodies. (**d**) Quantification of phosphorylation. Lane numbers correspond to the conditions given in **c**. Phosphorylation of each residue was normalized to total Akt. Data are represented as relative mean values to the mean of no treatment condition (lane 1) from three

independent experiments. Error bars are s.d.

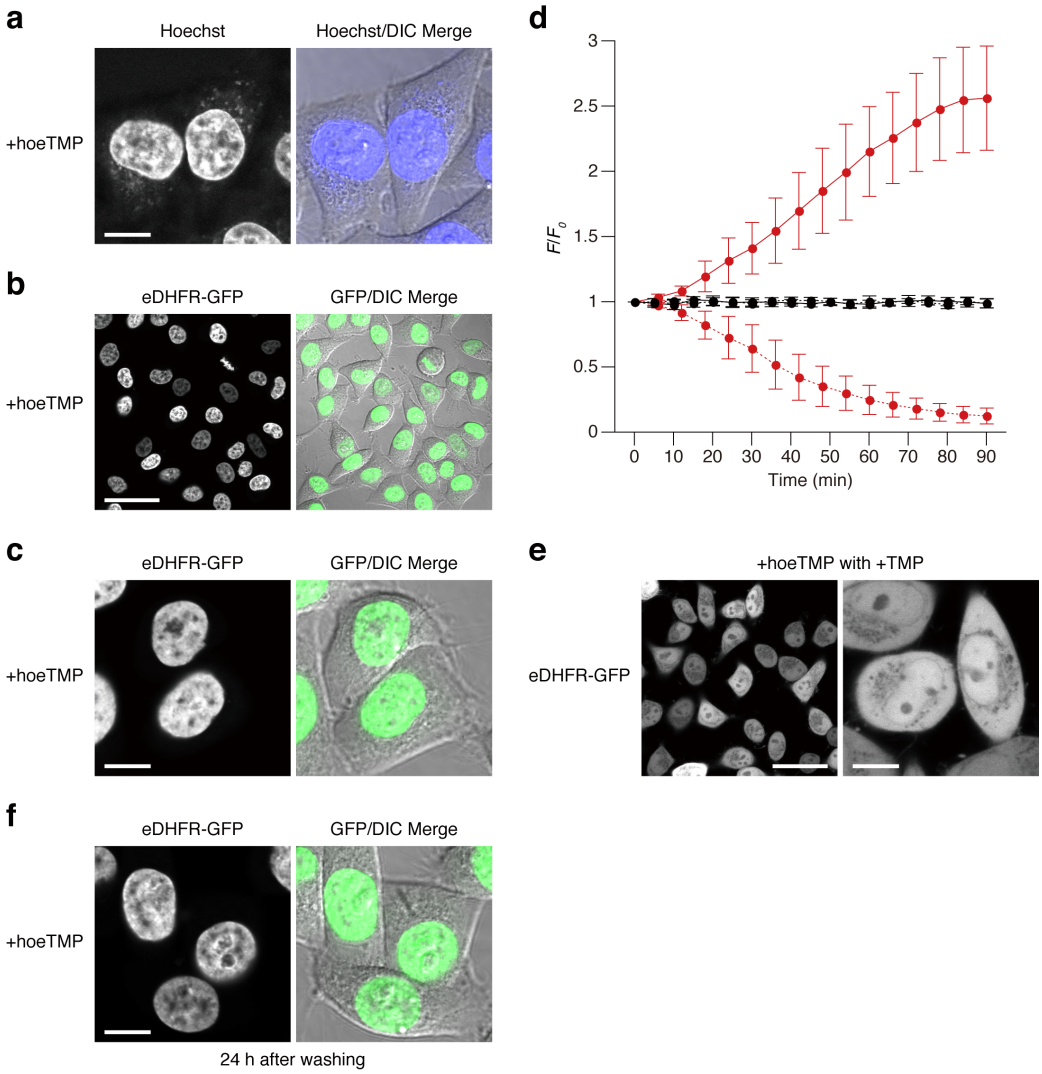


Figure S5. hoeTMP-induced eDHFR-GFP translocation. (**a**) Confocal fluorescence images of HeLa cells (non-transfected) after 90 min incubation with 5 μM hoeTMP. Left, Hoechst fluorescence; right, DIC image merged with Hoechst fluorescence. Scale bar, 10 μm. These data demonstrate that hoeTMP localizes to the nucleus. (**b**,**c**) L/H confocal fluorescence images of HeLa cells expressing eDHFR-GFP after 90 min incubation with 5 μM hoeTMP. Left, GFP fluorescence; right, DIC image merged with GFP fluorescence. Scale bars, 40 μm (**b**) and 10 μm (**c**). (**d**) Time course of eDHFR-GFP translocation induced by 5 μM hoeTMP (red) or 5 μM free TMP (black). Relative fluorescence signal intensities (F/F_0) in the nucleus (solid line) and cytoplasm (dashed line) of cells were plotted as a function of time after the ligand addition. Data are represented as mean ± s.d. (n = 10 cells). (**e**) L/H confocal fluorescence images of

eDHFR-GFP-expressing HeLa cells after 90 min incubation with 5 μM hoeTMP in the presence of 50 μM TMP. Scale bars, 40 μm (left) and 10 μm (right). (**f**) The hoeTMP-mediated nuclear translocation is stable. After inducing nuclear translocation of eDHFR-GFP with hoeTMP in HeLa cells (**Fig. 3a** and **Fig. S5b**,**c**), the cells were washed and incubated for 24 h. Confocal fluorescence images of the cells were then obtained. Scale bar, 10 μm.

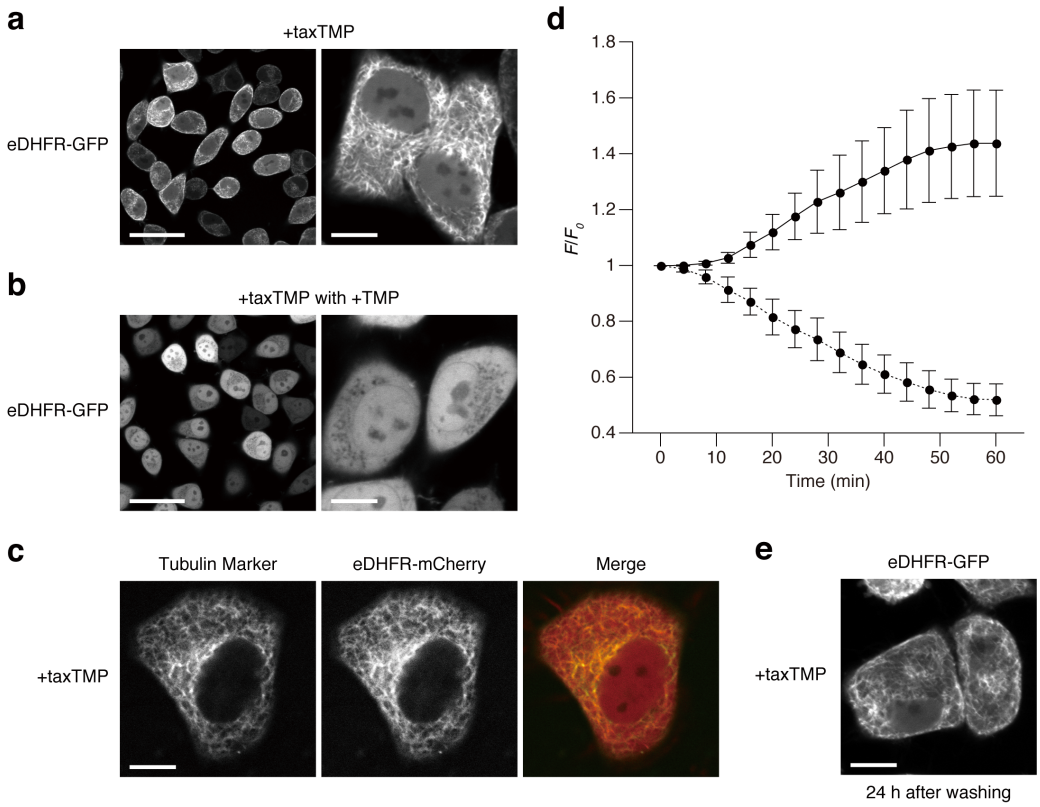


Figure S6. taxTMP-induced eDHFR-GFP translocation. (**a**,**b**) L/H confocal fluorescence images of HeLa cells expressing eDHFR-GFP after 60 min incubation with 5 μM taxTMP in the absence (**a**) or presence of 50 μM TMP (**b**). Scale bars, 40 μm (left) and 10 μm (right). (**c**) Co-localization assay. For this experiment, an eDHFR-mCherry fusion protein was used. Assembled eDHFR-mCherry fluorescence after taxTMP addition co-localizes with the microtubule marker TubulinTracker Green (as seen in yellow in the merged image). Left, tubulin marker fluorescence; center, mCherry fluorescence; right, merged image. Scale bar, 10 μm. (**d**) Time course of eDHFR-GFP translocation induced by 5 μM taxTMP. Relative fluorescence signal intensities (F/F_0) in the microtubule (solid line) and nucleus (dashed line) of cells were plotted as a function of time after the ligand addition (the nuclear fluorescence decreases upon taxTMP-induced eDHFR-GFP translocation). Data are represented as mean ± s.d. (n = 10 cells). The F/F_0 values in the microtubule are underestimated due to the difficulty of separating the fluorescence signal in this (filamentous and dynamic) region from that in the cytoplasm. (**e**) The taxTMP-mediated microtubule translocation is stable. After inducing microtubule translocation of eDHFR-GFP with taxTMP in

HeLa cells (**Fig. 3b** and **Fig. S6a**), the cells were washed and incubated for 24 h. Confocal fluorescence images of the cells were then obtained. Scale bar, 10 μm.

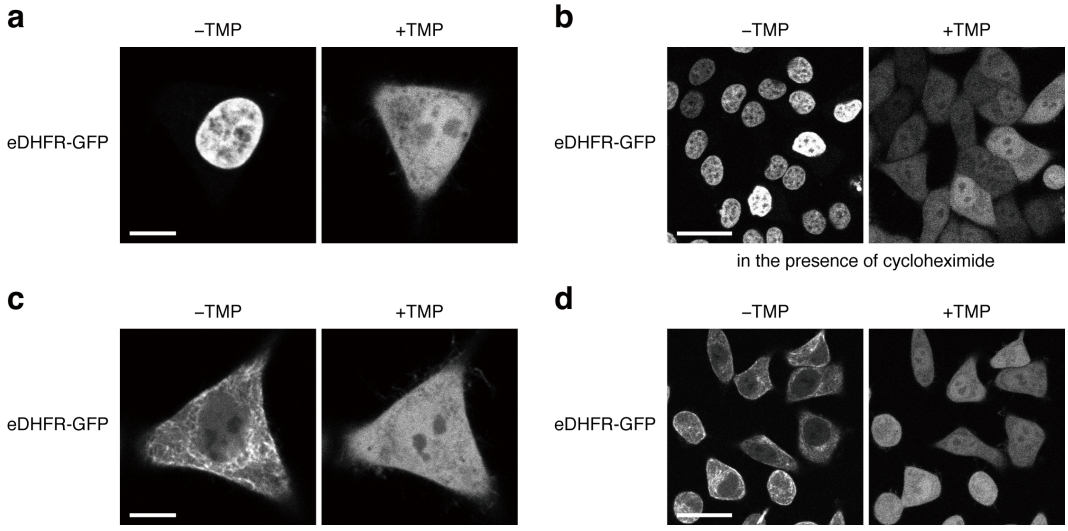


Figure S7. Reversible protein translocation. (**a**) From the nucleus back to the cytoplasm. After inducing nuclear translocation of eDHFR-GFP with hoeTMP in HeLa cells (**Fig. 3a** and **Fig. S5b**,**c**), confocal fluorescence images of the cells were obtained before and 90 min after addition of 50 μM TMP. (**b**) Control experiment for **a**. The same experiment as in **a** was performed in the presence of 50 μg/mL cycloheximide (protein synthesis inhibitor). The GFP fluorescence was redistributed throughout the cell 90 min after addition of 50 μM TMP. This result verifies that the observed fluorescence change is caused by eDHFR-GFP relocation to the cytoplasm, not by synthesis of new eDHFR-GFP. (**c**,**d**) From the microtubule back to the cytoplasm. After inducing microtubule translocation of eDHFR-GFP with taxTMP in HeLa cells (**Fig. 3b** and **Fig. S6a**), L/H confocal fluorescence images of the cells were obtained before and 60 min after addition of 50 μM TMP. Scale bars, 10 μm (**a**,**c**) and 40 μm (**b**,**d**).

