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Supplemental information

Fatty acylation enhances the cellular internalization and cytosolic distribution of a cystine-knot peptide

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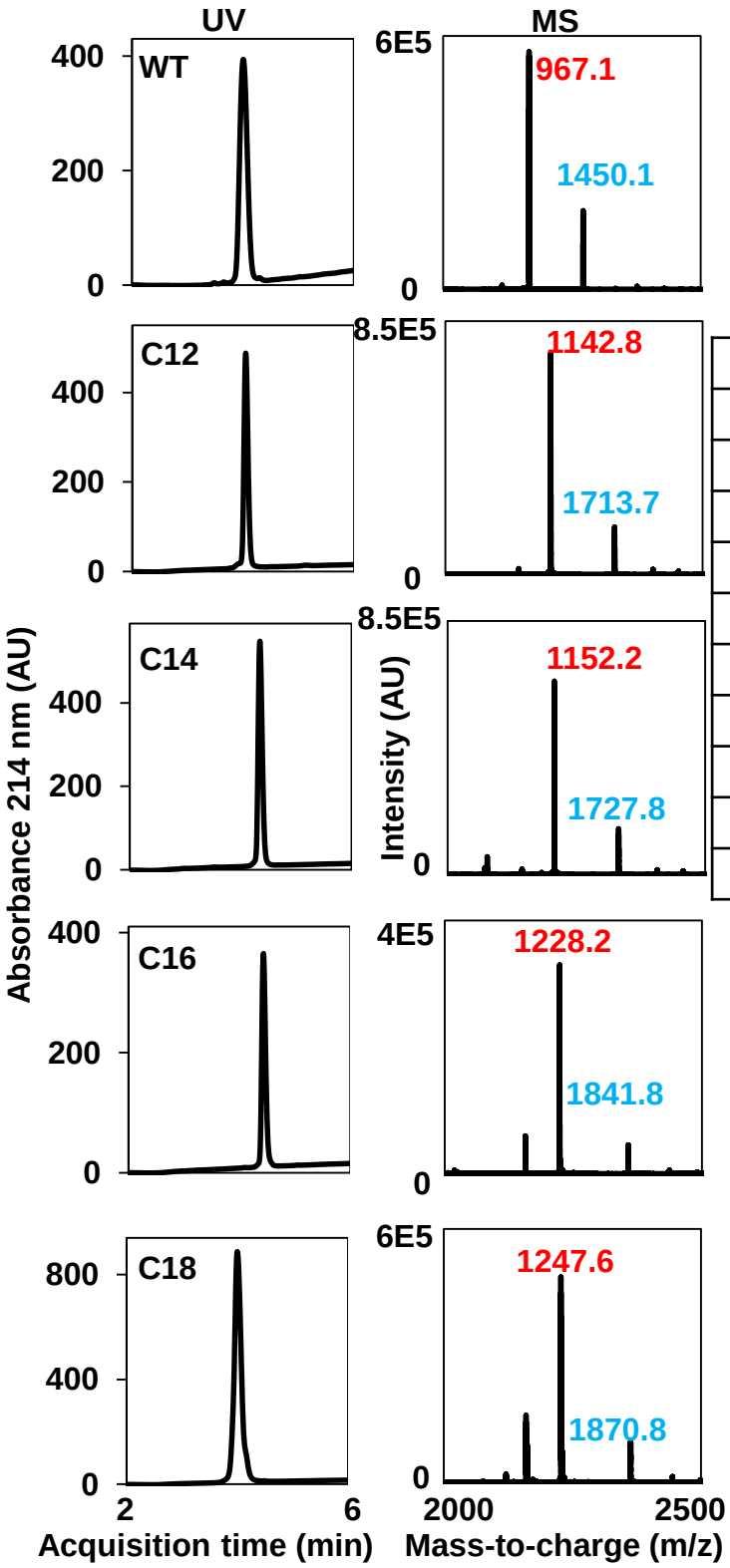
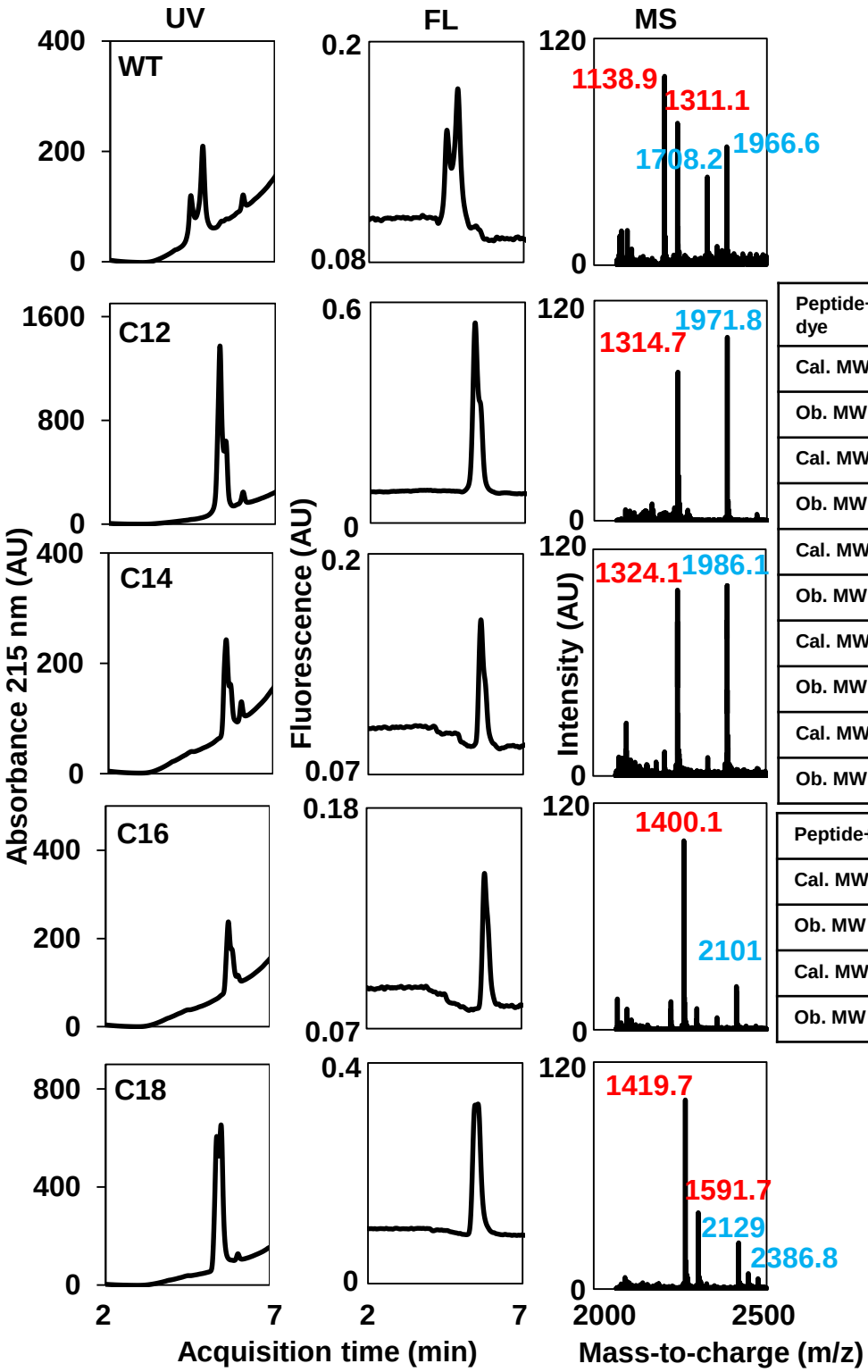


	$[M+2H]^{2+}$	$[M+3H]^{3+}$
Cal. MW (WT)	1450.2	967.1
Ob. MW (WT)	1450.1	967.1
Cal. MW (C12)	1713.3	1142.6
Ob. MW (C12)	1713.7	1142.8
Cal. MW (C14)	1726.7	1151.5
Ob. MW (C14)	1727.8	1152.2
Cal. MW (C16)	1842.7	1228.8
Ob. MW (C16)	1841.8	1228.2
Cal. MW (C18)	1871.2	1247.8
Ob. MW (C18)	1870.8	1247.6

