Supplementary Information

ORP9-PH domain-based fluorescent reporters for visualizing phosphatidylinositol 4-phosphate dynamics in living cells

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Supplementary Figures

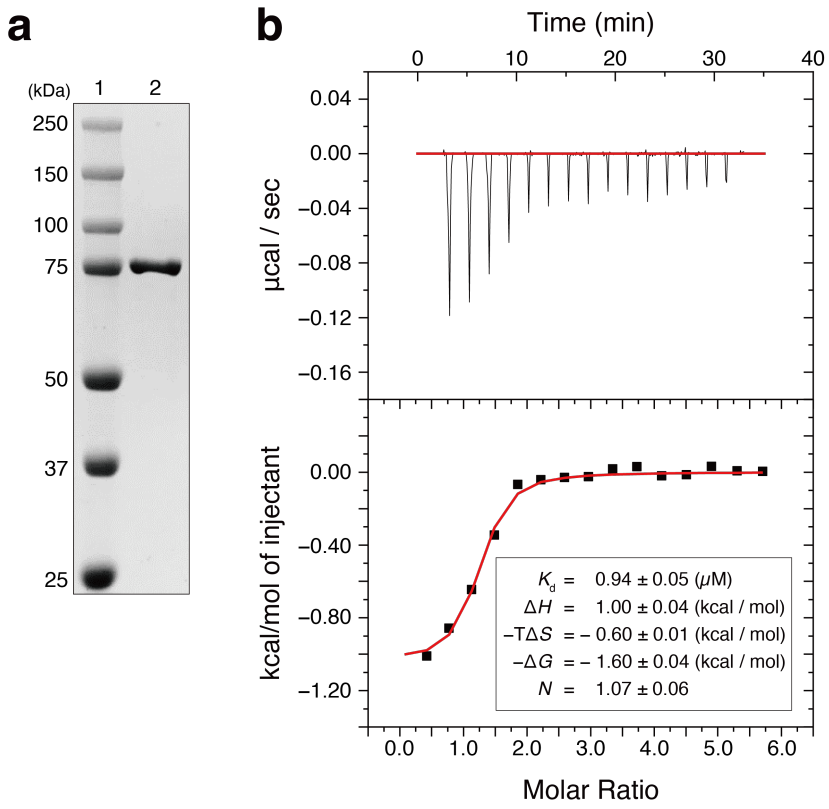

Figure S1. Affinity measurement of the ORP9-PH domain to PI4P by ITC. (**a**) SDS-PAGE image of the recombinant ORP9-PH domain used for ITC experiments. Lane 1, molecular weight marker; lane 2, the purified ORP9-PH domain fused to NusA-His6-tag (ORP9-PH-NusA-His6) (calculated M.W.: 72 kDa). (**b**) ITC titration kinetics (top) and integrated binding isotherm (bottom). Measurement conditions: [ORP9-PH-NusA-His6] = 20 µM, [08:0 PI4P] = 600 µM (15 × 2.3 µL injections), 20 mM HEPES buffer, 150 mM NaCl, pH 7.5, 25ºC. Thermodynamic parameters corresponding to the binding of ORP9-PH-NusA-His6 to PI4P are given in the inserted table.