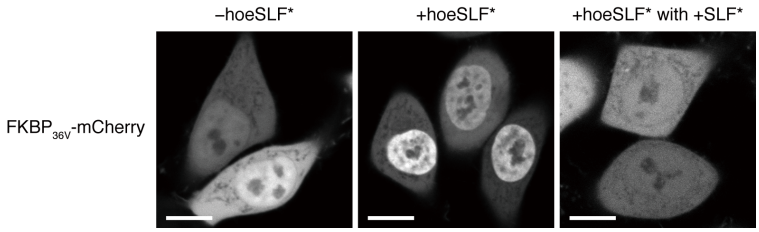


Figure S8. hoeSLF*-induced $FKBP_{36V}$-mCherry translocation. Confocal fluorescence images of HeLa cells expressing $FKBP_{36V}$-mCherry after the following incubations: left, none; center, 5 μM hoeSLF* for 90 min; right 5 μM hoeSLF* in the presence of 50 μM free SLF* (compound **21** shown in p.S33) for 90 min. Scale bars, 10 μm.

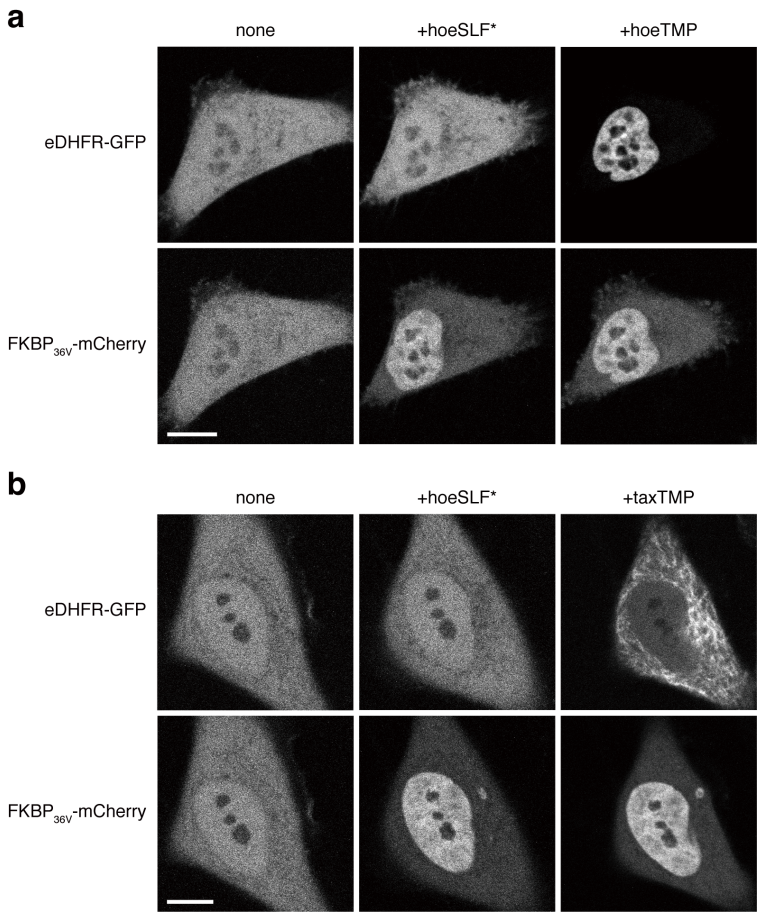


Figure S9. Simultaneous control of two distinct proteins in the same cell with orthogonal SLLs. (**a**,**b**) Confocal fluorescence images of HeLa cells co-expressing eDHFR-GFP and $FKBP_{36V}$-mCherry were obtained before (left), 90 min after the first addition of 2.5 μM hoeSLF* (center), and (**a**) 90 min after the second addition of 2.5 μM hoeTMP or (**b**) 60 min after the second addition of 2.5 μM taxTMP (right). Top, GFP fluorescence; bottom, mCherry fluorescence. Scale bars, 10 μm.

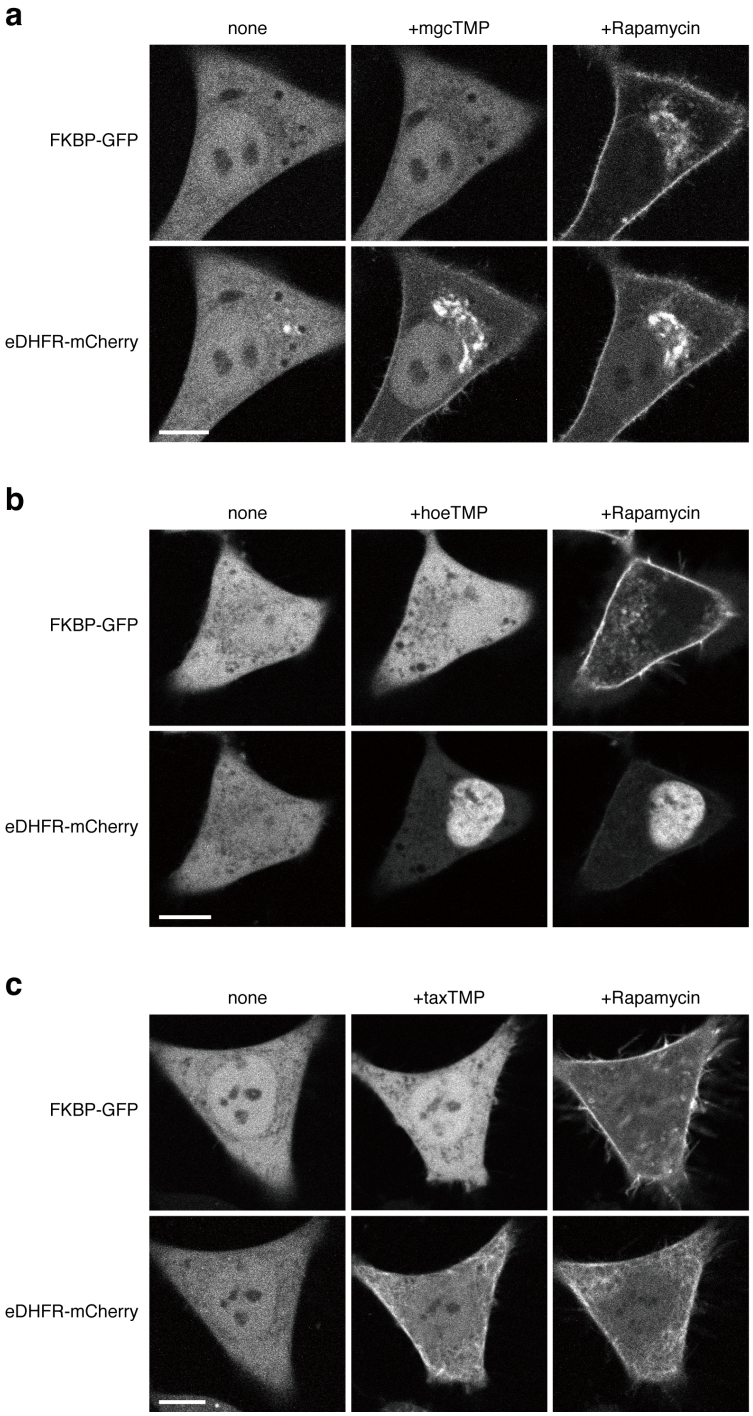


Figure S10. Simultaneous control of two distinct proteins in the same cell by a combination of SLLs and the rapamycin CID system. (**a**–**c**) Confocal fluorescence images of HeLa cells co-expressing Lyn11-targeted FRB (LDR),[S3] GFP-tagged FKBP12 (FKBP-GFP) and eDHFR-mCherry. Cells were treated first with 2.5 μM SLL for the indicated incubation time and then with 2.5 μM rapamycin for 20 min: **a**, mgcTMP, 20

min; **b**, hoeTMP, 90 min; **c**, taxTMP, 60 min. Images were obtained before treatment (left), after the first translocation (center) and after the second translocation (right). Top, GFP fluorescence; bottom, mCherry fluorescence. Scale bars, 10 μm.

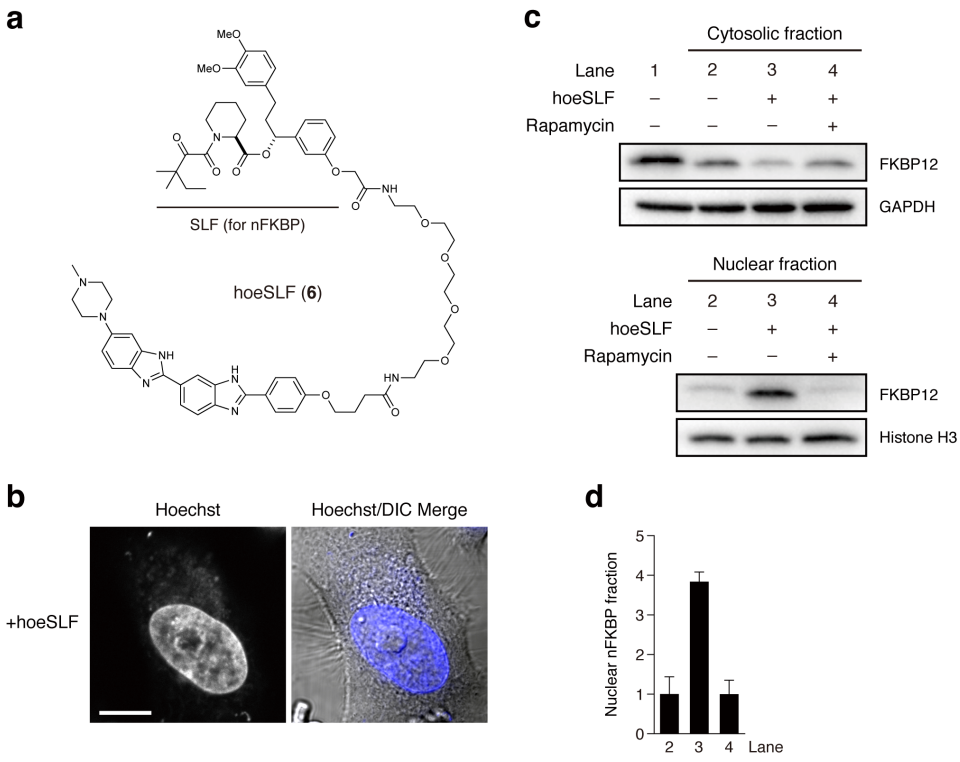


Figure S11. Spatial control of an endogenous protein (nFKBP) in intact cells. (**a**) Structure of hoeSLF (**6**). (**b**) Confocal fluorescence images of HeLa cells (non-transfected) after 2 h incubation with 5 μM hoeSLF. Left, Hoechst fluorescence; right, DIC image merged with Hoechst fluorescence. Scale bar, 10 μm. These data demonstrate that hoeSLF localizes to the nucleus. (**c**) Immunoblot analysis of nFKBP translocation. HeLa cells were incubated for 2 h under the following conditions: lane 1 and 2, none; lane 3, 5 μM hoeSLF; lane 4, 5 μM hoeSLF and 20 μM rapamycin. Cell lysates were separated into nuclear and cytosolic fractions, which were immunoblotted with the indicated antibodies. Lane 1, total lysate. (**d**) Quantification of nuclear nFKBP fraction. Lane numbers correspond to the conditions given in **c**. Nuclear nFKBP fraction was defined as the ratio of nuclear nFKBP (normalized to histone) to cytosolic nFKBP (normalized to GAPDH). Data are represented as mean values relative to the mean of no treatment condition (lane 2) from three independent experiments. Error bars are s.d.