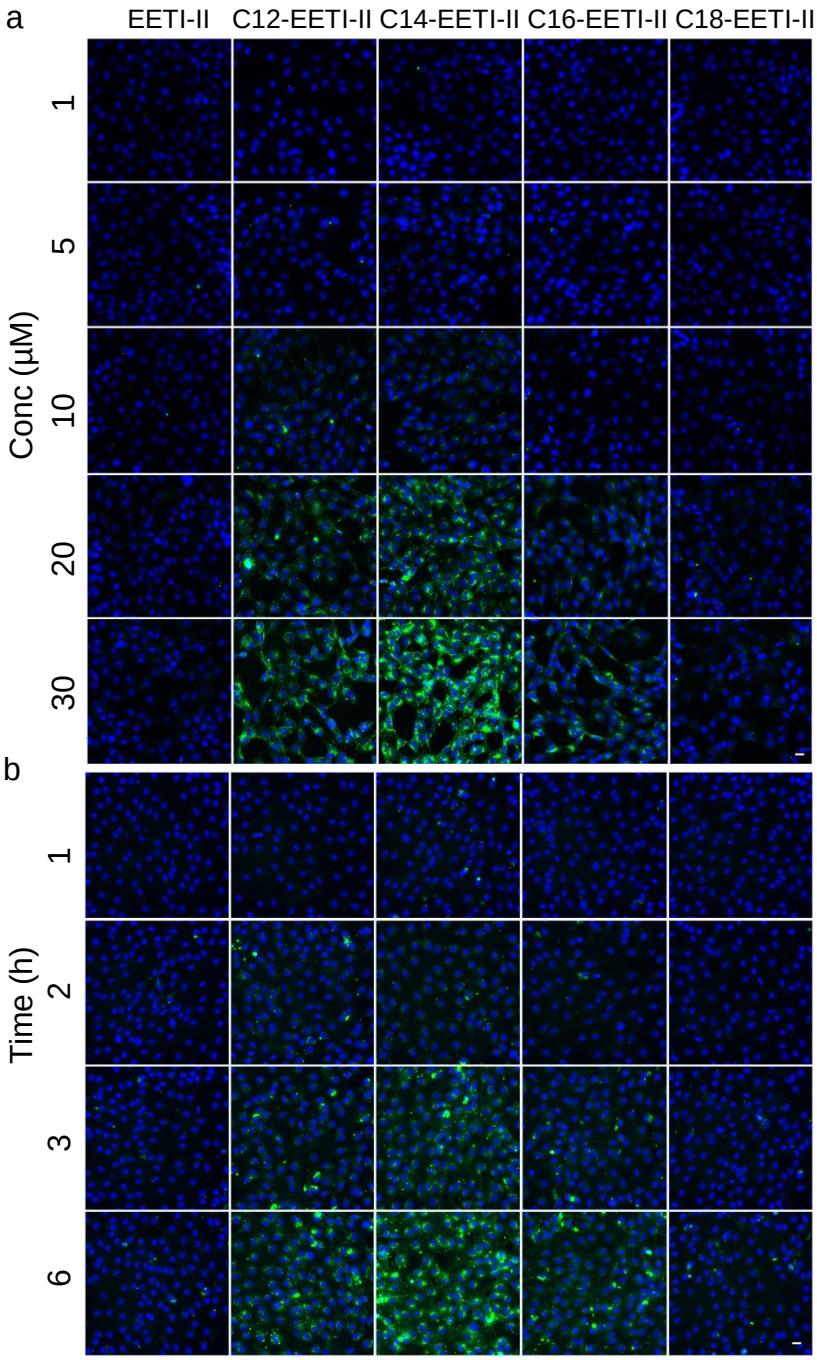
Supplementary Figure 1. LC-MS analysis of purified folded lipid EETI-II, related to STAR Methods. Representative HPLC trace (UV) and mass spectrometry analysis of WT and lipid EETI-II after RP-HPLC purification. Red: $[M+3H]^{3+}$, blue: $[M+2H]^{2+}$



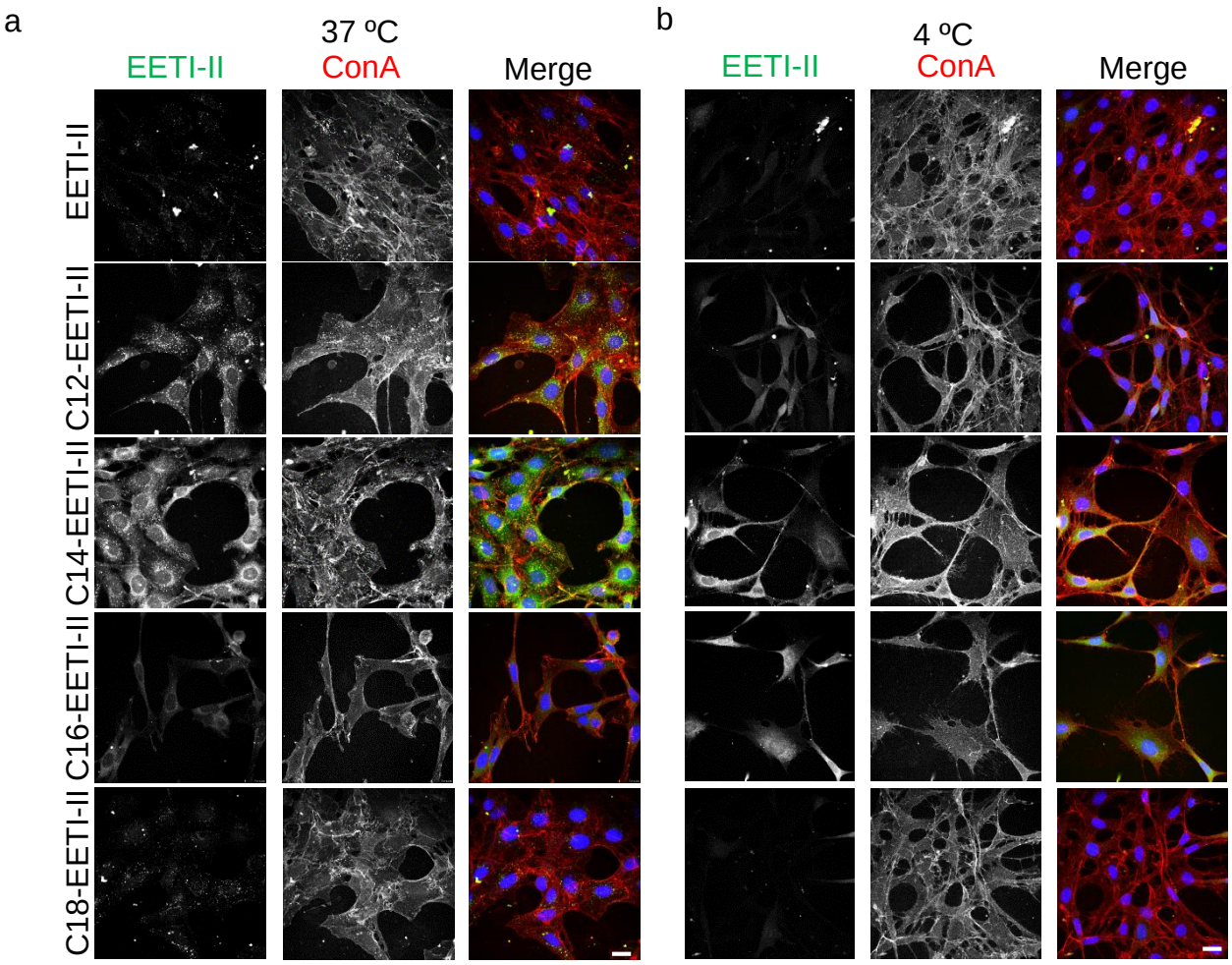
Peptide+1 dye	$[M+2H]^{2+}$	$[M+3H]^{3+}$
Cal. MW (WT)	1708.2	1138.8
Ob. MW (WT)	1708.2	1138.9
Cal. MW (C12)	1971.3	1314.2
Ob. MW (C12)	1971.8	1314.7
Cal. MW (C14)	1984.7	1323.5
Ob. MW (C14)	1986.1	1324.1
Cal. MW (C16)	2100.2	1400.1
Ob. MW (C16)	2101	1400.1
Cal. MW (C18)	2128.9	1419.1
Ob. MW (C18)	2129	1419.7

Peptide+2 dye	$[M+2H]^{2+}$	$[M+3H]^{3+}$
Cal. MW (WT)	1967.2	1311.8
Ob. MW (WT)	1966.6	1311.1
Cal. MW (C18)	2387.2	1591.8
Ob. MW (C18)	2386.8	1591.7