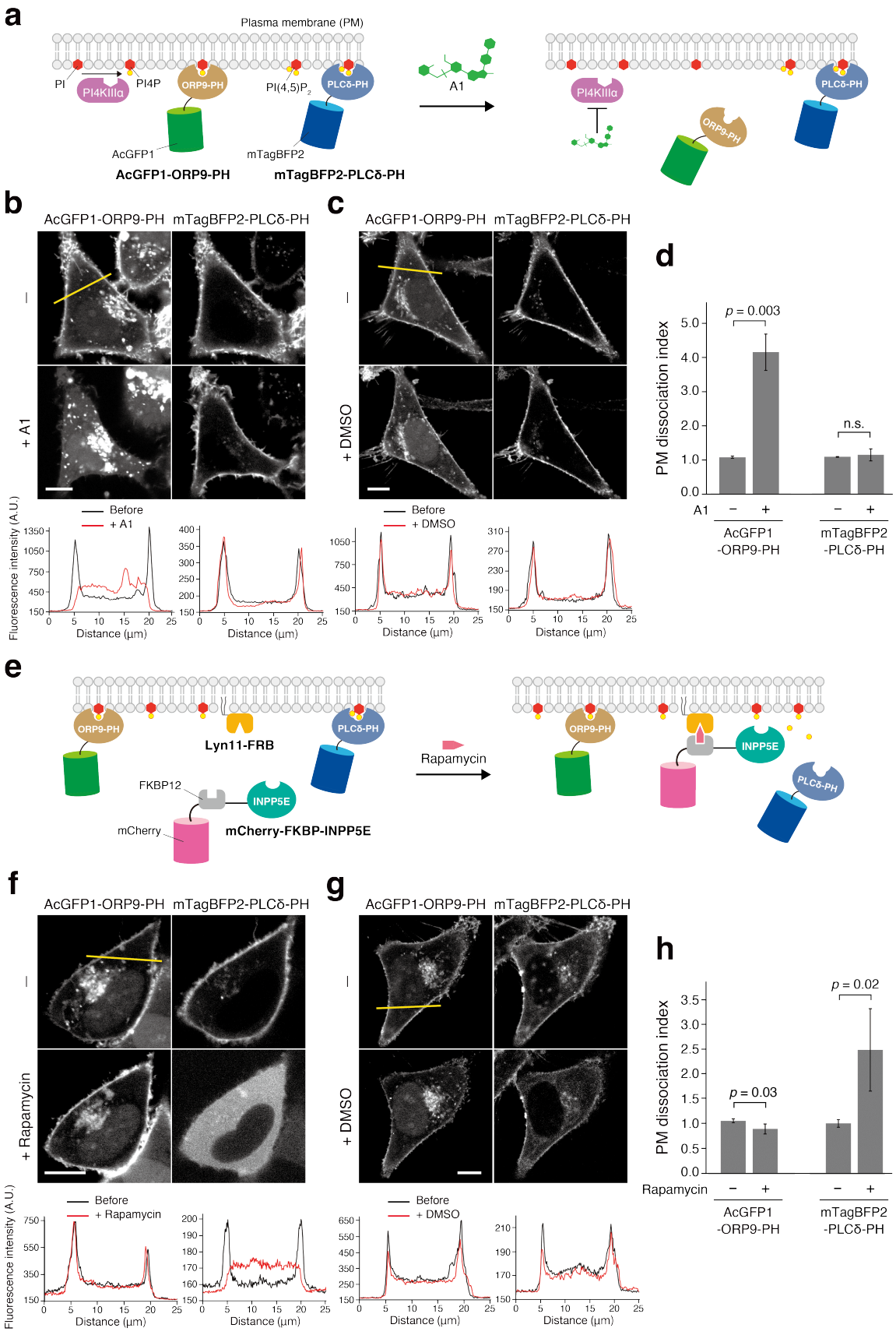

Figure S2. Investigation of PI4P specificity of AcGFP1-ORP9-PH. (**a**) Schematic illustration of the PI4P depletion experiment using the PI4KIIIα inhibitor A1. (**b**) Confocal fluorescence images of HeLa cells

coexpressing AcGFP1-ORP9-PH (left) and mTagBFP2-PLCδ-PH [PI(4,5)P_2 reporter] (right) were taken before (top) and 20 min after the addition of A1 (100 nM) (bottom). Fluorescence intensity profiles of AcGFP1-ORP9-PH and mTagBFP2-PLCδ-PH across the yellow line before (black) and after the A1 addition (red) are shown below the image. Scale bar, 10 μm. (**c**) Control experiment for **b** using DMSO. Scale bar, 10 μm. (**d**) Quantification of PM dissociation of AcGFP1-ORP9-PH and mTagBFP2-PLCδ-PH by PM PI4P depletion. The PM dissociation index is given by $(F_{cyto}/F_{PM})/(F_{cyto}/F_{PM})_0$, where (F_{cyto}/F_{PM}) and $(F_{cyto}/F_{PM})_0$ are the ratios of the cytosolic to the PM fluorescence intensity after and before lipid depletion, respectively. Data are presented as the mean ± SD (n = 5 cells). P values indicate the results of Student's *t*-test analysis: n.s., $p > 0.05$. (**e**) Schematic illustration of the PI(4,5)P_2 depletion experiment using the rapamycin-induced protein dimerization system. In this experiment, PM PI(4,5)P_2 was depleted by recruiting FKBP12-tagged INPP5E (mCherry-FKBP-INPP5E) to the PM-localized Lyn11-FRB by the addition of rapamycin. (**f**) Confocal fluorescence images of HeLa cells coexpressing AcGFP1-ORP9-PH (left) and mTagBFP2-PLCδ-PH [PI(4,5)P_2 reporter] (right) were taken before (top) and 1 min after the addition of rapamycin (200 nM) (bottom). Fluorescence intensity profiles of AcGFP1-ORP9-PH and mTagBFP2-PLCδ-PH across the yellow line before (black) and after the rapamycin addition (red) are shown below the image. Scale bar, 10 μm. (**g**) Control experiment for **f** using DMSO. Scale bar, 10 μm. (**h**) Quantification of PM dissociation of AcGFP1-ORP9-PH and mTagBFP2-PLCδ-PH by PM PI(4,5)P_2 depletion. The details are as given in panel **d**. Data are presented as the mean ± SD (n = 5 cells).