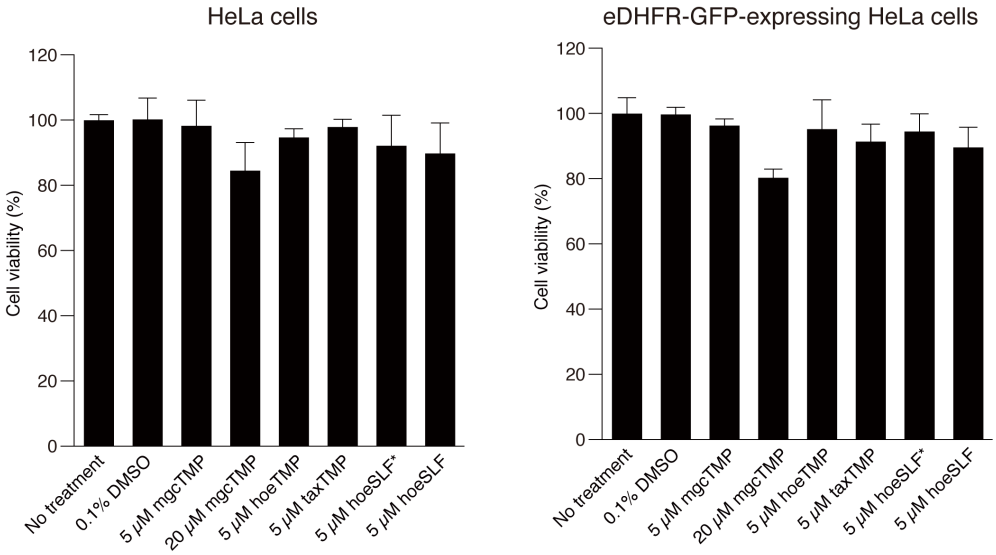


Figure S12. Cytotoxity assays. Intact HeLa cells (left) or eDHFR-GFP-expressing HeLa cells were incubated with none, DMSO (vehicle) or one of SLLs at the indicated concentrations for 2 h. The cells were then assayed for viability using Cell Counting Kit-8. Data are represented as mean ± s.d. of at least three independent experiments. No significant toxicity was observed with all the SLLs under these conditions.

Note: We observed the cytotoxic effect after a long-term treatment with taxTMP and hoeSLF (data not shown). These results are likely due to the following reasons. Taxol is originally a potent microtubule-stabilizing agent that blocks cell cycle and causes cell death.[S4] Endogenous FKBP12 is involved in diverse biological processes such as protein folding and cell signaling,[S5] and SLF inhibits the function of this protein.

Supplementary Methods: Chemical Synthesis

Chemicals were purchased from Tokyo Chemical Industry, Wako Pure Chemical Industries, Watanabe Chemical Industries, Kanto Chemical, Sigma-Aldrich or Quanta Biodesign, and were used without further purification.

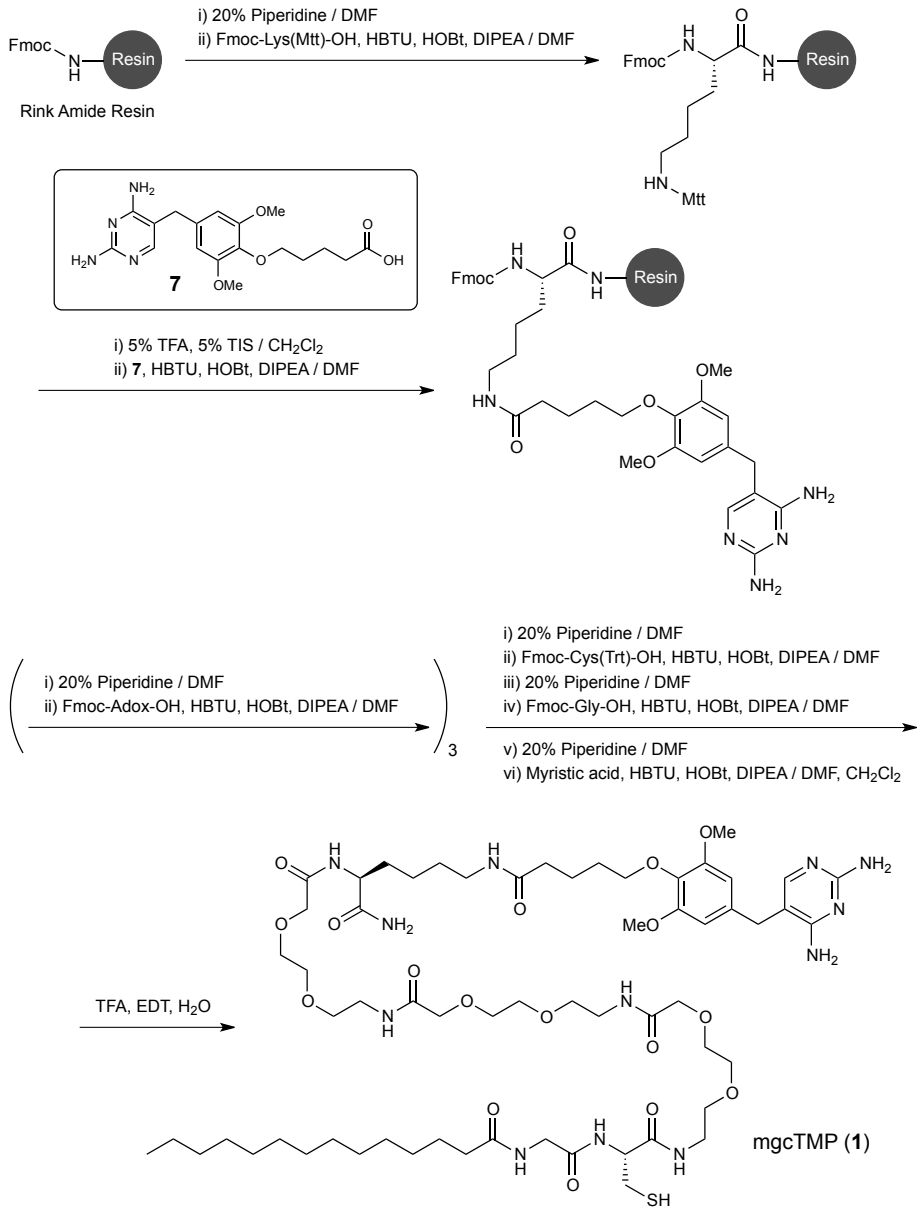
Thin layer chromatography (TLC) was performed on silica gel 60 F_{254} precoated aluminium sheets (Merck). TLC plates were visualized by fluorescence quenching and/or ninhydrin staining. Flash column chromatography was performed using silica gel 60 N (neutral, 40–50 μm) (Kanto Chemical). Reversed-phase 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 Varian Mercury 400 (400 MHz) or JEOL AL-400 (400 MHz) spectrometer. ^{1}H NMR spectra are represented as follows: chemical shift, integration, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad), coupling constant (*J*) in Hertz (Hz). ^{1}H NMR chemical shifts were referenced to tetramethylsilane (0 ppm). High-resolution mass spectra were measured by Dr. Keiko Kuwata (Institute of Transformative Bio-Molecules, Nagoya University). Low-resolution MALDI-TOF mass spectra were acquired on an Applied Biosystems Voyager Elite mass spectrometer.

Reagent abbreviations

DIC: *N*,*N*'-diisopropylcarbodiimide
DIPEA: *N*,*N*-diisopropylethylamine
DMAP: 4-(dimethylamino)pyridine
DMF: *N*,*N*-dimethylformamide
EDT: 1,2-ethanedithiol
Fmoc-Adox-OH: Fmoc-8-amino-3,6-dioxaoctanoic acid
HBTU: *O*-(benzotriazol-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium hexafluorophosphate
HOBt: 1-hydroxybenzotriazole (monohydrate)
TBAF: tetrabutylammonium fluoride
TFA: trifluoroacetic acid
THF: tetrahydrofuran
TIS: triisopropylsilane

Synthesis of mgcTMP (1)



Scheme S1. Synthetic route of mgcTMP (1).

mgcTMP (**1**) was synthesized manually on Rink 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 (4.1 eq.), HBTU (4.0 eq.), HOBt (4.0 eq.) and DIPEA (8.0 eq.) in DMF. All Fmoc

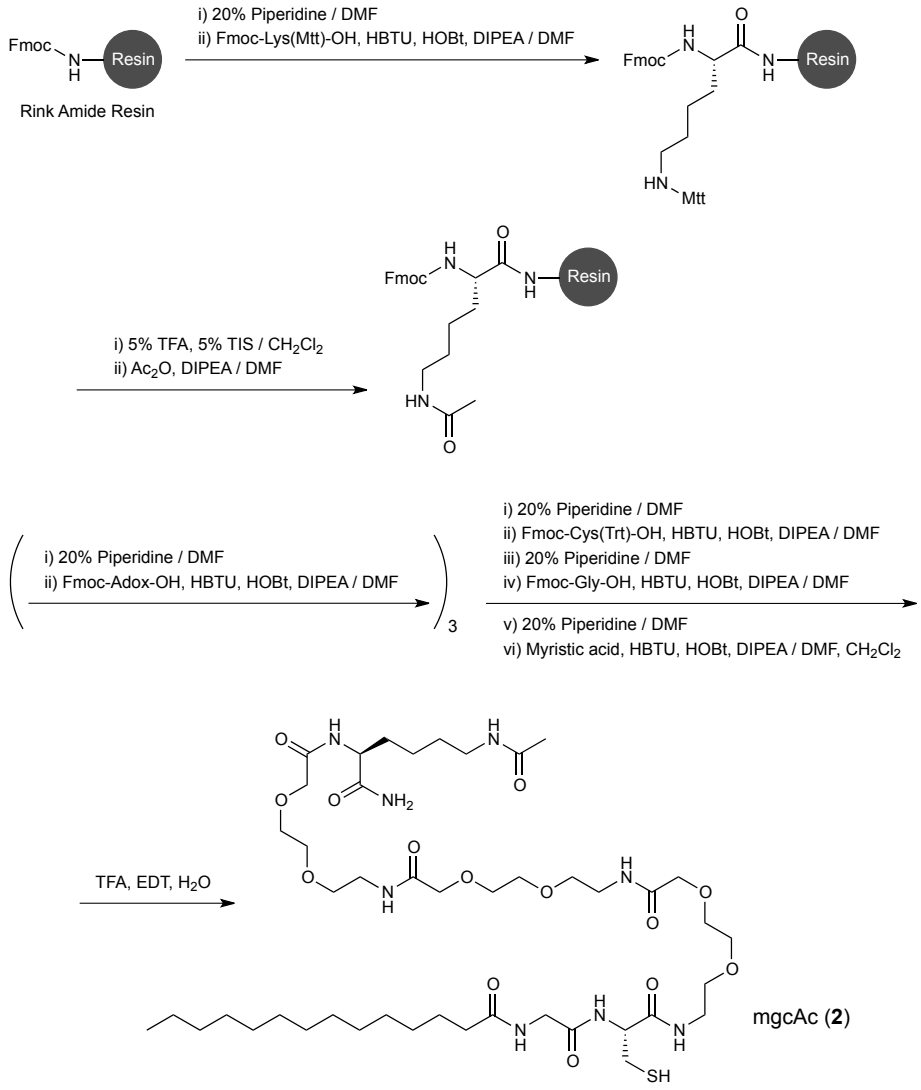
deprotection and coupling steps were monitored by the Kaiser test.[S6] Unless otherwise stated, all washing procedures were performed with DMF. Compound **7** was synthesized as described previously.[S7]

Rink Amide resin (0.65 mmol/g) (185 mg, 120 μmol) was Fmoc-deprotected and washed. Fmoc-Lys(Mtt)-OH was coupled to the resin. After washing with DMF, MeOH and then CH_2Cl_2, the Mtt group was selectively deprotected by treatment with CH_2Cl_2 containing 5% TFA and 5% TIS. The resin was washed with CH_2Cl_2, MeOH and then DMF. **7** was coupled to the side chain of the lysine with a mixture of **7** (3.1 eq.), HBTU (3.0 eq.), HOBt (3.0 eq.) and DIPEA (8.0 eq.) in DMF. Subsequently, Fmoc deprotection and coupling reactions were repeated using Fmoc-Adox-OH, Fmoc-Cys(Trt)-OH and Fmoc-Gly-OH as building blocks. The N-terminus was myristoylated using a mixture of myristic acid (4.1 eq.), HBTU (4.0 eq.), HOBt (4.0 eq.) and DIPEA (8.0 eq.) in DMF/CH_2Cl_2 (1:1). Trt deprotection and cleavage from the resin was performed with TFA containing 5% EDT and 2.5% H_2O. The crude products were precipitated by Et_2O and 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 mgcTMP (**1**) as a white solid (43 mg, 25% as a mono-TFA salt).