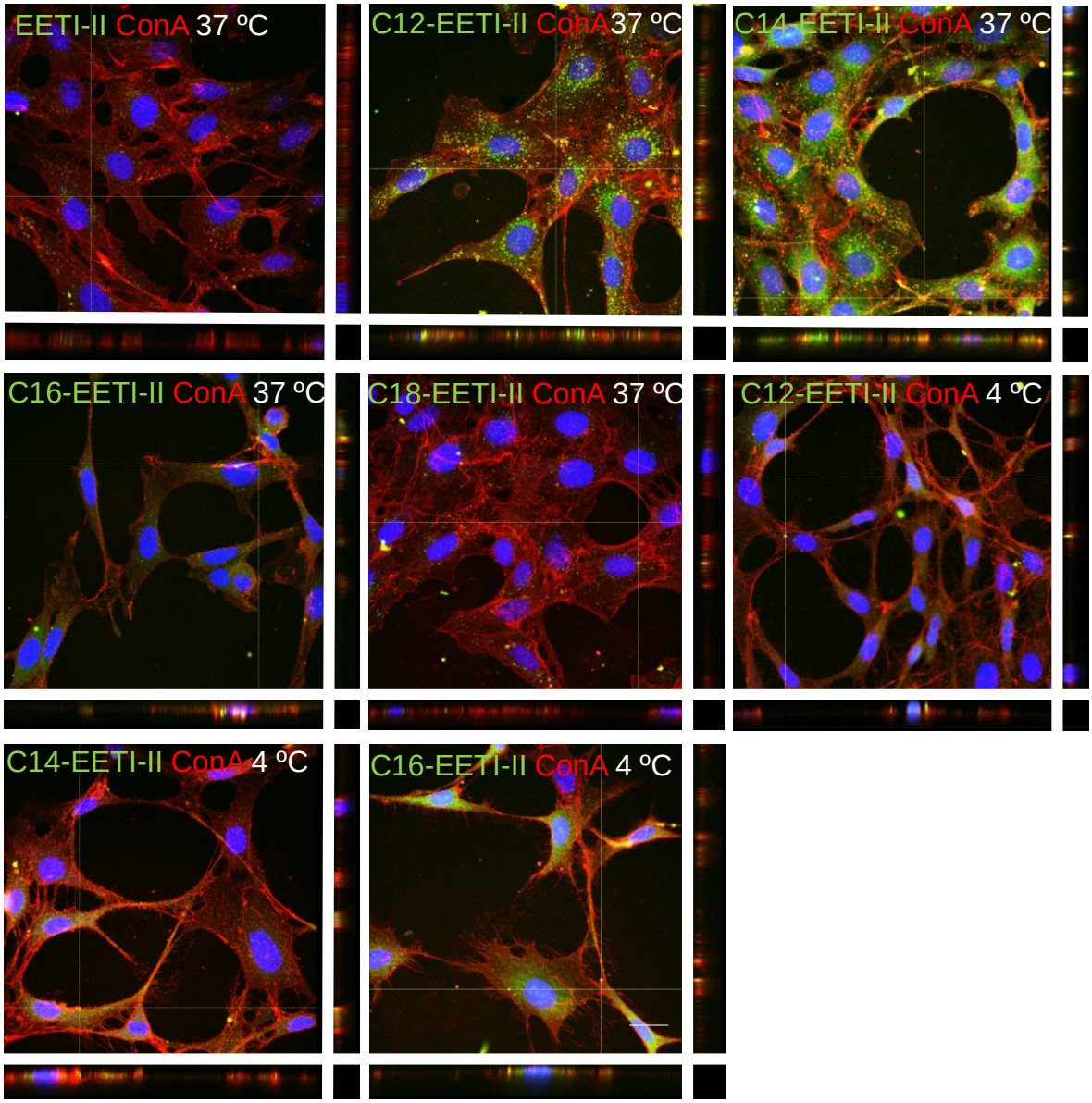
Supplementary Figure 2. LC-MS analysis of Alexa488 labeled lipid EETI-II, related to STAR Methods. Representative HPLC trace (UV and fluorescence) and mass spectrometry analysis of Alexa488 labeled WT and lipid EETI-II after RP-HPLC purification. Red: $[M+3H]^{3+}$, blue: $[M+2H]^{2+}$.



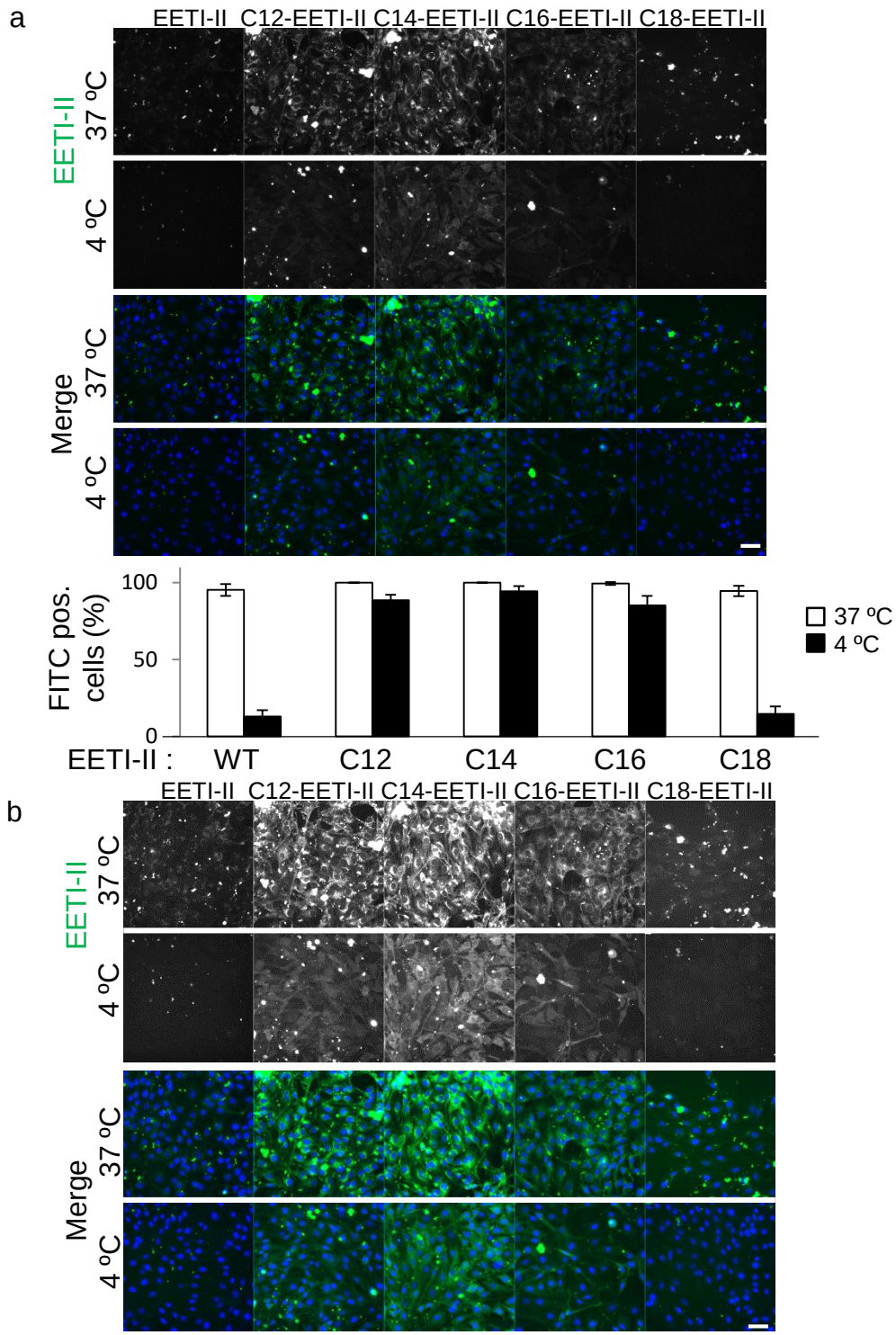
Supplementary Figure 3. Lipid EETI-II peptides are efficiently internalized into mammalian cells, related to Figure 1. NIH 3T3 cells were treated with (a) increasing concentrations (1, 5, 10, 20, 30 µM) of Alexa488 labeled peptides for 2 h, or (b) 5 µM of Alexa488 labeled WT and lipid EETI-II for 1, 2, 3, 6 h. Cells were processed as described in methods. All samples were imaged on a high throughput ImageXpress Micro XL imaging system (Molecular Devices) with a 40x objective and images were analyzed by MetaXpress 4.0. Integrated fluorescence intensity values above a threshold defined using the DMSO-treated samples were measured and normalized to samples with the highest signal. Mean ± SD. n = 1,000 cells. Representative images from at least three independent experiments are shown. Scale bar, 20 µm.