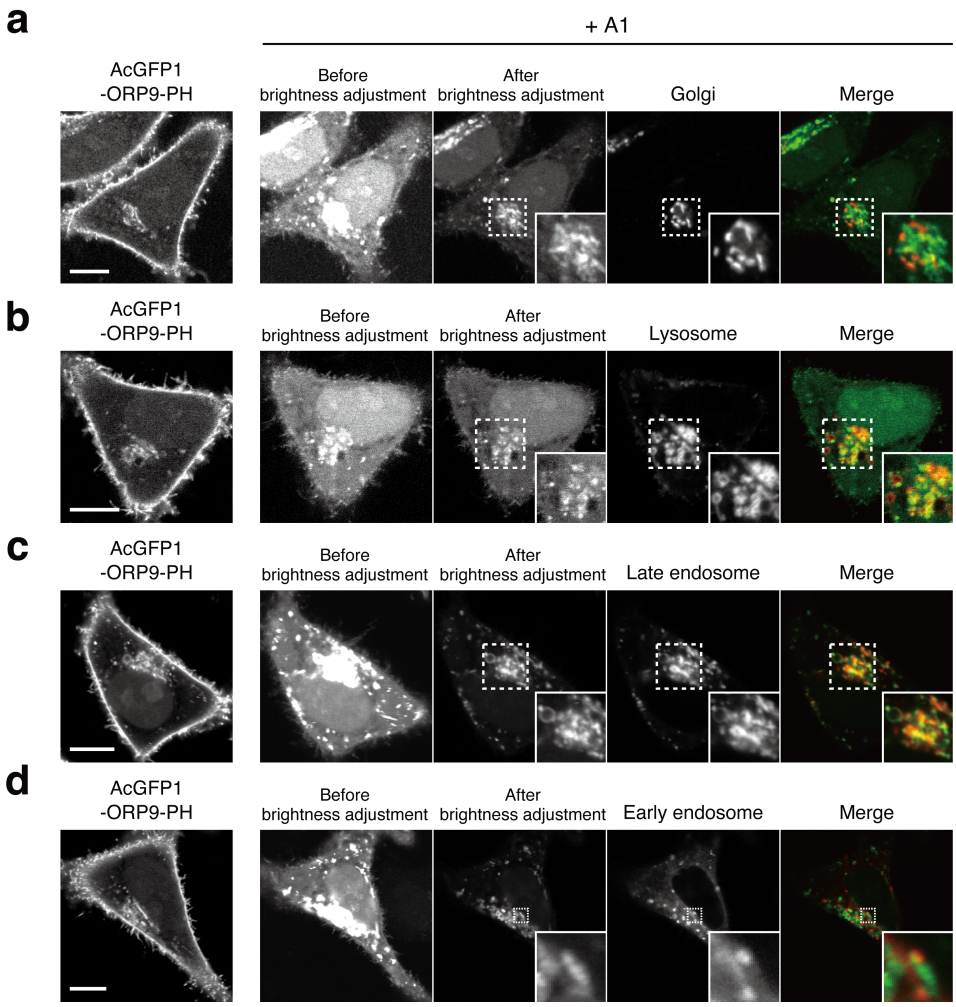

Figure S3. Colocalization assays for AcGFP1-ORP9-PH upon PM PI4P depletion. Confocal fluorescence images of HeLa cells expressing AcGFP1-ORP9-PH and organelle markers were taken before (left) and 20 min after the addition of A1 (100 nM): (**a**) the Golgi apparatus, GalT-mCherry; (**b**) lysosome, LAMP1-mCherry; (**c**) late endosome, iRFP713-Rab7; (**d**) early endosome: iRFP713-Rab5. Insets show the regions indicated by white dashed boxes at higher magnification. Scale bars, 10 μm.