^{1}H NMR (400 MHz, CD_3OD): δ 7.23 (1H, s), 6.56 (2H, s), 4.51 (1H, m), 4.42 (1H, m), 4.03 (2H, s), 4.00 (4H, s), 3.92 (2H, t, J = 6.0 Hz), 3.88 (2H, s, s), 3.80 (6H, s), 3.66 (14H, m), 3.59 (6H, m), 3.45 (4H, m), 3.41 (2H, m), 3.18 (2H, t, J = 6.8 Hz), 2.86 (2H, m), 2.26 (4H, m), 1.80 (2H, m), 1.71 (2H, m), 1.62 (2H, m), 1.52 (2H, m), 1.38 (2H, m), 1.28 (22H, m), 0.89 (3H, t, J = 6.6 Hz).

HRMS (ESI): calcd for $[M+H]^+$, 1309.7436; found, 1309.7442.

Synthesis of mgcAc (2)



Scheme S2. Synthetic route of mgcAc (2).

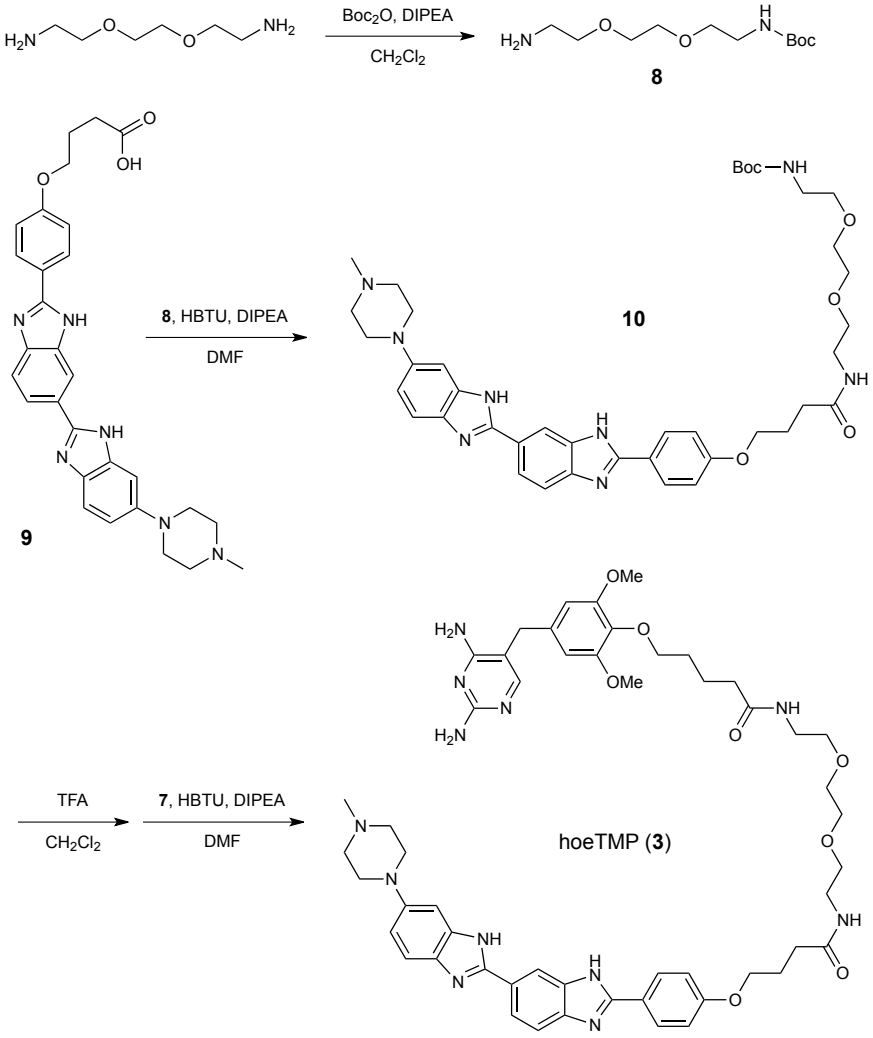
mgcAc (**2**) was synthesized in a similar manner to that described for mgcTMP (**1**). Rink Amide resin (0.55 mmol/g) (55 mg, 30 μmol) was Fmoc-deprotected and washed. Fmoc-Lys(Mtt)-OH was coupled to the resin. After washing with DMF, MeOH and then CH_2Cl_2, the Mtt group was selectively deprotected by treatment with CH_2Cl_2 containing 5% TFA and 5% TIS. The resin was washed with CH_2Cl_2, MeOH and then DMF. The deprotected side chain of the lysine was acetylated with a mixture of acetic anhydride (3.1 eq.) and DIPEA (8.0 eq.) in DMF. Subsequently, Fmoc deprotection and coupling

reactions were repeated using Fmoc-Adox-OH, Fmoc-Cys(Trt)-OH and Fmoc-Gly-OH as building blocks. The N-terminus was myristoylated using a mixture of myristic acid (4.1 eq.), HBTU (4.0 eq.), HOBt (4.0 eq.) and DIPEA (8.0 eq.) in DMF/CH_2Cl_2 (1:1). Trt deprotection and cleavage from the resin was performed with TFA containing 5% EDT and 2.5% H_2O. The crude products were precipitated by Et_2O and 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 mgcTMP (**2**) as a white solid (8.5 mg, 29%).

^{1}H NMR (400 MHz, CD_3OD): δ 4.51 (1H, m), 4.43 (1H, m), 4.04 (2H, s), 4.01 (4H, s), 3.88 (2H, s, s), 3.67 (12H, m), 3.60 (6H, m), 3.47 (4H, m), 3.42 (2H, m), 3.15 (2H, t, *J* = 7.0 Hz), 2.86 (2H, m), 2.27 (2H, t, *J* = 7.6 Hz), 1.92 (3H, s), 1.85 (1H, m), 1.72 (1H, m), 1.63 (2H, m), 1.52 (2H, m), 1.39 (2H, m), 1.29 (20H, m), 0.90 (3H, t, *J* = 7.0 Hz).

HRMS (ESI): calcd for $[M+H]^+$, 993.5901; found, 993.5872.

Synthesis of hoeTMP (3)



Scheme S3. Synthetic route of hoeTMP (3).

Compound **8**

To a solution of 1,2-bis(2-aminoethoxy)ethane (4.07 g, 27.46 mmol) and DIPEA (1.56 mL, 9.17 mmol) in anhydrous CH_2Cl_2 (50 mL) at room temperature was added a solution of Boc_2O (2.0 g, 9.16 mmol) in anhydrous CH_2Cl_2 (20 mL) dropwise. After stirring for 1 h, the mixture was concentrated and purified by column chromatography (silica, 4:4:1 AcOEt/MeOH/25% aqueous ammonia). The collected fraction was concentrated, dissolved in $CHCl_3$ and filtered. The filtrate was concentrated again to afford **8** as a colorless oil (2.25 g, 99%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 5.16 (1H, brs), 3.62 (4H, m), 3.57–3.51 (4H, m), 3.32 (2H, m), 2.89 (2H, t, *J* = 5.2 Hz), 1.65 (2H, s), 1.45 (9H, s).

Compound **10**

Compound **9** was synthesized as described previously.[S8] To a suspension of **9** (500 mg, 0.98 mmol) in anhydrous DMF (10 mL) at room temperature were added DIPEA (0.50 mL, 2.94 mmol) and HBTU (409 mg, 1.08 mmol). The mixture was stirred for 5 min, at which time the suspension became a clear solution. To this was added a solution of **8** (292 mg, 1.18 mmol) in anhydrous DMF (3 mL). After stirring for 2 h, the mixture was concentrated and purified by column chromatography (silica, 1:1 AcOEt/MeOH). The collected fraction was concentrated and triturated with Et_2O to afford **10** as a pale yellow solid (610 mg, 84%).

^{1}H NMR (400 MHz, CD_3OD): δ 8.23 (1H, s), 8.06 (2H, d, *J* = 9.2 Hz), 7.96 (1H, brd), 7.70 (1H, brd), 7.51 (1H, d, *J* = 8.8 Hz), 7.15 (1H, s), 7.10 (2H, d, *J* = 8.8 Hz), 7.06 (1H, dd, *J* = 2.4, 8.8 Hz), 4.10 (2H, t, *J* = 6.2 Hz), 3.58 (4H, s), 3.54 (2H, t, *J* = 5.4 Hz), 3.49 (2H, m, overlapped with the residual ether peak), 3.39 (2H, t, *J* = 5.6 Hz), 3.24 (4H, m), 3.20 (2H, t, *J* = 5.6 Hz), 2.69 (4H, m), 2.44 (2H, t, *J* = 7.2 Hz), 2.39 (3H, s), 2.12 (2H, m), 1.42 (9H, s).

hoeTMP (**3**)

To a solution of **10** (47 mg, 0.063 mmol) in CH_2Cl_2 (2 mL) at room temperature was added TFA (1 mL). After stirring for 1 h, the mixture was co-evaporated with toluene (5 mL × 2). The residue was dissolved in anhydrous DMF (0.6 mL), and then DIPEA (65 μL, 0.382 mmol) was added.

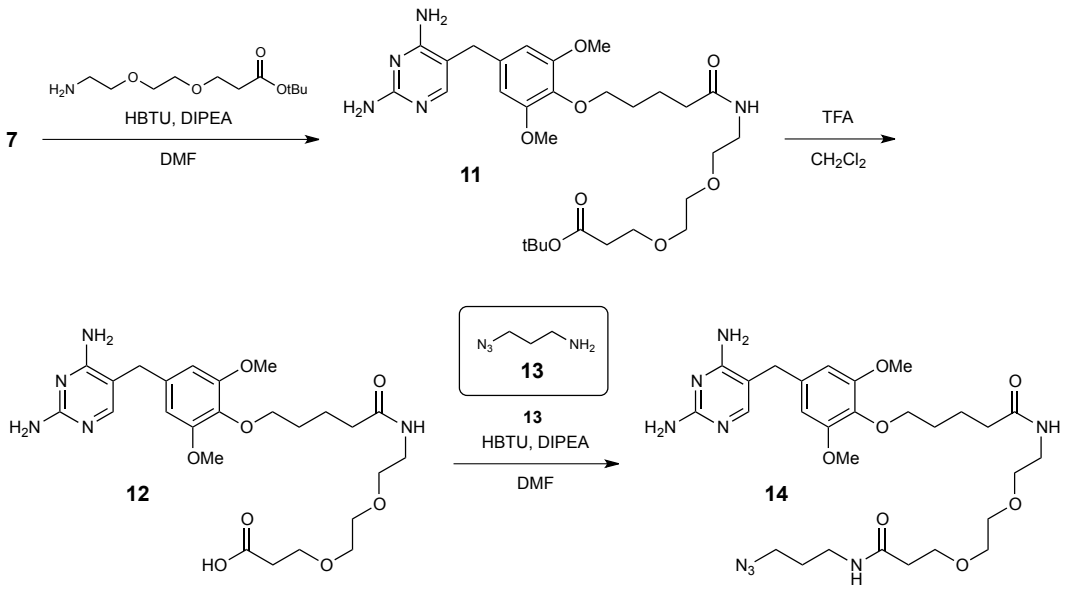
To a suspension of **7** (20 mg, 0.053 mmol) in anhydrous DMF (0.6 mL) at room temperature were added DIPEA (18 μL, 0.106 mmol) and HBTU (22.2 mg, 0.059 mmol). The mixture was stirred for 5 min, at which time the suspension became a clear solution. To this was added the solution of deprotected **10** prepared above. After stirring for 2 h, the mixture was concentrated and purified by column chromatography (silica, 10:3 $CHCl_3$/MeOH → 10:3:0.2 $CHCl_3$/MeOH/25% aqueous ammonia). The collected fraction was concentrated and triturated with Et_2O to afford hoeTMP (**3**) as a pale yellow solid (42 mg, 79%).