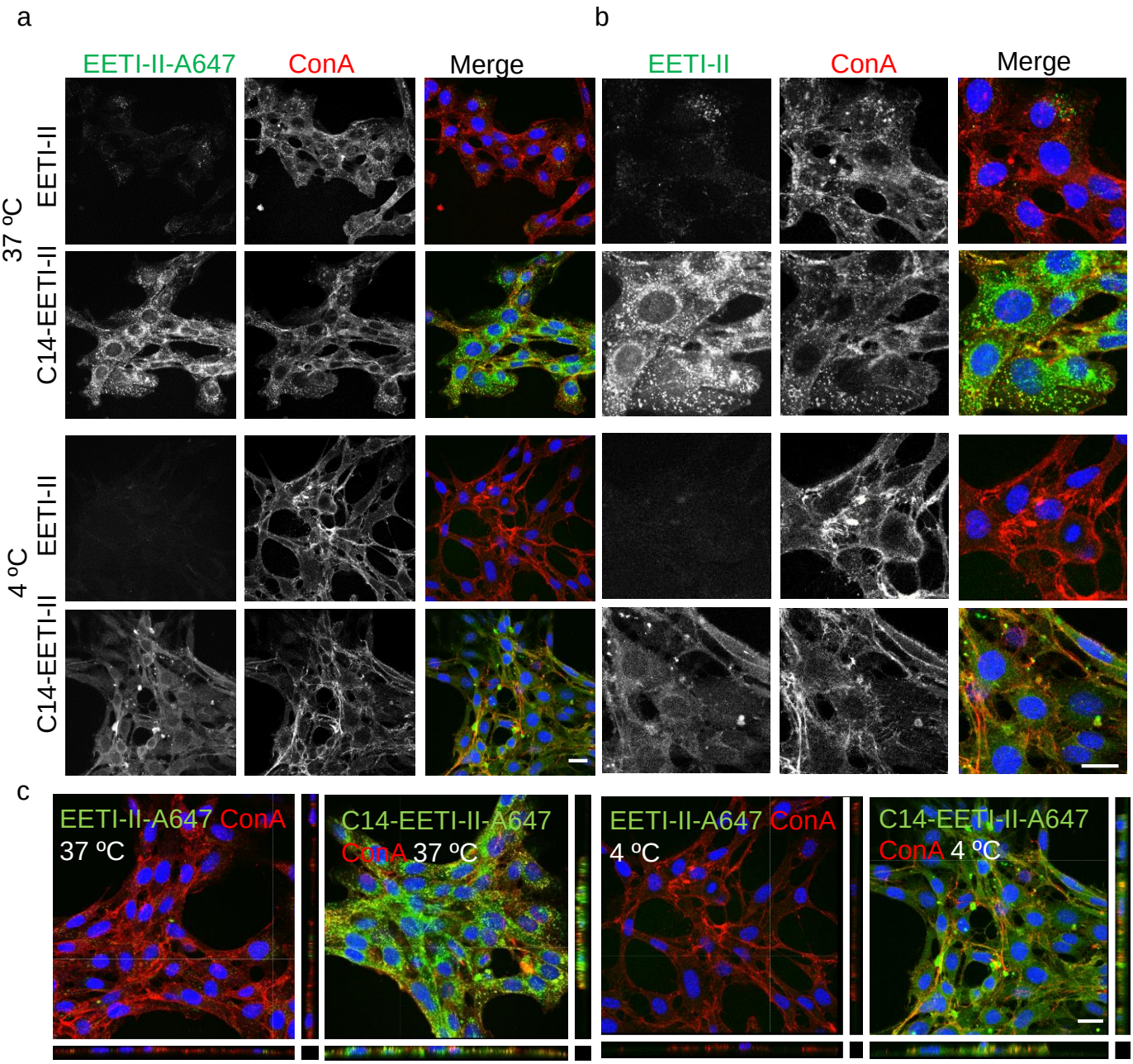
Supplementary Figure 4. Lipid EETI-II peptides localize in cellular vesicles, plasma membrane and cytosol, related to Figure 2. Full site view of cell images. NIH 3T3 cells were treated with the indicated peptides (5 µM) for 2 h at 37 ºC (a) or 4 ºC (b). Cells were washed with PBS and fixed with 4% PFA (20 min at RT for 37 ºC treated cells, and 20 min at 4 ºC then 20 min at RT for 4 ºC treated cells to avoid internalization of membrane bound peptides). Cells were then incubated with tetramethylrhodamine Conjugate Concanavalin A (10 µg/ml, shown in red) for 10 min at RT to label the plasma membrane. Images of samples on coverslips were captured on an upright AX10 LEICA SPE laser scanning confocal microscope and images were analyzed with the LAS AF image processing software (Leica Microsystems). Representative images from two independent experiments are shown. Scale bar, 20 µm.



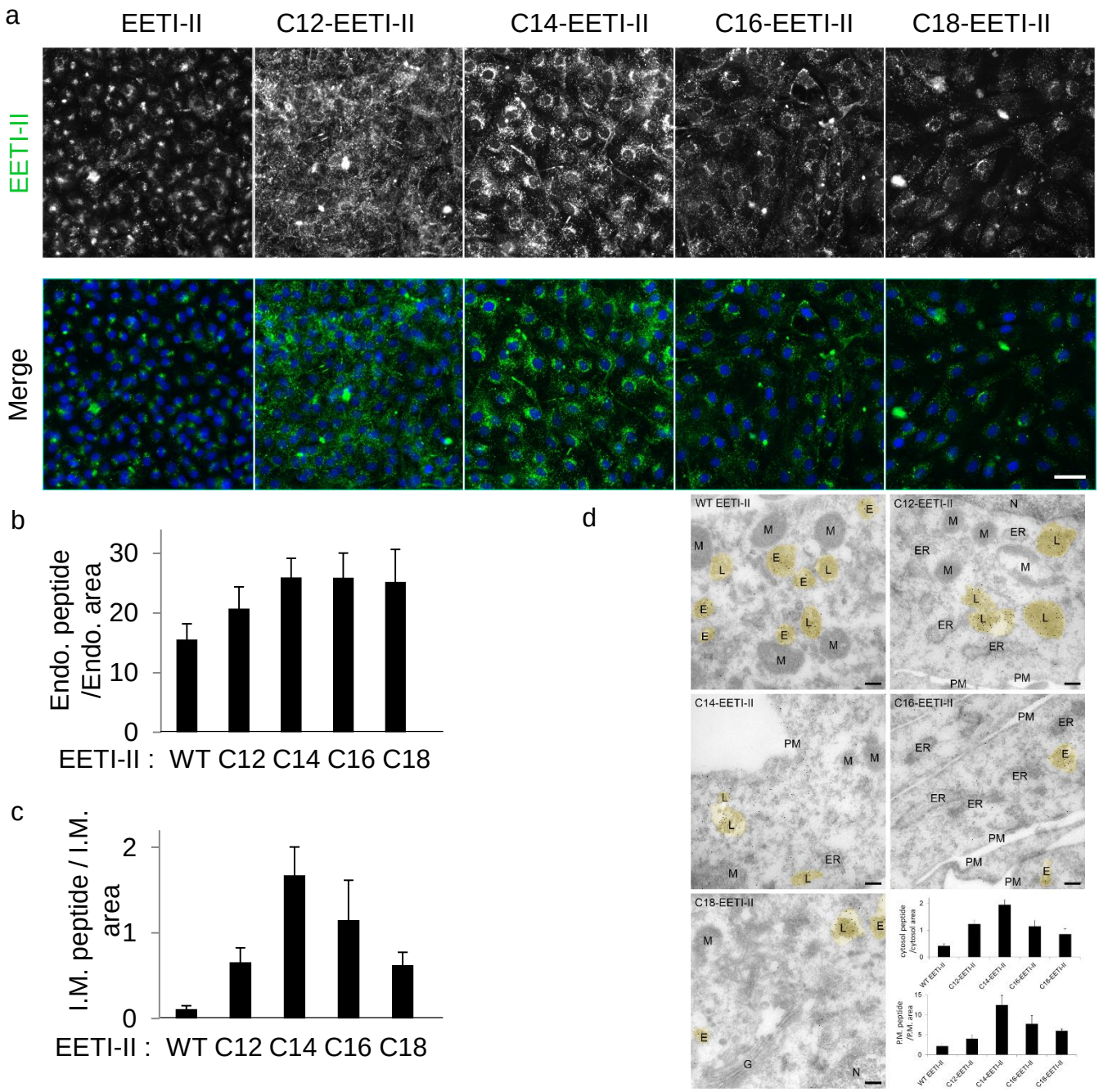
Supplementary Figure 5. Lipid EETI-II peptides localize in cellular vesicles, plasma membrane and cytosol, related to Figure 2. Representative images showing 3D sections of cellular distribution of acylated EETI-II peptides. NIH 3T3 cells were treated with the peptides (5 µM) for 2 h at 37 ºC or 4 ºC. Z sections were acquired with 0.44-µm spacing. For samples treated with WT and C18-EETI-II at 4 ºC , Z sections were not acquired (low fluorescence levels in the FITC channel). Scale bar, 20 µm.