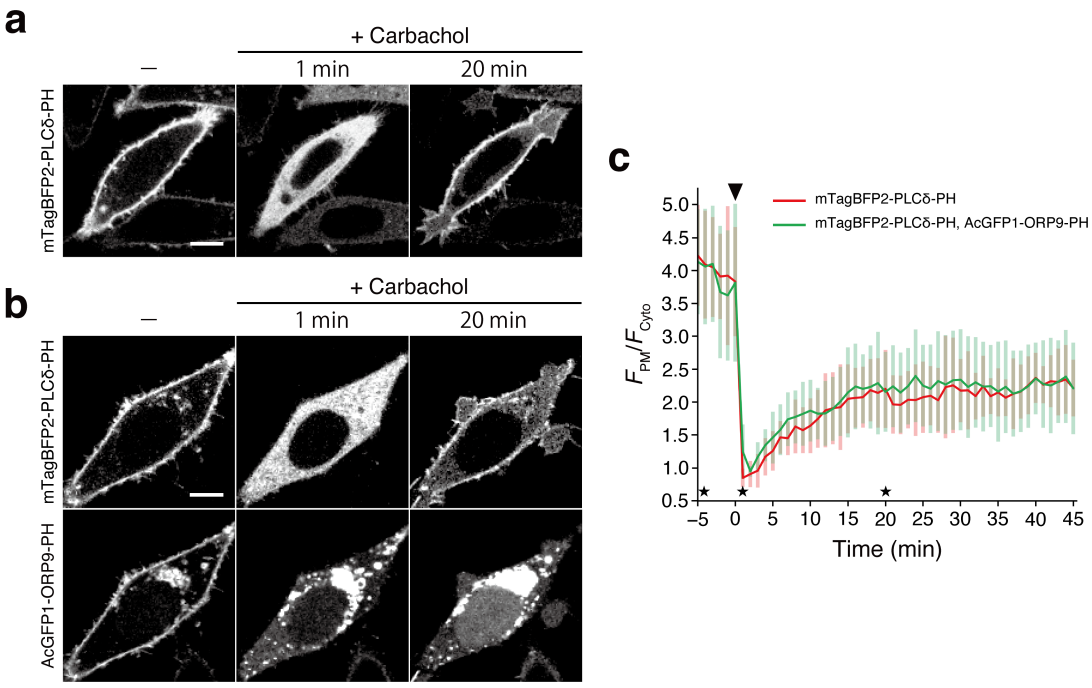

Figure S4. Evaluation of PM PI(4,5)P_2 dynamics upon M1R stimulation. (**a**) Confocal fluorescence images of HeLa cells coexpressing M1R and mTagBFP2-PLCδ-PH [PI(4,5)P_2 reporter]. Images were taken at the time points indicated by the asterisks in panel **c**: before (left), and 1 min (center) and 20 min (right) after the addition of carbachol (1 mM). Scale bar, 10 μm. (**b**) Confocal fluorescence images of HeLa cells coexpressing M1R, mTagBFP2-PLCδ-PH, and AcGFP1-ORP9-PH. The experiments were performed in the same manner as in **a**. Scale bar, 10 μm. (**c**) Time course of the PM PI(4,5)P_2 level monitored by mTagBFP2-PLCδ-PH. The ratios of the PM to the cytosolic fluorescence intensity (F_{PM}/F_{cyto}) of mTagBFP2-PLCδ-PH are plotted as a function of time. The arrowheads in the graph indicate the time of carbachol addition. Data are presented as the mean ± SD (n = 6 cells).