^{1}H NMR (400 MHz, CD_3OD): δ 8.27 (1H, s), 8.06 (2H, d, *J* = 8.8 Hz), 7.96 (1H, d, *J* =

9.2 Hz), 7.70 (1H, brd, J = 8.4 Hz), 7.51 (1H, d, J = 8.8 Hz), 7.49 (1H, s), 7.15 (1H, s), 7.09 (2H, d, J = 8.8 Hz), 7.06 (1H, dd, J = 2.4, 8.8 Hz), 6.47 (2H, s), 4.08 (2H, t, J = 6.2 Hz), 3.87 (2H, t, J = 6.2 Hz), 3.74 (6H, s), 3.60 (2H, s), 3.57 (4H, s), 3.54–3.50 (4H, m), 3.39–3.33 (4H, m), 3.24 (4H, m), 2.69 (4H, m), 2.42 (2H, t, J = 7.4 Hz), 2.38 (3H, s), 2.25 (2H, t, J = 7.4 Hz), 2.11 (2H, m), 1.79 (2H, m), 1.68 (2H, m).

HRMS (FAB): calcd for $[M]^+$, 998.5122; found, 998.5119.

Synthesis of taxTMP (4)



Scheme S4. Synthetic route of azide-functionalized TMP.

Compound **11**

To a suspension of **7** (300 mg, 0.80 mmol) in anhydrous DMF (5 mL) at room temperature were added DIPEA (0.41 mL, 2.41 mmol) and HBTU (332 mg, 0.88 mmol). The mixture was stirred for 5 min, at which time the suspension became a clear solution. To this was added a solution of amino-dPEG$_2$-*t*-butyl ester (223 mg, 0.96 mmol) in anhydrous DMF (1 mL). After stirring for 1 h, the mixture was concentrated. The residue was dissolved in $CHCl_3$ (20 mL) and washed with saturated aqueous $NaHCO_3$ (10 mL × 2) and brine (10 mL × 1). The organic layer was collected, dried over anhydrous Na_2SO_4 and concentrated. The residue was purified by column chromatography (silica, 10:1 → 10:2 AcOEt/MeOH) to afford **11** as a white solid (350 mg, 74%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.78 (1H, s), 6.37 (2H, s), 6.21 (1H, brt), 4.70 (2H, s), 4.57 (2H, s), 3.95 (2H, t, *J* = 6.0 Hz), 3.78 (6H, s), 3.71 (2H, t, *J* = 6.4 Hz), 3.65 (2H, s), 3.59 (4H, s), 3.54 (2H, t, *J* = 5.0 Hz), 3.44 (2H, m), 2.50 (2H, t, *J* = 6.4 Hz), 2.29 (2H, t, *J* = 7.2 Hz), 1.85–1.76 (4H, m), 1.45 (9H, s).

Compound **12**

To a solution of **11** (350 mg, 0.59 mmol) in anhydrous CH_2Cl_2 (2 mL) at room temperature was added TFA (2 mL). After stirring for 1 h, the mixture was co-evaporated with toluene (50 mL × 2). The residue was dissolved in MeOH (5 mL) and concentrated again to afford **12** as a light tan wax (318 mg, 100%).

^{1}H NMR (400 MHz, CD_3OD): δ 7.22 (1H, s), 6.56 (2H, s), 3.93 (2H, t, *J* = 6.0 Hz), 3.81 (6H, s), 3.73 (2H, t, *J* = 6.2 Hz), 3.66 (2H, s), 3.59 (4H, s), 3.53 (2H, t, *J* = 5.4 Hz), 3.36 (2H, t, *J* = 5.4 Hz), 2.54 (2H, t, *J* = 6.2 Hz), 2.29 (2H, t, *J* = 7.4 Hz), 1.81 (2H, m), 1.72 (2H, m).

Compound **14**

Compound **13** was synthesized as described previously.[S9] To a solution of **12** (31.6 mg, 0.059 mmol) in anhydrous DMF at room temperature were added DIPEA (30 μL, 0.176 mmol), HBTU (26.9 mg, 0.071 mmol) and **13** (8.9 mg, 0.089 mmol). After stirring for 4 h, the mixture was concentrated and purified by column chromatography (silica, 20:1 → 10:1 → 5:1 $CHCl_3$/MeOH) to afford **14** as a colorless oil (16.7 mg, 46%).

^{1}H-NMR (400 MHz, CD_3OD): δ 7.51 (1H, s), 6.51 (2H, s). 3.92 (2H, t, *J* = 6.0 Hz), 3.78 (6H, s), 3.71 (2H, t, *J* = 6.0 Hz), 3.64 (2H, s), 3.58 (4H, m), 3.52 (2H, t, *J* = 5.6 Hz), 3.37–3.34 (4H, m), 3.22 (2H, t, *J* = 6.8 Hz), 2.42 (2H, t, *J* = 6.0 Hz), 2.28 (2H, t, *J* = 7.2 Hz), 1.84–1.69 (4H, m), 1.26–1.22 (2H, m).

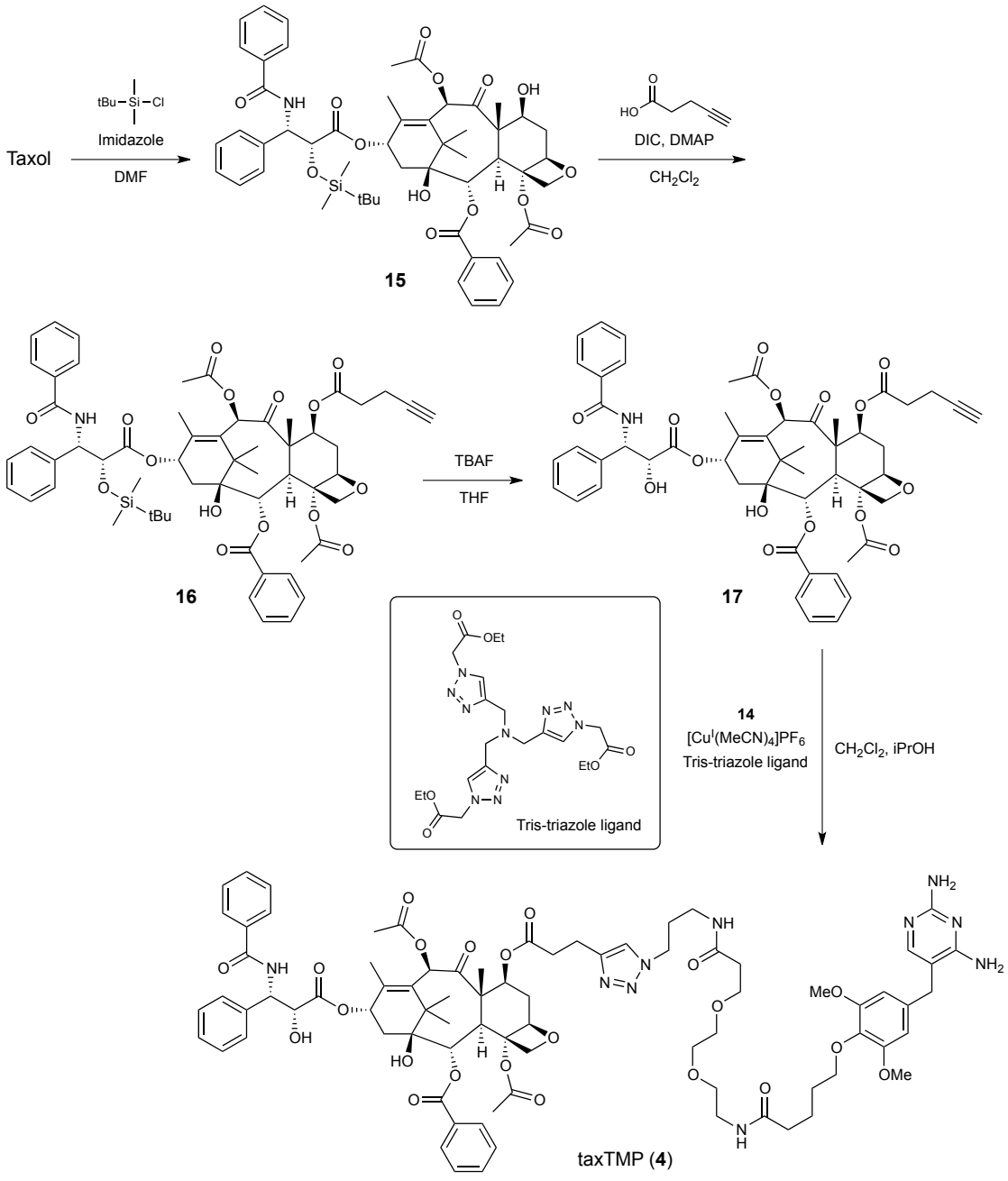
LRMS (MALDI): calcd for $[M+H]^+$, 618.34; found, 618.

Compound **15** (see next page for scheme)

This compound was synthesized by modifying a previously reported procedure.[S10] To a solution of taxol (50 mg, 0.059 mmol) in anhydrous DMF (0.2 mL) at room temperature were added imidazole (9.8 mg, 0.144 mmol) and *tert*-butyldimethylchlorosilane (26.3 mg, 0.174 mmol). After stirring for 17 h, the mixture was diluted with CH_2Cl_2 (20 mL) and washed with saturated aqueous NH_4Cl (10 mL × 1) and water (5 mL × 1). The organic layer was collected, dried over anhydrous Na_2SO_4 and concentrated. The residue was triturated with hexane to afford **15** as a white solid (55.5 mg, 98%).

^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.14 (2H, d, *J* = 7.6 Hz), 7.74 (2H, d, *J* = 6.8 Hz),

7.63–7.58 (1H, m), 7.54–7.49 (3H, m), 7.43–7.37 (4H, m), 7.34–7.32 (3H, m), 7.08 (1H, d, *J* = 8.8 Hz), 6.30 (2H, s), 5.74 (2H, m), 4.98 (1H, dd, *J* = 2.4, 7.6 Hz), 4.66 (1H, d, *J* = 2.0 Hz), 4.46–4.41 (1H, m), 4.32 (1H, d, *J* = 8.4 Hz), 4.22 (1H, d, *J* = 8.4 Hz), 3.83 (1H, d, *J* = 7.2 Hz), 2.57–2.38 (5H, m), 2.23 (3H, s), 2.17–2.10 (1H, m), 1.93–1.69 (7H, m), 1.25 (3H, s), 1.14 (3H, s), 0.80 (9H, s), –0.03 (3H, s), –0.29 (3H, s).



Scheme S5. Synthetic route of taxTMP (4).

Compound **16**

To a solution of **15** (55 mg, 0.057 mmol) in anhydrous CH_2Cl_2 at room temperature were added 4-pentynoic acid (6.9 mg, 0.070 mmol), DMAP (0.7 mg, 0.006 mmol) and DIC (11 μL, 0.071 mmol). After stirring for 26 h, the mixture was concentrated and purified by column chromatography (silica, 2:1 → 3:2 hexane/AcOEt) to afford **16** as a white solid (48.4 mg, 81%).

^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.13 (2H, d, *J* = 7.2 Hz), 7.75 (2H, d, *J* = 7.2 Hz), 7.63–7.59 (1H, m), 7.54-7.48 (4H, m), 7.44–7.39 (4H, m), 7.34–7.32 (3H, m), 7.09 (1H, d, *J* = 8.8 Hz), 6.24 (2H, s), 5.76–5.61 (2H, m), 4.98 (1H, d, *J* = 8.0 Hz), 4.68 (1H, d, *J* = 2.0 Hz), 4.46–4.41 (1H, m), 4.34 (1H, d, *J* = 8.4 Hz), 4.22 (1H, d, *J* = 8.4 Hz), 3.97 (1H, d, *J* = 7.6 Hz), 2.63–2.39 (11H, m), 2.23 (3H, s), 2.04–1.83 (7H, m), 1.22 (3H, s), 1.16 (3H, s), 0.80 (9H, s), –0.02 (3H, s), –0.29 (3H, s).