Supplementary Figure 6. Lipid EETI-II peptides localize in cellular vesicles, plasma membrane and cytosol. Related to Figure 2. (a) NIH 3T3 cells were treated with the peptides (5 µM) for 2 h at 37 ºC or 4 ºC. Cells were washed with PBS at the end of the study and fixed with 4% PFA (20 min at RT for 37 ºC treated cells, and 20 min at 4 ºC then 20 min at RT for 4 ºC treated cells to avoid internalization of membrane bound peptides). Fluorescence images were captured on ImageXpress Micro XL (Molecular Devices). Images were analyzed by MetaXpress 4.0. Mean ± SD. n = 800 cells. Representative images from at least four independent experiments are shown. (b) Samples were imaged using longer exposure time to show the cellular distribution of 4 ºC samples. Green: peptides; blue: nuclei. Scale bar, 50 µm.

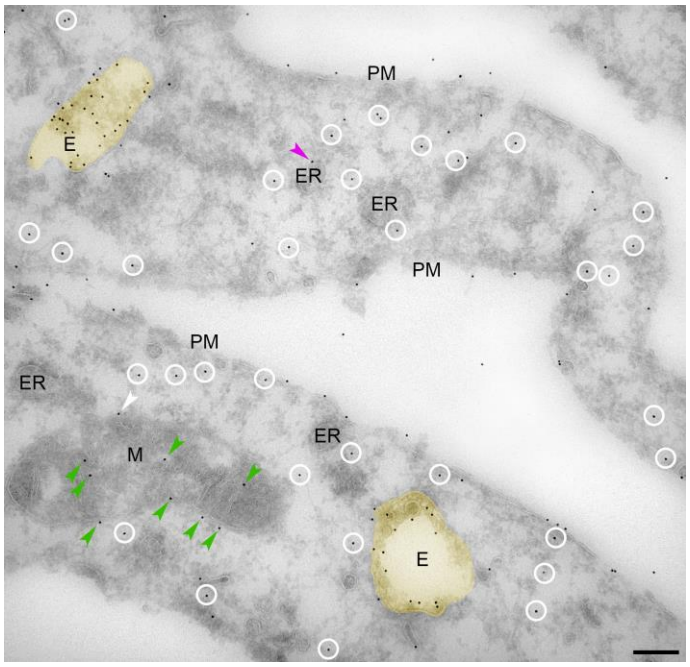


Supplementary Figure 7. Lipid EETI-II peptides localize in cellular vesicles, plasma membrane and cytosol. Related to Figure 2. NIH 3T3 cells were treated with Alexa647 labeled WT or C14-EETI-II (5 µm, shown in green, false colored) for 2 h at 37 ºC or 4 ºC**.** Representative images from two independent experiments are shown. Scale bar, 20 µm. (a), full site view of cell images; (b), zoom-ins; (c) 3D sections (0.44-µm spacing).

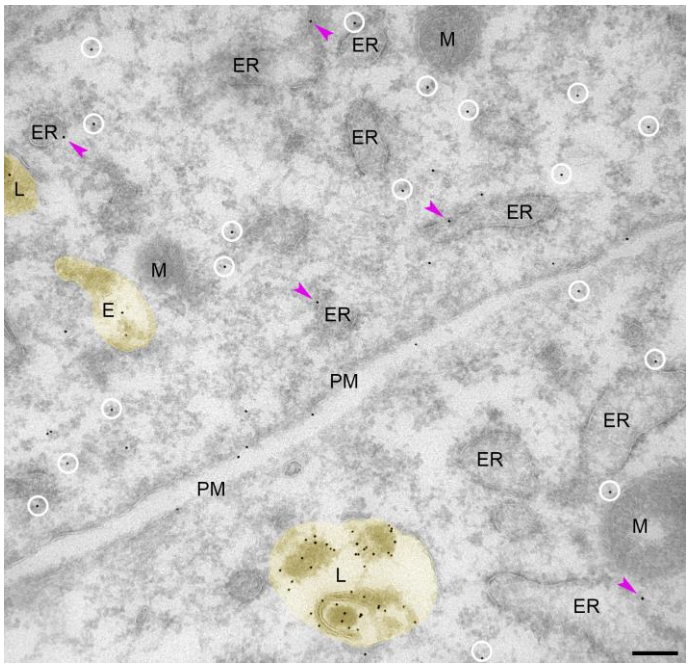


Supplementary Figure 8. Lipid EETI-II peptides show improved membrane association. Related to Figure 3. (a) Fluorescence images of cells treated with the same peptide solutions as in Fig 3 (50 µM for WT EETI-II; 10 µM for C12- and C14-EETI-II; 20 µM for C16-EETI-II; 40 µM for C18-EETI-II; 6 h incubation at 37 °C). Scale bar, 50 µm. (b) WT, C12-, C14-, C16-, and C18-EETI-II show similar levels of endosomal/lysosomal localization. The numbers of gold particles in endosomes and lysosomes (lumen and limiting membrane) were counted and divided by the compartment area (in arbitrary units). Error bars represent S.E.M. (c) Lipid EETI-II peptides show higher levels of intracellular membrane localization than WT EETI-II. I.M.: intracellular membrane. The numbers of 10 nm gold particles in ER, Golgi and mitochondrial compartments were counted and divided by the compartment area (in arbitrary units). n > 10 images per treatment. Error bars represent S.E.M. (d) Original micrographs as shown in Figure 3 without annotations. Scale bars, 200 nm.