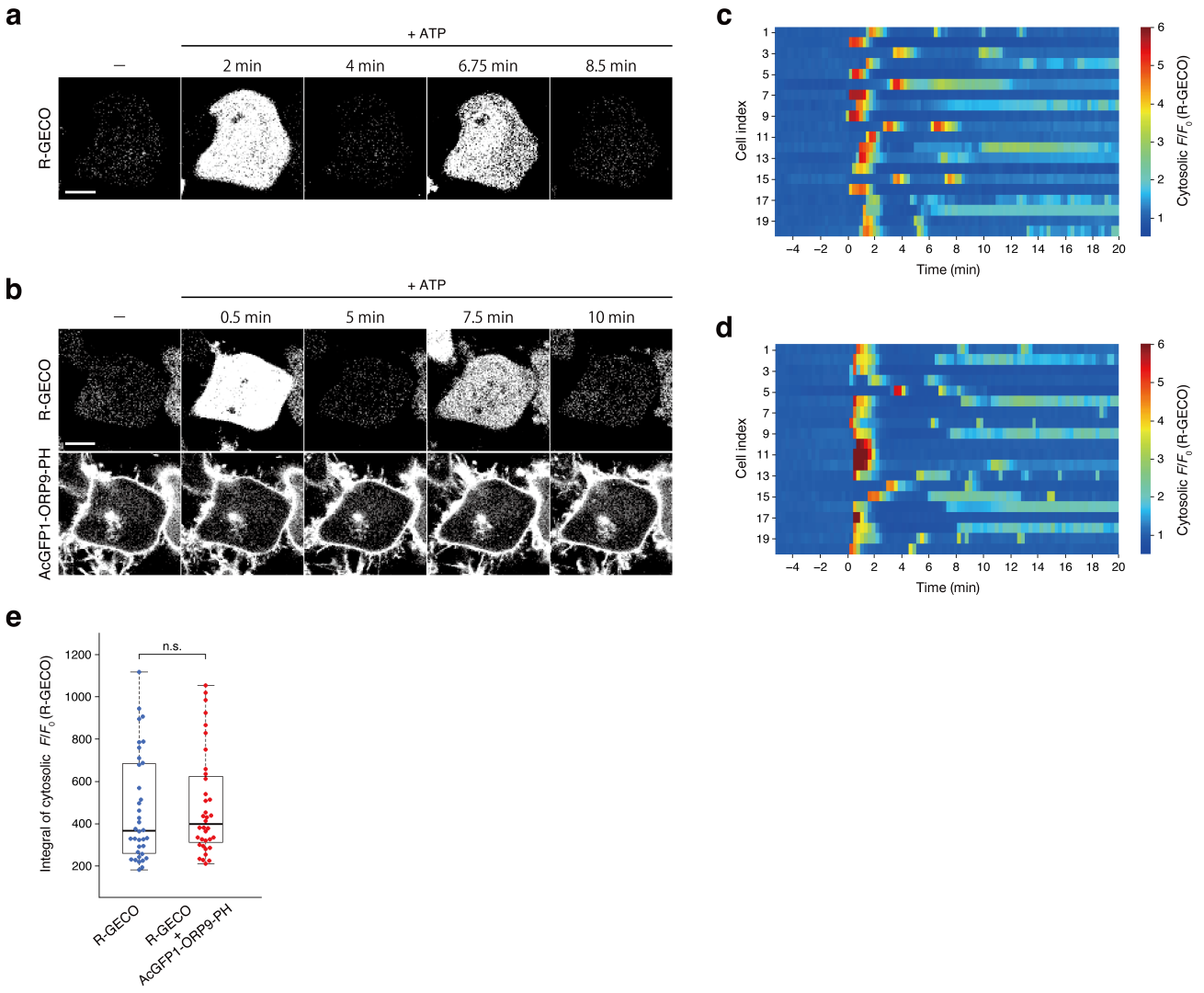

Figure S5. Evaluation of Ca^{2+} signaling dynamics upon GPCR stimulation. (**a**) Confocal fluorescence images of a HeLa cell expressing R-GECO (Ca^{2+} reporter). Images were taken before (left) and after the addition of ATP (100 μM) at the time points indicated above the images. Scale bar, 10 μm. (**b**) Confocal fluorescence images of a HeLa cell expressing R-GECO and AcGFP1-ORP9-PH. The experiments were performed in the same manner as in **a**. Scale bar, 10 μm. (**c**,**d**) Heatmaps depicting ATP-induced Ca^{2+} oscillations for 20 randomly selected cells expressing (**c**) R-GECO only (corresponding to **a**) or (**d**) R-GECO and AcGFP1-ORP9-PH (corresponding