LRMS (MALDI): calcd for $[M+Na]^+$, 1070.43; found, 1070.

Compound **17**

To a solution of **16** (48 mg, 0.046 mmol) in anhydrous THF (1 mL) at room temperature was added a solution of TBAF (1.0 M) in THF (48 μL, 0.048 mmol). After stirring for 15 min, the mixture was diluted with CH_2Cl_2 (30 mL) and washed with water (10 mL × 1). The organic layer was collected, dried over anhydrous Na_2SO_4 and concentrated. The residue was triturated with hexane to afford **17** as a white solid (28.6 mg, 67%).

^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.11 (2H, d, *J* = 7.2 Hz), 7.77 (2H, d, *J* = 7.2 Hz), 7.63–7.59 (1H, m), 7.52–7.48 (5H, m), 7.42–7.39 (5H, m), 7.35–7.26 (1H, m), 7.20 (1H, d, *J* = 9.2 Hz), 6.19 (2H, s), 5.80–5.55 (2H, m), 4.93 (1H, d, *J* = 8.4 Hz), 4.80 (1H, d, *J* = 2.8 Hz), 4.31 (1H, d, *J* = 8.0 Hz), 4.28 (1H, d, *J* = 8.4 Hz), 3.91 (1H, d, *J* = 7.6 Hz), 2.64–2.12 (13H, m), 1.82–1.81 (8H, m), 1.20 (3H, s), 1.16 (3H, s).

LRMS (MALDI): calcd for $[M+K]^+$, 972.32; found, 972.

taxTMP (**4**)

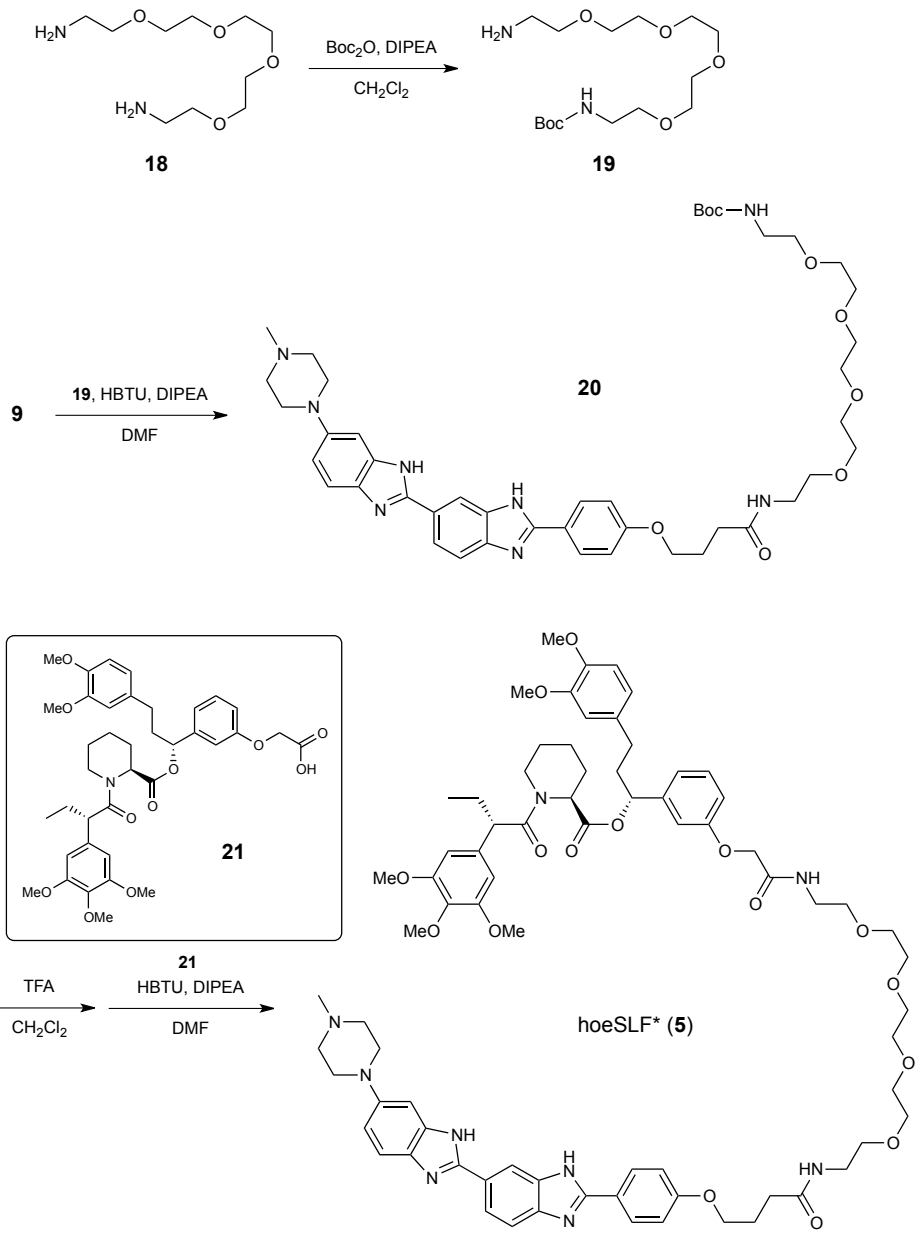
Tris-triazole ligand was synthesized as described previously.[S11] To a solution of **17** (28.6 mg, 0.031 mmol) in CH_2Cl_2 (0.25 mL) and *i*-PrOH (0.25 mL) at room temperature were added **14** (28.1 mg, 0.045 mmol) and a solution of $[Cu(MeCN)_4]PF_6$/tris-triazole ligand (1.0 M) in CH_2Cl_2 (46 μL, 0.046 mmol). After stirring for 12 h, the mixture was

concentrated and purified by reversed-phase HPLC using a semi-preparative C18 column (a linear gradient of MeCN and 10 mM aqueous ammonium acetate) to afford taxTMP (**4**) as a white solid (15.5 mg, 33%).

^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.10 (2H, d, *J* = 7.2 Hz), 7.80 (2H, d, *J* = 7.6 Hz), 7.64–7.60 (1H, m), 7.53–7.46 (7H, m), 7.41–7.37 (5H, m), 7.32–7.28 (1H, m), 6.34 (2H, s), 6.21–6.10 (2H, m), 5.77–5.65 (2H, m), 5.58–5.53 (1H, m), 5.46 (1H, s), 4.90 (1H, d, *J* = 8.4 Hz), 4.80 (1H, d, *J* = 2.8 Hz), 4.29 (2H, d, *J* = 6.4 Hz), 4.18–4.15 (1H, m), 3.95 (2H, t, *J* = 6.0 Hz), 3.98 (2H, t, *J* = 7.2 Hz), 3.76 (6H, s), 3.58 (2H, s), 3.54 (6H, s), 3.53 (2H, t, *J* = 5.6 Hz), 3.42 (2H, q, *J* = 5.6 Hz), 3.23–3.17 (2H, m), 2.98 (2H, m), 2.78–2.61 (2H, m), 2.51–1.99 (18H, m), 1.82–1.73 (11H, m), 1.64–1.59 (1H, m), 1.41–1.36 (1H, m), 1.20 (3H, s), 1.15 (3H, s).

HRMS (ESI): calcd for $[M+H]^+$, 1551.6930; found, 1551.7032.

Synthesis of hoeSLF* (5)



Scheme S6. Synthetic route of hoeSLF* (5).

Compound **19**

Compound **18** was synthesized as described previously.[S12] To a solution of **18** (1.64 g, 6.94 mmol) and DIPEA (0.59 mL, 3.47 mmol) in anhydrous CH_2Cl_2 (20 mL) at room temperature was added a solution of Boc_2O (0.76 g, 3.48 mmol) in anhydrous CH_2Cl_2

(10 mL) dropwise. After stirring for 1 h, the mixture was concentrated and purified by column chromatography (silica, 4:4:1 AcOEt/MeOH/25% aqueous ammonia). The collected fraction was concentrated, dissolved in $CHCl_3$ and filtered. The filtrate was concentrated again to afford **19** as a colorless oil (0.75 g, 64%).
^{1}H NMR (400 MHz, $CDCl_3$): δ 5.33 (1H, brs), 3.69–3.61 (12H, m), 3.57–3.54 (4H, m), 3.31 (2H, m), 2.90 (2H, t, *J* = 5.2 Hz), 2.46 (2H, brs), 1.44 (9H, s).

Compound **20**

To a suspension of **9** (50 mg, 0.098 mmol) in anhydrous DMF (1 mL) at room temperature were added DIPEA (67 μL, 0.394 mmol) and HBTU (41 mg, 0.108 mmol). The mixture was stirred for 5 min, at which time the suspension became a clear solution. To this was added a solution of **19** (40 mg, 0.119 mmol) in anhydrous DMF (0.5 mL). After stirring for 2 h, the mixture was concentrated and purified by column chromatography (silica, 1:1 AcOEt/MeOH). The collected fraction was concentrated and triturated with Et_2O to afford **20** as a pale yellow solid (65 mg, 80%).
^{1}H NMR (400 MHz, CD_3OD): δ 8.27 (1H, s), 8.07 (2H, d, *J* = 8.8 Hz), 7.96 (1H, brd), 7.70 (1H, brd), 7.52 (1H, d, *J* = 8.4 Hz), 7.15 (1H, s), 7.11 (2H, d, *J* = 8.8 Hz), 7.06 (1H, dd, *J* = 2.4, 8.8 Hz), 4.11 (2H, t, *J* = 6.2 Hz), 3.61–3.56 (12H, m), 3.53 (2H, t, *J* = 5.4 Hz), 3.46 (2H, t, *J* = 5.6 Hz), 3.38 (2H, t, *J* = 5.6 Hz), 3.24 (4H, m), 3.18 (2H, t, *J* = 5.6 Hz), 2.69 (4H, m), 2.43 (2H, t, *J* = 7.4 Hz), 2.39 (3H, s), 2.12 (2H, m), 1.41 (9H, s).

hoeSLF* (**5**)

To a solution of **20** (50.2 mg, 0.061 mmol) in CH_2Cl_2 (2 mL) at room temperature was added TFA (1 mL). After stirring for 1 h, the mixture was co-evaporated with toluene (5 mL × 2). The residue was dissolved in anhydrous DMF (0.6 mL), and then DIPEA (62 μL, 0.365 mmol) was added.

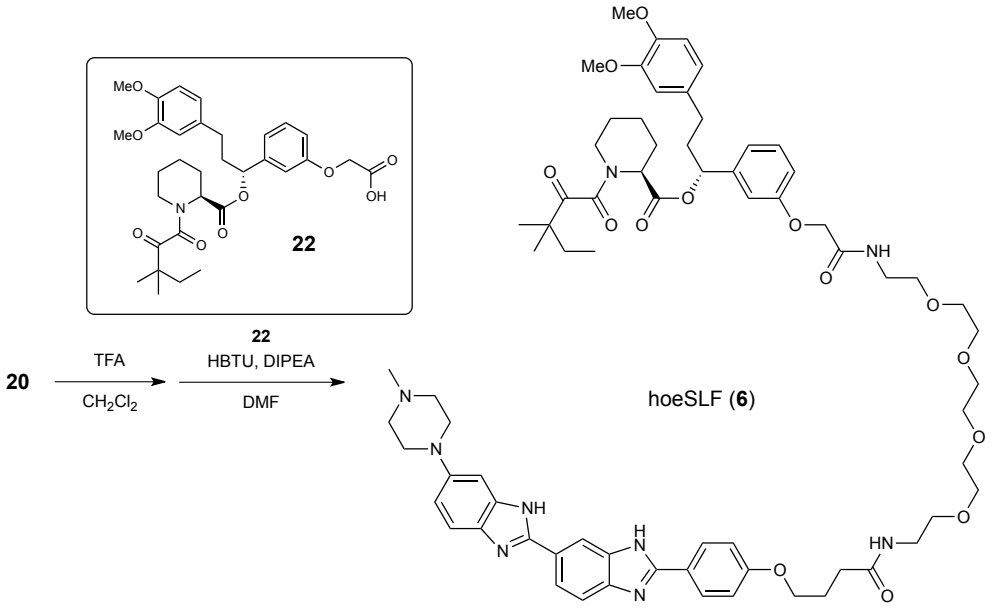
Compound **21** was synthesized as described previously.[S13] To a suspension of **21** (35 mg, 0.050 mmol) in anhydrous DMF (0.6 mL) at room temperature were added DIPEA (17 μL, 0.10 mmol) and HBTU (21 mg, 0.055 mmol). The mixture was stirred for 5 min, at which time the suspension became a clear solution. To this was added the solution of deprotected **20** prepared above. After stirring for 2 h, the mixture was concentrated and purified by column chromatography (silica, 10:1 → 10:2 $CHCl_3$/MeOH). The collected fraction was concentrated and triturated with Et_2O to afford hoeSLF* (**5**) as a pale

yellow solid (55 mg, 78%).