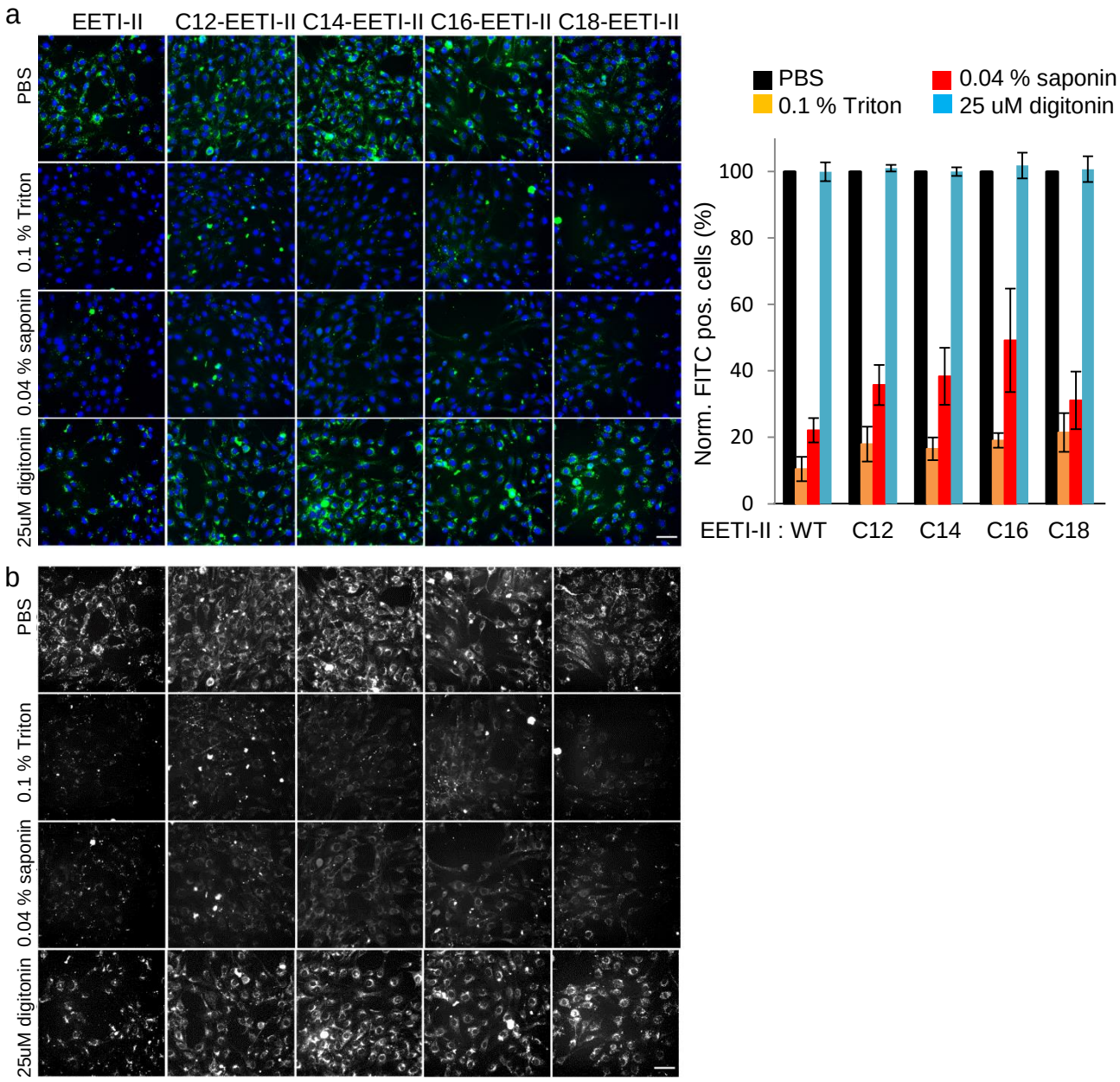
a



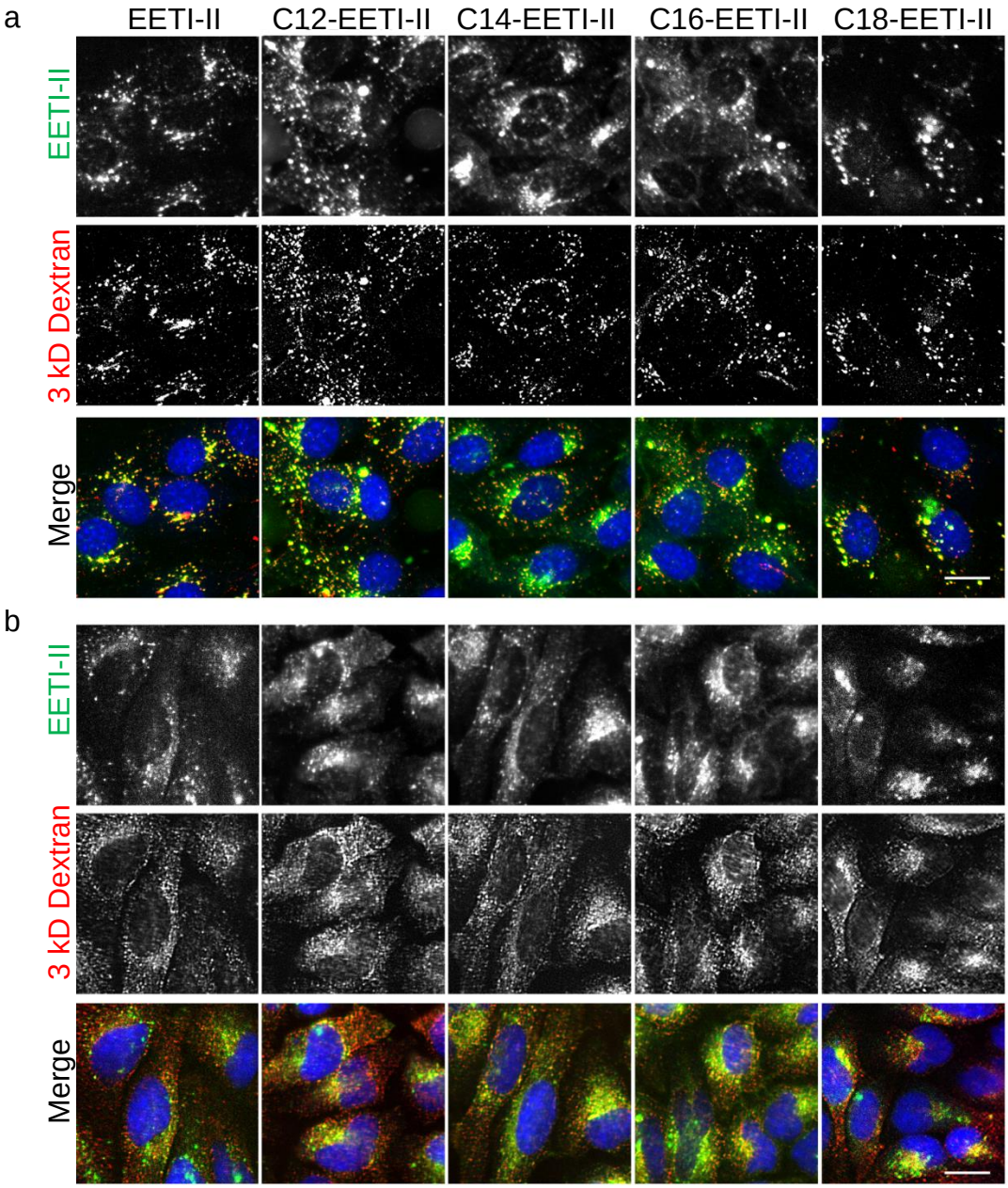
b



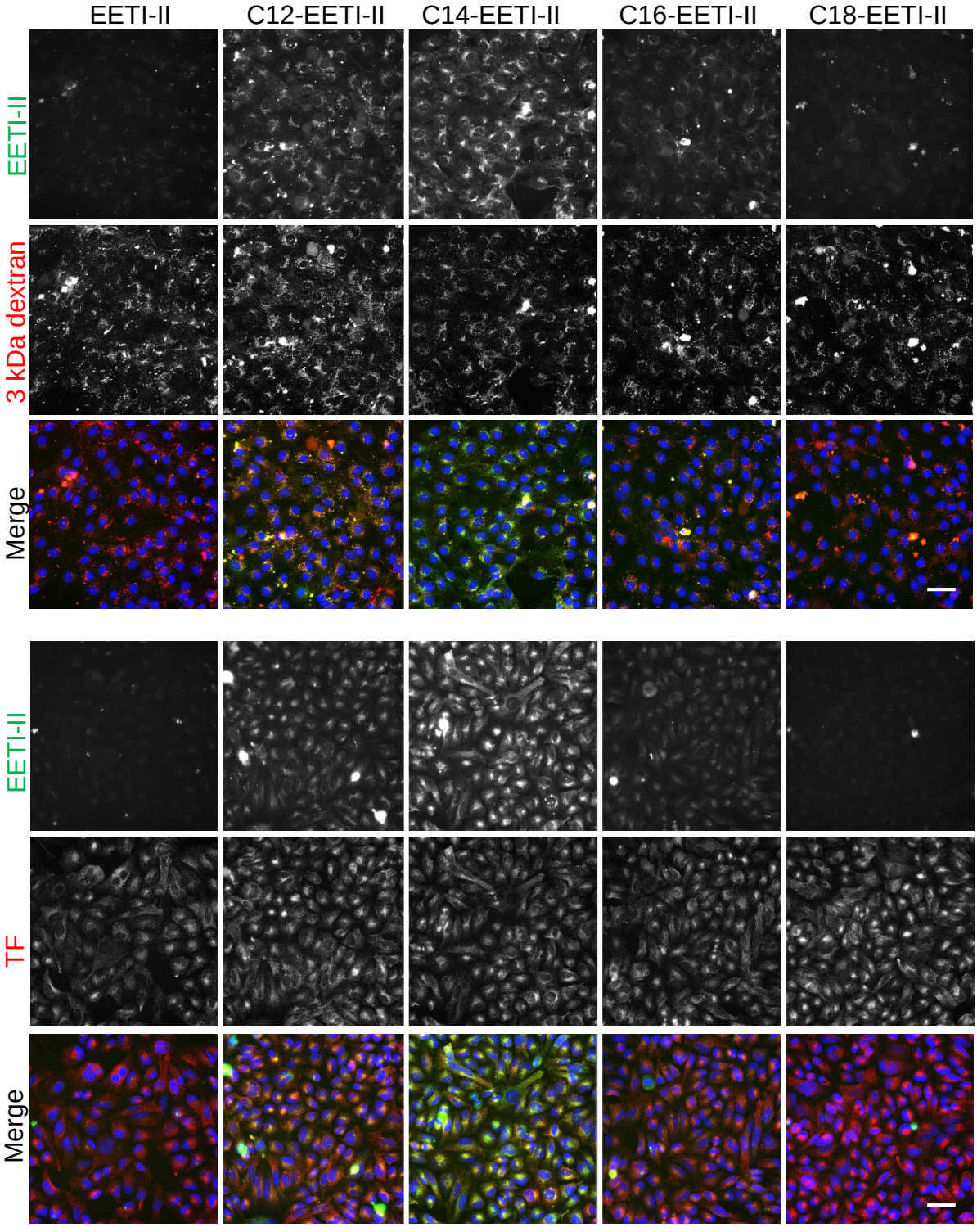
Supplementary Figure 9. Lipid EETI-II peptides (C14-EETI-II, a; C16-EETI-II, b) show improved membrane association. Related to Figure 3. Circles: Alexa488 gold particles in the cytosol. green arrows: Alexa488 gold particles associated with mitochondrion; purple arrows: Alexa488 gold particles associated with ER; Light yellow patches: endosomal / lysosomal vacuoles; PM: plasma membrane; L: lysosomes; E: endosomes; M: mitochondrion; ER: endoplasmic reticulum. Scale bar, 200 nm.