to **b**). (**e**) Quantification of the Ca^{2+} responses. The Ca^{2+} signaling activities were evaluated by quantifying the integrals of the normalized R-GECO fluorescence intensity in the cytoplasm (cytosolic F/F_0) for individual cells expressing (left) R-GECO only (corresponding to **a**) or (right) R-GECO and AcGFP1-ORP9-PH (corresponding to **b**). Student's *t*-test analysis indicated no statistically significant difference: n.s., $p > 0.05$.

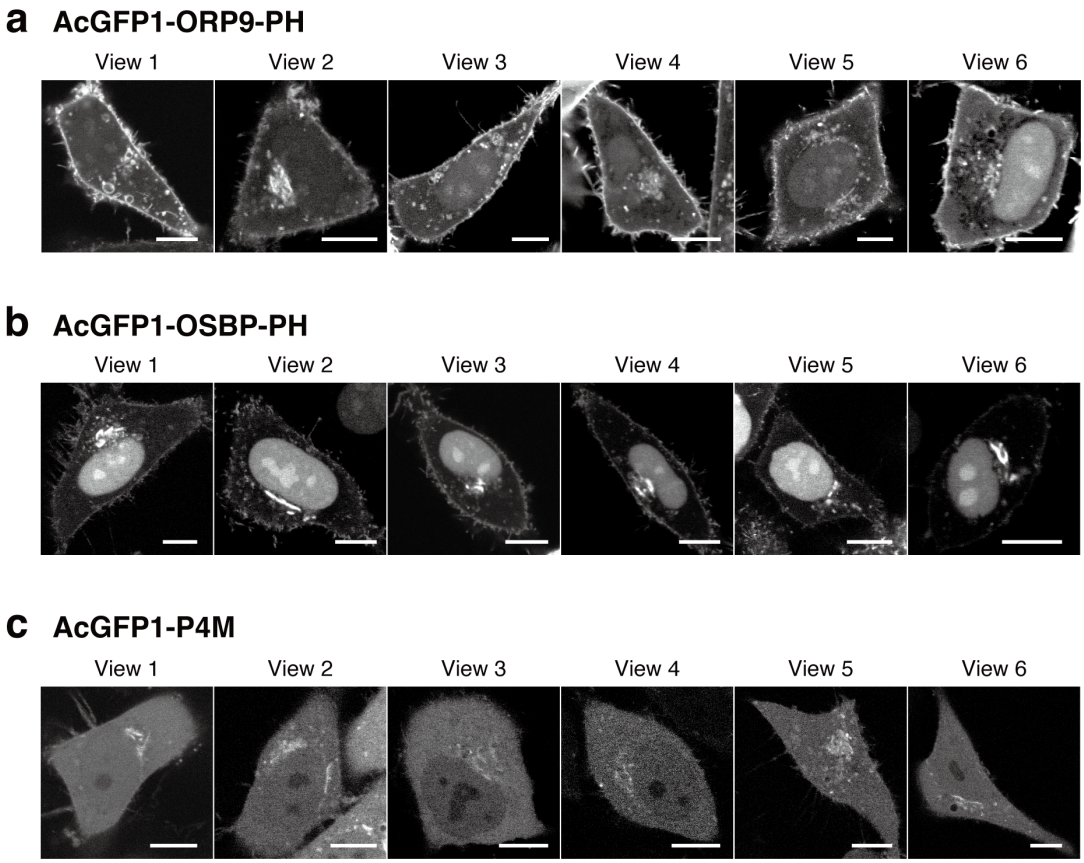

Figure S6. Additional confocal fluorescence images of HeLa cells expressing AcGFP1-ORP9-PH (**a**), AcGFP1-OSBP-PH (**b**), and AcGFP1-P4M(**c**), with main images shown in **Fig. 1b**. Scale bars, 10 μm.