^{1}H NMR (400 MHz, CD_3OD): δ 8.28 (1H, s), 8.07 (2H, d, *J* = 8.8 Hz), 7.96 (1H, d, *J* = 7.6 Hz), 7.70 (1H, d, *J* = 8.8 Hz), 7.52 (1H, d, *J* = 8.8 Hz), 7.17–7.13 (2H, m), 7.10 (2H, d, *J* = 9.2 Hz), 7.07 (1H, dd, *J* = 2.4, 8.8 Hz), 6.84–6.81 (2H, m), 6.77 (1H, s), 6.71 (1H, d, *J* = 1.6 Hz), 6.64 (1H, dd, *J* = 1.6, 8.2 Hz), 6.56 (2H, s), 6.53 (1H, d, *J* = 7.6 Hz), 5.55 (1H, m), 5.37 (1H, m), 4.48 (2H, m), 4.09 (2H, t, *J* = 6.2 Hz), 3.80–3.73 (2H, m), 3.78 (3H, s), 3.77 (3H, s), 3.68 (3H, s), 3.65 (6H, s), 3.56–3.42 (18H, m), 3.36 (2H, t, *J* = 5.4 Hz), 3.29 (4H, m, overlapped with the solvent peak), 2.87 (4H, m), 2.71 (1H, m), 2.59–2.36 (2H, m), 2.52 (3H, s), 2.42 (2H, t, *J* = 7.2 Hz), 2.26 (1H, m), 2.11 (2H, m), 2.00 (2H, m), 1.86 (1H, m), 1.73–1.57 (4H, m), 1.48–1.23 (2H, m), 0.88 (3H, t, *J* = 7.2 Hz).

HRMS (MALDI): calcd for $[M+H]^+$, 1404.7127; found, 1404.7117.

Synthesis of hoeSLF (6)



Scheme S7. Synthetic route of hoeSLF (6).

hoeSLF (**6**)

To a solution of **20** (39.0 mg, 0.047 mmol) in CH_2Cl_2 (2 mL) at room temperature was added TFA (1 mL). After stirring for 1 h, the mixture was co-evaporated with toluene (5 mL × 2). The residue was dissolved in anhydrous DMF (0.5 mL), and then DIPEA (48 μL, 0.282 mmol) was added.

Compound **22** was synthesized as described previously.[S14] To a solution of **22** (23 mg, 0.039 mmol) in anhydrous DMF (0.5 mL) at room temperature were added DIPEA (13 μL, 0.076 mmol) and HBTU (16.4 mg, 0.043 mmol). The mixture was stirred for 5 min. To this was added the solution of deprotected **20** prepared above. After stirring for 2 h, the mixture was concentrated and 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). The collected fraction was concentrated and triturated with Et_2O to afford hoeSLF (**6**) as a pale yellow solid (14 mg, 22% as a tri-TFA salt).

^{1}H NMR (400 MHz, d_6-DMSO): δ 9.76 (1H, brs), 8.41 (1H, s), 8.17 (2H, d, *J* = 8.0 Hz), 8.09 (1H, m), 8.03 (1H, d, *J* = 8.4 Hz), 7.94 (1H, m), 7.85 (1H, d, *J* = 8.4 Hz), 7.67 (1H, d, *J* = 8.8 Hz), 7.32–7.25 (2H, m), 7.19 (1H, s), 7.16 (2H, d, *J* = 8.4 Hz), 6.96 (1H, m),

6.95 (1H, s), 6.90 (1H, d, J = 8.8 Hz), 6.83 (1H, d, J = 7.6 Hz), 6.75 (1H, s), 6.66 (1H, d, J = 6.8 Hz), 5.68 (1H, m), 5.15 (1H, m), 4.48 (2H, s), 4.08 (2H, t, J = 6.4 Hz), 3.91 (2H, m), 3.72 (3H, s), 3.70 (3H, s), 3.59 (2H, m), 3.48 (12H, m), 3.44–3.37 (4H, m), 3.33–3.17 (6H, m), 3.04 (4H, m), 2.91 (3H, s), 2.33–2.20 (4H, m), 2.18–2.01 (2H, m), 1.97 (2H, m), 1.76–1.52 (6H, m), 1.40–1.19 (2H, m), 1.15 (3H, s), 1.13 (3H, s), 0.79 (3H, t, J = 7.4 Hz).

HRMS (MALDI): calcd for $[M+H]^+$, 1294.6759; found, 1294.6766.

Supplementary Methods: Plasmid Construction

Restriction and DNA-modifying enzymes were purchased from New England Biolabs or Takara Bio. DNA primers were purchased from Operon Biotechnologies.

Plasmid construction was performed using standard molecular cloning techniques. All PCR amplified sequences were verified by DNA sequencing. Complete plasmid sequences are available upon request.

DNA primers

DNA primer sequences used in this study are listed below.

P1 (5' for eDHFR-GFP):
5'-AAAAC TGCAG ACCAT GGCTA TCAGT CTGAT TGCGG CGTTA GCG-3'

P2 (3' for eDHFR-GFP):
5'-CGCGG ATCCG AAGCG GCCGC CCGCC GCTCC AGAAT-3'

P3 (5' for YFP-eDHFR):
5'-ATGGA CGAGC TGTAC AAGAG TGCTG GTGGT ATCAG TCTGA TTGCG-3'

P4 (3' for YFP-eDHFR):
5'-AGCTT GAGCT CGAGG ACCAC CAGCA CTACC ACCAG CACTA CCACC AGCAC TACCA CCAGC ACTAC CACCA GCACT CCGCC GCTCC AGAAT-3'

P5 (5' for YFP-eDHFR-Akt_{KD}):
5'-GTCGA CGGTA CCGCG GGCCT CAAGA AGCAG-3'

P6 (3' for YFP-eDHFR-Akt_{KD}):
5'-GATCA GTTAG GATCC TTAGG CCGTG CTGCT GGC-3'

P7 (5' for mCherry-eDHFR-$p85_{iSH}$):
5'-pTCGAC AAGCT GGTGG TAGTG CTGGT GGTTC-3' (p = phosphate)

P8 (3' for mCherry-eDHFR-$p85_{iSH}$):
5'-pTCGAG AACCA CCAGC ACTAC CACCA GCTTG-3' (p = phosphate)

P9 (5' for $FKBP_{36V}$-mCherry):
5'-GCGGA ATTCA CCATG GGAGT GCAGG TGGAG ACTAT CTCCC CAGGA-3'

P10 (3' for $FKBP_{36V}$-mCherry):
5'-CGCGG ATCCG AAGCG GCCGC TTCCA GTTTT AGAAG CTCCA CATCG AAGAC-3'

eDHFR-GFP (pDG)

The eDHFR gene was amplified by PCR from pBAD-$DHFR^{S15}$ (provided by Prof. Teruyuki Nagamune) using P1 and P2 primers, digested with PstI and BamHI, and

inserted into the same sites of pEGFP-N1 (Clontech) to yield the pDG vector.

YFP-eDHFR (pYD)

The eDHFR gene was amplified by PCR from pDG using P3 and P4 primers, digested with BsrGI and XhoI, and inserted into the same sites of pEYFP-C1 (Clontech) to yield the pYD vector.

YFP-eDHFR-Akt$_{KD}$ (pYD-Akt$_{KD}$)

The Akt$_{KD}$ gene was amplified by PCR from the human Akt1 cDNA (provided by Prof. Yukiko Gotoh) using P5 and P6 primers, digested with SacII and BamHI, and inserted into the same sites of pEYFP-C1 to yield the pY-Akt$_{KD}$ vector.

The eDHFR gene was digested form pYD with BsrGI and XhoI and inserted into the same sites of the above pY-Akt$_{KD}$ to yield the pYD-Akt$_{KD}$ vector.

YFP-eDHFR-Tiam1$_{DH-PH}$ (pYD-Tiam1$_{DH-PH}$)

The Tiam1$_{DH-PH}$ gene was digested from pYF-Tiam1^{S3} (Addgene plasmid 20154, provided by Prof. Tobias Meyer) with XhoI and SacII and inserted into the same sites of pYD to yield the pYD-Tiam1$_{DH-PH}$ vector.

GFP-eDHFR (pGD)

The eDHFR gene was digested from pYD with BsrGI and XhoI and inserted into the same sites of pEGFP-C1 to yield the pGD vector.

GFP-eDHFR-Tiam1$_{DH-PH}$ (pGD-Tiam1$_{DH-PH}$)

The eDHFR-Tiam1$_{DH-PH}$ gene was digested from pYD-Tiam1$_{DH-PH}$ with BsrGI and SacII and inserted into the same sites of pEGFP-C1 (Clontech) to yield the pGD-Tiam1$_{DH-PH}$ vector.

mCherry-eDHFR (pRD)

The mCherry gene was digested from pmCherry-N1 (Clontech) with NheI and BsrGI and inserted into the same sites of pYD to yield the pRD vector.

mCherry-eDHFR-p85$_{iSH}$ (pRD-p85$_{iSH}$)

The $p85_{iSH}$ gene was digested from pCF-iSH[S16] (Addgene plasmid 20159, provided by Prof. Tobias Meyer) with EcoRI and XhoI and inserted into the same sites of pEYFP-C1 to yield the pY-iSH vector.

The eDHFR gene was digested from pYD with BsrGI and XhoI and inserted into the above pY-iSH. Then, a DNA fragment encoding a flexible linker sequence (QAGGSAGGSR), which was prepared by annealing P7 and P8 primers, was two tandemly inserted into the XhoI site between the eDHFR and the iSH domain to yield the pYD-iSH vector.

The mCherry gene was digested from pmCherry-N1 with NheI and BsrGI and inserted into the same sites of the above pYD-iSH to yield the pRD-iSH vector.

$FKBP_{36V}$-mCherry ($pF_{36V}R$)

The $FKBP_{36V}$ gene was amplified by PCR from pC_4-F_V1E (provided by ARIAD pharmaceuticals Inc.) using P9 and P10 primers, digested with EcoRI and BamHI, and inserted into the same sites of pmCherry-N1 to yield the $pF_{36V}R$ vector.

FKBP-GFP (pFG)

The pFG vector was originally prepared for another purpose. The detail of its construction will be reported elsewhere.

eDHFR-mCherry (pDR)

The eDHFR gene was digested from pDG with HindIII and BamHI and inserted into the same sites of pmCherry-N1 to yield the pDR vector.

Supplementary Methods: Cell Biological Experiments

Cell culture, transfection, and materials

HeLa and NIH3T3 cells were obtained from Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin at 37 ºC under 5% CO_2 atmosphere. Transfections were performed using Lipofectamine LTX (Invitrogen) according to the manufacture's protocol. HeLa cells stably expressing eDHFR-GFP and NIH3T3 cells stably expressing YFP-eDHFR-Akt_{KD} were obtained by G418 selection. For serum starvation, cells were washed and incubated in serum-free DMEM supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin. Compounds were aliquoted and stored in DMSO as 1,000× stock solutions.

Microscopy

Cells for imaging were plated in 35 mm glass-bottomed dishes (IWAKI). For NIH3T3 cells, the dishes were precoated with 0.01% poly-L-lysine solution (Sigma). All imaging was performed on an Olympus IX81-ZDC/FV1000 laser scanning confocal microscope. Fluorescence and DIC images were acquired using a 60× 1.42 NA PlanApo oil immersion objective and lasers at 405 (for Hoechst), 488 (for GFP), 515 (for YFP) and 543 nm (for TRITC and mCherry). Time-lapse imaging was conducted in an air-conditioned room at 30 ± 2 ºC. Fluorescence images were analyzed using the ImageJ software (National Institute of Health).