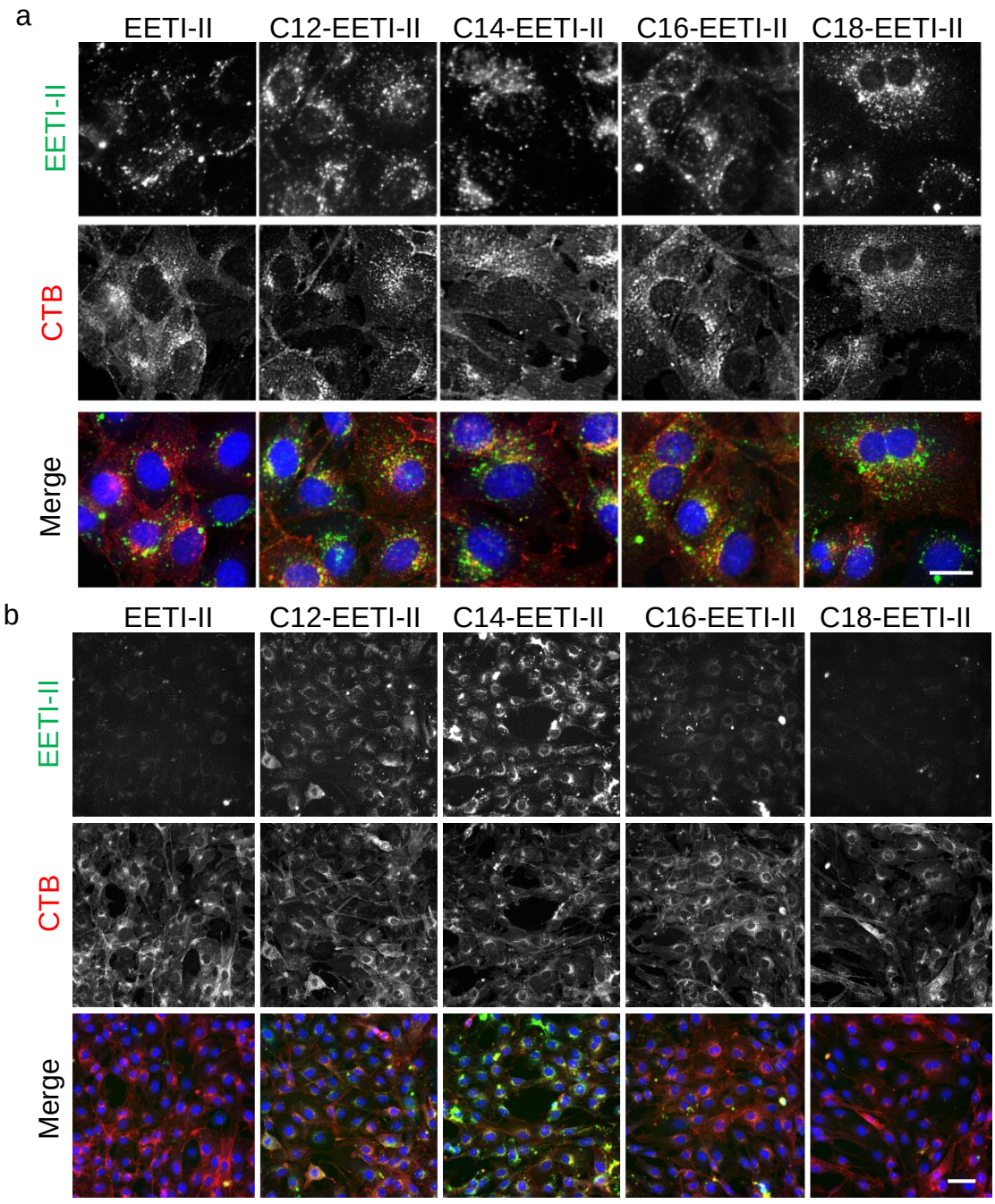
Supplementary Figure 10. Lipid EETI-II peptides are more resistant to detergent extraction. Related to STAR Methods. NIH 3T3 cells were treated with 5 μM WT or lipid EETI-II-A488 for 60 min then fixed with 4% PFA as described in methods. Cells were then incubated with PBS, 0.1 % Triton X-100, 0.04 % Saponin, or 25 μM Digitonin in PBS for 5 min at room temperature, washed and imaged. Fluorescence images were captured on a high throughput ImageXpress Micro XL imaging system (Molecular Devices) (a, overlay; b, FITC only). Fluorescence intensity values above a threshold defined using the DMSO-treated samples were measured and used to quantify percentage of dye-positive cells. The same threshold was used for samples treated with the same peptides then the values were normalized to PBS treated samples and averaged from three independent experiments. Mean ± SD. n = 1000 cells. Representative images from three independent experiments are shown. Fluorescence intensity for each peptide was normalized independently to show its cellular distribution. Scale bar, 50 μm.



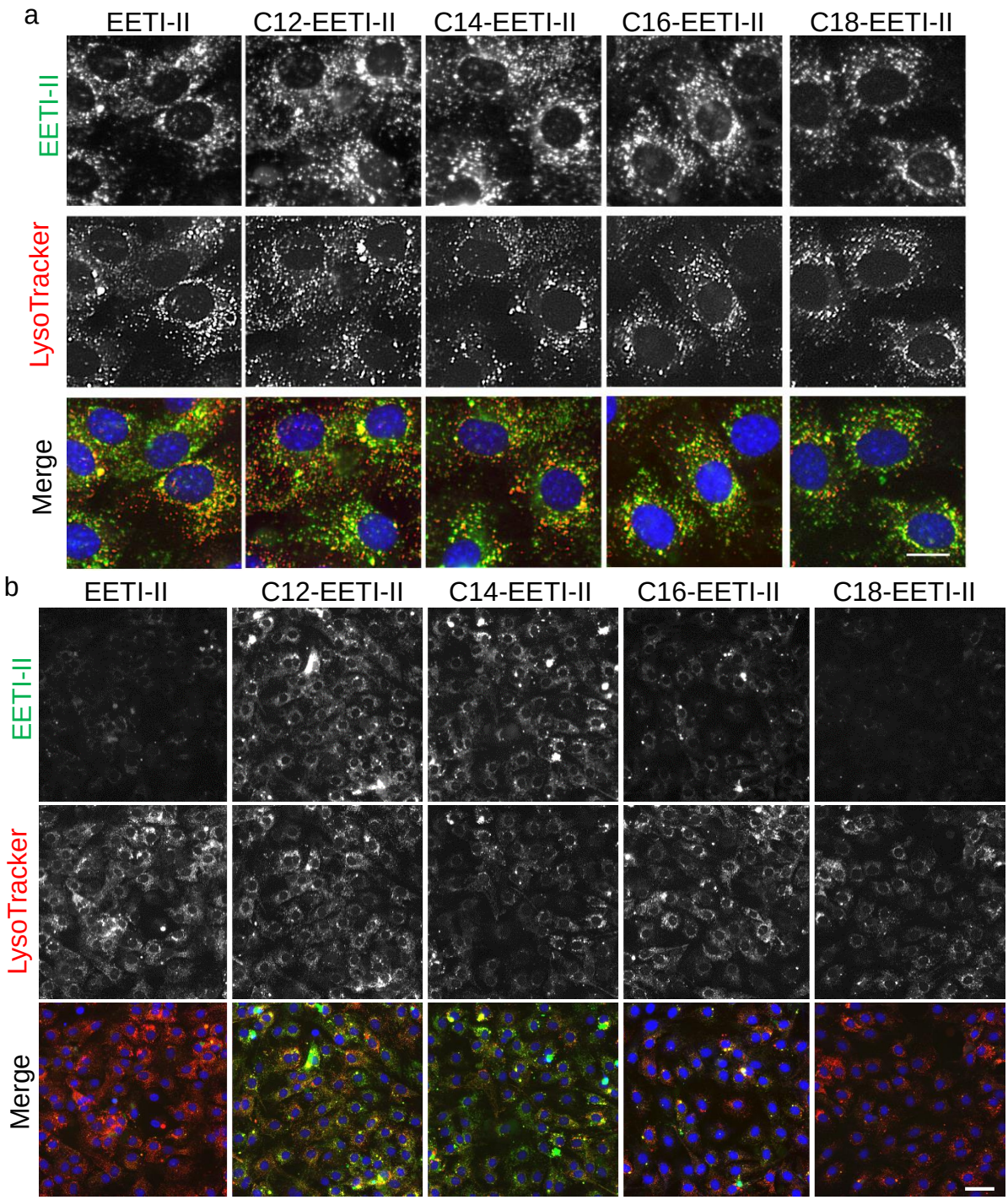
Supplementary Figure 11. WT or lipid EETI-II peptides are internalized in part via a macropinocytosis and a clathrin-mediated endocytic pathway. Related to STAR Methods. WT or lipid EETI-II-A488 co-localizes with internalized 3 kDa dextran (Texas red-conjugated, shown in red) (a), or transferrin-positive (TF-A555, shown in red) vesicles in the early /recycling endosomes (b). NIH 3T3 cells were incubated with WT or lipid EETI-II-A488 (5 µM, 60 min) at 37 °C and markers (Tf-A555, 0.2 mg/ml, added in the last 10 min of incubation; 3 kDa dextran-TR, 0.1 mg/ml, 1 h incubation. The short incubation with transferrin was used to mainly target the probes to early endosome compartments). Cells were processed as described in methods. Fluorescence images were captured on a high throughput ImageXpress Micro XL imaging system (Molecular Devices). Representative images from at least two independent experiments are shown. In (a) and (b), fluorescence intensity for each peptide was normalized independently to show its cellular distribution. Scale bar, 20 µm.