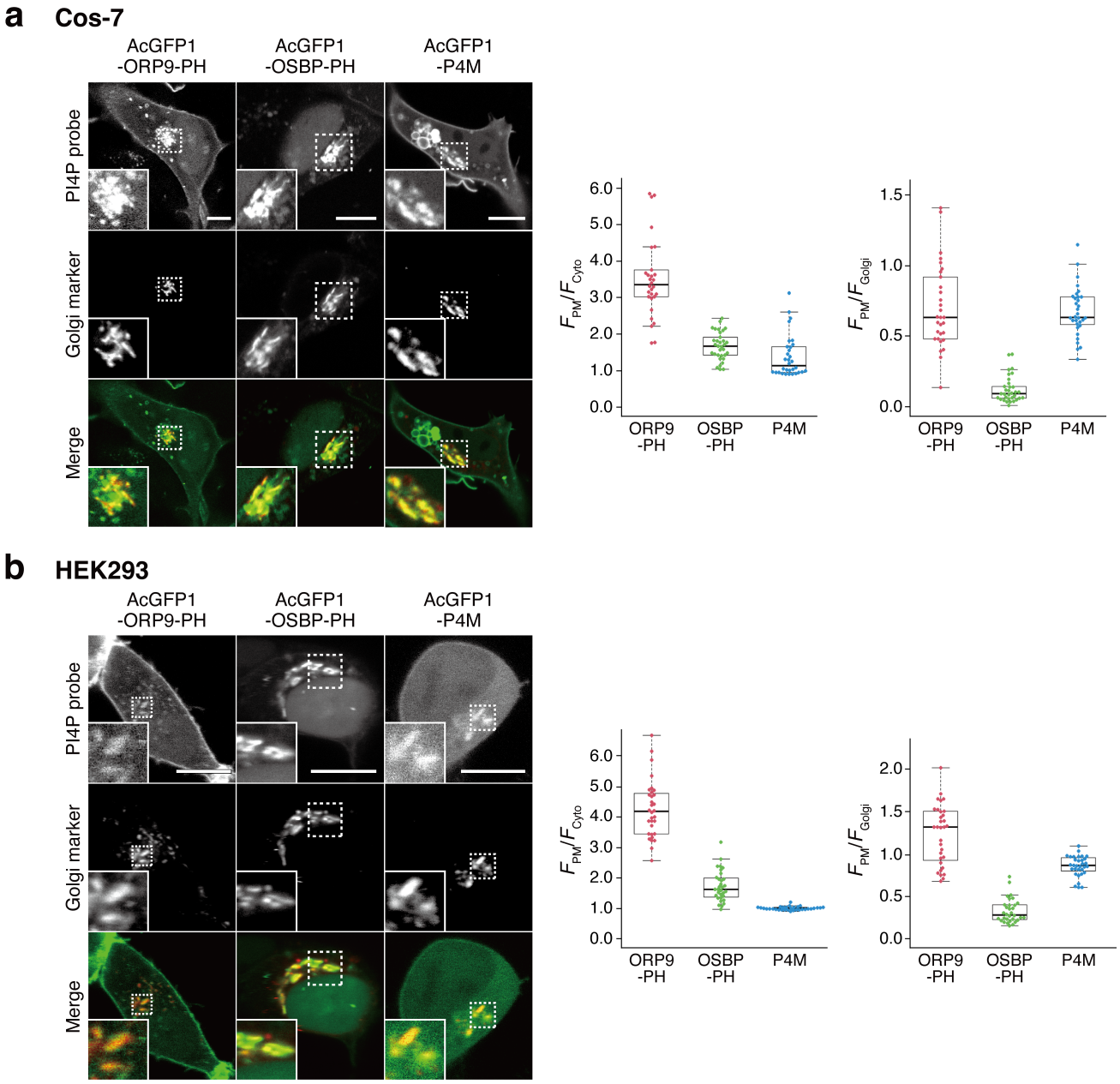

Figure S7. Application of fluorescent PI4P reporters (AcGFP1-ORP9-PH, AcGFP1-OSBP-PH, and AcGFP1-P4M) to other cell lines. (**a**) Cos-7 cells. (**b**) HEK293 cells. (Left) Representative confocal fluorescence images of cells coexpressing the indicated PI4P reporter and Golgi marker (GalT-mCherry) are shown. Insets show the regions indicated by white dashed boxes at higher magnification. Scale bars, 10 μm. (Right) Quantification of the PI4P detection sensitivity and Golgi bias. Data analysis was performed as described in **Fig. 1c** ($n \geq 30$ cells).