Immunoblotting

Cells for immunoblotting were plated in 40 mm culture dishes (TPP). Cells were washed twice with cold phosphate-buffered saline (PBS) and lysed at 4 ºC (on ice) in RIPA buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1.0% Nonidet P-40] with freshly added Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific). The cell lysates were cleared by centrifugation, mixed with 2× SDS-PAGE sample buffer [0.1 M Tris-HCl (pH 6.8), 4% SDS, 12% 2-mercaptoethanol (2ME), 20% glycerol, 0.02% bromophenol blue] and

heated at 95 ºC for 5 min. The samples were resolved by SDS-PAGE and transferred to Immun-Blot PVDF membranes (Bio-Rad Laboratories). After blocking with 5% skim milk in Tris-buffered saline containing 0.1% Tween-20 (TBST), the membranes were probed with appropriate antibodies. TBST was used for all washing steps. The immunoblots were developed with SuperSignal West Pico Chemiluminescent Substrate or SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific) and detected using a ChemiDoc MP system (Bio-Rad Laboratories). For reprobing, the membranes were incubated in stripping buffer [62.7 mM Tris-HCl (pH 6.8), 2% SDS, 100 mM 2ME] at 50 ºC for 30 min and washed. Band intensities were quantified using the Image Lab software (Bio-Rad Laboratories).

Antibodies used in this work are listed below (all from Cell Signaling Technology, except for FKBP12 from Abcam).

Primary antibodies: anti-phospho-Akt (Thr308) rabbit mAb (C31E5E), anti-phospho-Akt (Ser473) rabbit mAb (D9E), anti-Akt1 rabbit mAb (C73H10), anti-phospho-GSK-3α/β (Ser21/9) rabbit mAb (GSK-3α preferred) (37F11), anti-phospho-GSK-3β (Ser9) rabbit mAb (D85E12), anti-GSK-3α/β rabbit mAb (D75D3), anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) rabbit mAb (D13.14.4E), anti-p44/42 MAPK (Erk1/2) rabbit mAb (137F5), anti-GAPDH rabbit mAb (14C10), anti-Histone H3 rabbit mAb (D1H2), and anti-FKBP12 rabbit pAb.

Secondary antibody: anti-rabbit IgG, HRP-linked antibody.

eDHFR-GFP translocation

HeLa cells expressing eDHFR-GFP were cultured for 24–30 h. The cell culture medium was changed to an imaging medium (serum- and phenol red-free DMEM supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin, $DMEM_i$) after two times of washing with the same medium. Fluorescence images were taken at various time points after addition of compounds.

Fig. S1c: HeLa cells expressing eDHFR-GFP were cultured for 24 h and then stained with BODIPY TR ceramide (the Golgi marker, Molecular Probes) according to the manufacture's protocol. The cells were incubated with 5 μM mgcTMP in $DMEM_i$ at 37 ºC for 20 min and subjected to imaging.

Fig. S5a: HeLa cells (non-transfected) were cultured for 12–18 h and then incubated with 5 μM hoeTMP in $DMEM_i$ at 37 ºC for 90 min. After washing with $DMEM_i$, the

cells were subjected to imaging.

Fig. S5f and **S6e**: After inducing eDHFR-GFP translocation using 5 μM hoeTMP or taxTMP, the cells were washed with $DMEM_i$ and incubated at 37 ºC for 24 h. The cells were then subjected to imaging.

Fig. S6c: HeLa cells were transfected with pDR for 24 h. The cells were incubated with 5 μM taxTMP together with 0.5 μM TubulinTracker Green (the microtubule maker, Molecular Probes) in $DMEM_i$ at 37 ºC for 40 min. After washing with $DMEM_i$, the cells were subjected to imaging.

Fig. S7a,**c**,**d**: After the first translocation, the cell culture medium was changed to fresh $DMEM_i$. The cells were subjected to the second addition of 50 μM free TMP and imaging.

Fig. S7b: After the first translocation, the cell culture medium was changed to fresh $DMEM_i$ containing 50 μg/mL cycloheximide (Wako). The cells were incubated at 37 ºC for 90 min (to stop protein synthesis) and then subjected to the second addition of 50 μM free TMP and imaging in the same cycloheximide-containing medium.

YFP-eDHFR-Akt_{KD} translocation and activation

NIH3T3 cells expressing YFP-eDHFR-Akt_{KD} were cultured for 12–18 h, followed by serum-starvation for 6–8 h. Live cell fluorescence imaging was carried out in the same manner as described in the "eDHFR-GFP translocation" section. For immunoblot analysis, the cells were incubated with 5 μM mgcTMP ± 50 μM TMP, or 10% FBS in $DMEM_i$ at 37 ºC for 0 or 15 min. The cells were subjected to immunoblotting as described in the "Immunoblotting" section.

YFP-eDHFR-$Tiam1_{DH\text{-}PH}$ translocation, Rac activation, and lamellipodia formation

Cell morphology analysis. NIH3T3 cells were transfected with none, pYD or pYD-$Tiam1_{DH\text{-}PH}$ for 10–12 h, followed by serum-starvation for 12–14 h. The cells were incubated with 0.1% DMSO (vehicle) or 5 μM mgcTMP ± 50 μM TMP in $DMEM_i$ at 37 ºC for 1 h. The cells were washed with PBS at room temperature, fixed in 4% paraformaldehyde in PBS for 15 min, and permeabilized in 0.2% Triton X-100 in PBS for 5 min. The cells were then stained with 0.5 μg/mL phalloidin-TRITC (Sigma) in PBS for 10 min. After washing with PBS, the cells were subjected to imaging.

Live cell Imaging. NIH3T3 cells were co-transfected with pPakGBD-mCherry[S1]

(Addgene plasmid 22280, pAL197, provided by Prof. Christopher Voigt) and either pGD or pGD-Tiam1$_{DH\text{-}PH}$ for 10–12 h, followed by serum-starvation for 12–14 h. Live cell fluorescence imaging was carried out in the same manner as described in the "eDHFR-GFP translocation" section.

mCherry-eDHFR-p85$_{iSH}$ translocation, PIP$_3$ formation, and Akt activation

Live cell imaging. HeLa cells were co-transfected with pAktPH-GFP[S2] (Addgene plasmid 18836, pcDNA3-AKT-PH-GFP, provided by Prof. Craig Montell) and either pRD or pRD-p85$_{iSH}$ for 12–18 h, followed by serum-starvation for 6–8 h. Live cell fluorescence imaging was carried out in the same manner as described in the "eDHFR-GFP translocation" section.

Immunoblot analysis. HeLa cells were transfected with pRD or pRD-p85$_{iSH}$ for 12–18 h, followed by serum-starvation for 6–8 h. The cells were incubated with 5 μM mgcTMP ± 50 μM TMP in DMEM$_i$ at 37 ºC for 0 or 15 min. The cells were subjected to immunoblotting as described in the "Immunoblotting" section.

FKBP$_{36V}$-mCherry translocation

HeLa cells were transfected with pF$_{36V}$R for 24 h. Live cell fluorescence imaging was carried out in the same manner as described in the "eDHFR-GFP translocation" section.

Orthogonal translocation of two distinct proteins in the same cell

Control by SLLs. HeLa cells were co-transfected with pDG and pF$_{36V}$R for 12–18 h. Live cell fluorescence imaging was carried out in the same manner as described in the "eDHFR-GFP translocation" section. hoeSLF* and either one of TMP-based SLLs (both 2.5 μM) were sequentially added to the same medium.

Control by SLLs and the rapamycin CID system. HeLa cells were co-transfected with pLDR[S3] (Addgene plasmid 20147, pLyn11-targeted FRB, provided by Prof. Tobias Meyer) and pFG and pDR for 12–18 h. Live cell fluorescence imaging was carried out in the same manner as described in the "eDHFR-GFP translocation" section. Either one of TMP-based SLLs and rapamycin (LC Laboratories) (both 2.5 μM) were sequentially added to the same medium.

Nuclear translocation of nFKBP

HeLa cells (non-transfected) were cultured for 12–18 h and then incubated with 5 μM hoeSLF ± 20 μM rapamycin in $DMEM_i$ at 37 ºC for 2 h. Live cell fluorescence imaging was carried out after washing the cells with $DMEM_i$. For immunoblot analysis, the cells were washed and separated into nuclear and cytosolic fractions using NE-PER Nuclear and Cytoplasmic Extraction Reagent Kit (Thermo Fisher Scientific) according to the manufacture's protocol. The nuclear and cytosolic fractions were subjected to immunoblotting as described in the "Immunoblotting" section.

Cytotoxity assays

HeLa cells (non-transfected) and eDHFR-GFP-expressing HeLa cells were cultured in 96-well plates (TPP) for 12–18 h and then incubated with none, 0.1% DMSO (vehicle), 5 μM mgcTMP, 20 μM mgcTMP, 5 μM hoeTMP, 5 μM taxTMP, 5 μM hoeSLF* or 5 μM hoeSLF in $DMEM_i$ at 37 ºC for 2 h. The cells were then subjected to viability assay using Cell Counting Kit-8 (Dojindo) according to the manufacture's protocol. Absorbance at 450 nm was measured using a Model 680 Microplate Reader (Bio-Rad Laboratories).

References

(S1) Levskaya, A.; Weiner, O. D.; Lim, W. A.; Voigt, C. A. *Nature* **2009**, *461*, 997.

(S2) Kwon, Y.; Hofmann, T.; Montell, C. *Mol. Cell* **2007**, *25*, 491.

(S3) Inoue, T.; Heo, W. D.; Grimley, J. S.; Wandless, T. J.; Meyer, T. *Nat. Methods* **2005**, *2*, 415.

(S4) Dumontet, C.; Jordan, M. A. *Nat. Rev. Drug Discov.* **2010**, *9*, 790.

(S5) Ahearn, I. M.; Tsai, F. D.; Court, H.; Zhou, M.; Jennings, B. C.; Ahmed, M.; Fehrenbacher, N.; Linder, M. E.; Philips, M. R. *Mol. Cell* **2011**, *41*, 173 and references cited therein.

(S6) Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. I. *Anal. Biochem.* **1970**, *34*, 595.

(S7) Ando, T.; Tsukiji, S.; Tanaka, T.; Nagamune, T. *Chem. Commun.* **2007**, *43*, 4995.

(S8) Rastogi, K.; Chang, J.-Y.; Pan, W.-Y.; Chen, C.-H.; Chou, T.-C.; Chen, L.-T.; Su, T.-L. *J. Med. Chem.* **2002**, *45*, 4485.

(S9) Tamanini, E.; Rigby, S. E. J.; Motevalli, M.; Todd, M. H.; Watkinson, M. *Chem. Eur. J.* **2009**, *15*, 3720.

(S10) Gibson, J. D.; Khanal, B. P.; Zubarev, E. R. *J. Am. Chem. Soc.* **2007**, *129*, 11653.

(S11) Zhou, Z.; Fahrni, C. J. *J. Am. Chem. Soc.* **2004**, *126*, 8862.

(S12) Ciuffarin, E.; Isola, M.; Leoni, P. *J. Org. Chem.* **1981**, *46*, 3064.

(S13) Yang, W.; Rozamus, L. W.; Narula, S.; Rollins, C. T.; Yuan, R.; Andrade, L. J.; Ram, M. K.; Phillips, T. B.; van Schravendijk, M. R.; Dalgarno, D.; Clackson, T.; Holt, D. A. *J. Med. Chem.* **2000**, *43*, 1135.

(S14) Keenan, T.; Yaeger, D. R.; Courage, N. L.; Rollins, C. T.; Pavone, M. E.; Rivera, V. M.; Yang, W.; Guo, T.; Amara, J. F.; Clackson, T.; Gilman, M.; Holt, D. A. *Bioorg. Med. Chem.* **1998**, *6*, 1309.

(S15) Tanaka, T.; Kamiya, N.; Nagamune, T. *FEBS Lett.* **2005**, *579*, 2092.

(S16) Suh, B.-C.; Inoue, T.; Meyer, T.; Hille, B. *Science* **2006**, *314*, 1454.