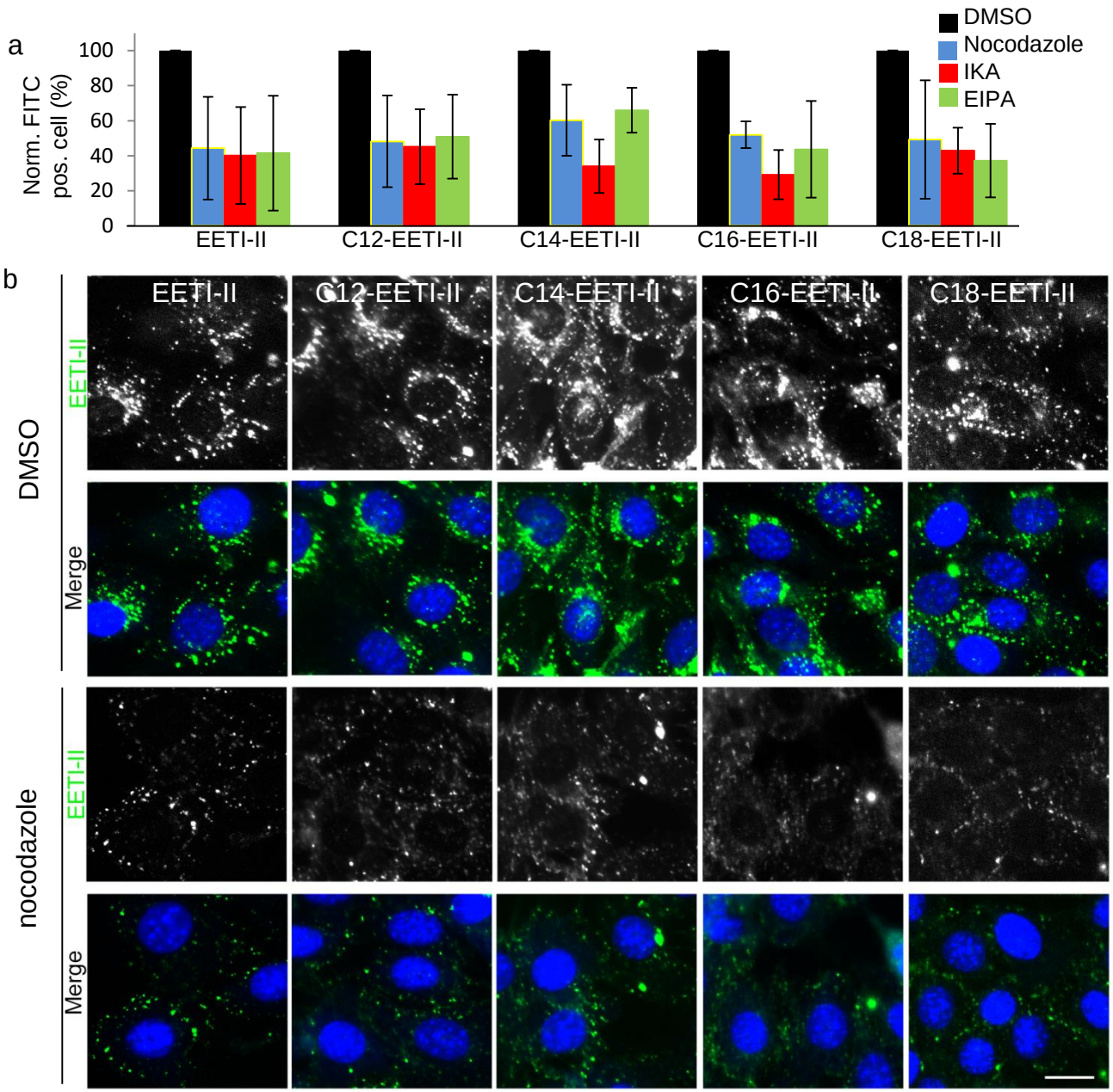
Supplementary Figure 12. WT or lipid EETI-II peptides are internalized in part via a macropinocytosis and a clathrin-mediated endocytic pathway. Related to STAR Methods. Full site view of cell images of **Fig. S11**. Scale bar, 50 µm.



Supplementary Figure 13. WT or lipid EETI-II-A488 does not co-localize with internalized cholera toxin. Related to STAR Methods. (a) NIH 3T3 cells were incubated with WT or lipid EETI-II-A488 (5 µM, 60 min) at 37 °C and CTxB-A647 (25 µg/ml, shown in red, false colored) was added in the last 10 min of incubation. Cells were washed, fixed with 4% PFA and processed as described in methods. Fluorescence images were captured on a high throughput ImageXpress Micro XL imaging system (Molecular Devices). Representative images from at least two independent experiments are shown. In (a), fluorescence intensity for each peptide was normalized independently to show its cellular distribution. Scale bar, 20 µm. (b) Full site view of cell images. Scale bar, 50 µm.



Supplementary Figure 14. WT or lipid EETI-II eventually accumulates in lysosomes. Related to STAR Methods. WT or lipid EETI-II-A488 co-localizes with LysoTracker Red DND-99 (lysosome marker). NIH 3T3 cells were incubated with WT or lipid EETI-II-A488 (5 µM, 5 h) at 37 °C and LysoTracker Red DND-99 (50 nM, added in the last 30 min of incubation). Cells were washed, fixed with 4% PFA and processed as described in methods. Fluorescence images were captured on a high throughput ImageXpress Micro XL imaging system (Molecular Devices). Representative images from at least two independent experiments are shown. In (a), fluorescence intensity for each peptide was normalized independently to show its cellular distribution. Scale bar, 20 µm. (b) Full site view of cell images. Scale bar, 50 µm.



Supplementary Figure 15. Cellular uptake of WT or lipid EETI-II is inhibited by nocodazole, an inhibitor of microtubule polymerization, ikarugamycin, an inhibitor of clathrin-mediated endocytosis, or EIPA, an inhibitor of Na^+/H^+ exchange. Related to STAR Methods. (a) NIH 3T3 cells were treated with DMSO, nocodazole (10 μM), ikarugamycin (4 μM), or EIPA (50 μM) for 30 min then with 5 μM WT or lipid EETI-II-A488, in the presence of DMSO or inhibitors for 60 min at 37 °C. Cells were washed with PBS then fixed with 4% PFA and processed as described in methods. Fluorescence images were captured on ImageXpress Micro XL (Molecular Devices) and analyzed by MetaXpress 4.0 (Molecular Devices). Fluorescence intensity values above a threshold defined using the DMSO-treated samples were measured and used to quantify percentage of dye-positive cells. The same threshold was used for samples treated with the same peptides then the values were normalized to the DMSO-treated samples and averaged from three independent experiments. Mean ± SD. n = 1000 cells. (b) Zoom-in images of nocodazole treated cells. Scale bar, 20 μm. Fluorescence intensity for each peptide was normalized independently to show its cellular distribution.