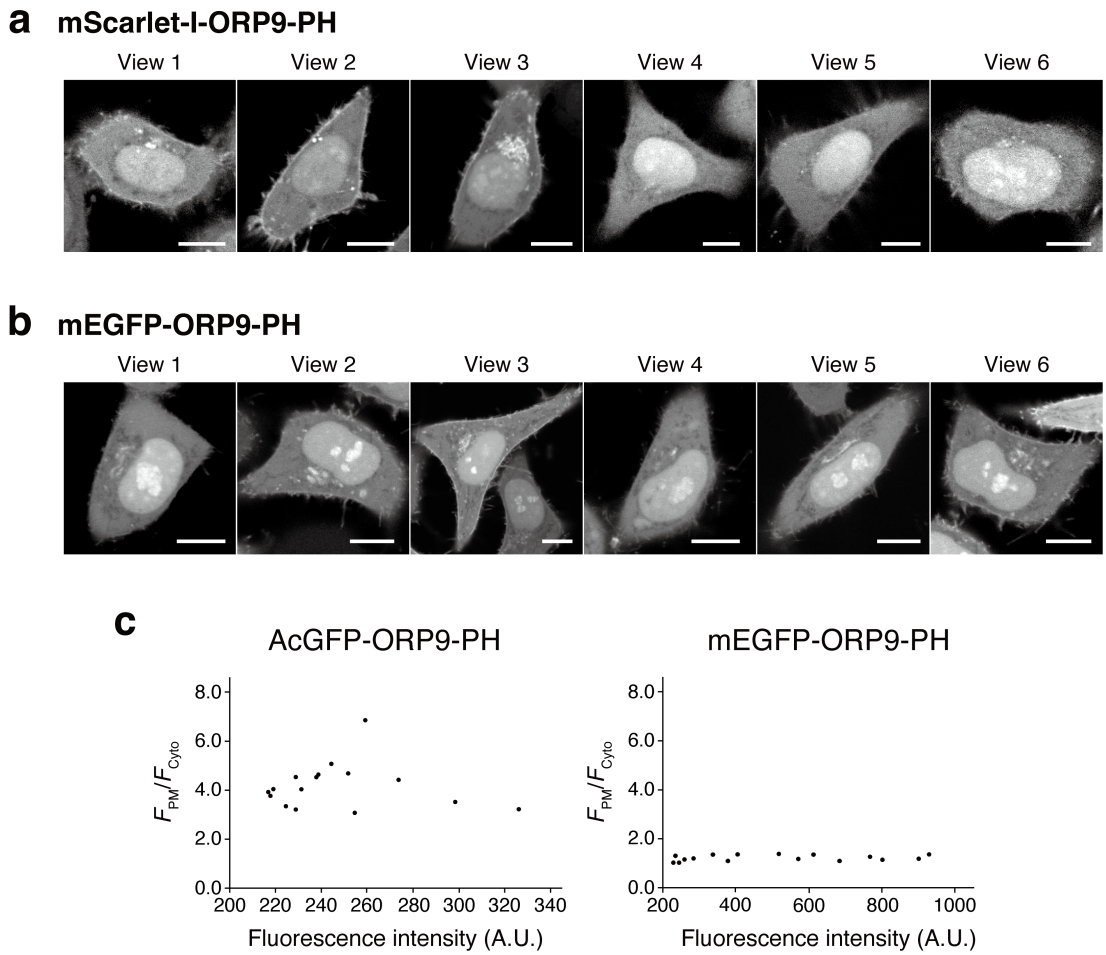

Figure S8. Confocal fluorescence images of HeLa cells expressing monomeric fluorescent protein-tagged ORP9-PH domains. (**a**) mScarlet-I-ORP9-PH. (**b**) mEGFP-ORP9-PH. Scale bars, 10 μm. (**c**) Quantification of the PI4P detection sensitivity and expression level of AcGFP1-ORP9-PH (left) and mEGFP-ORP9-PH (right). The PI4P detection sensitivity was evaluated as described in the caption of **Fig. 2c**, and the expression level was evaluated by quantifying the total fluorescence intensity of individual cells (n = 16 cells).

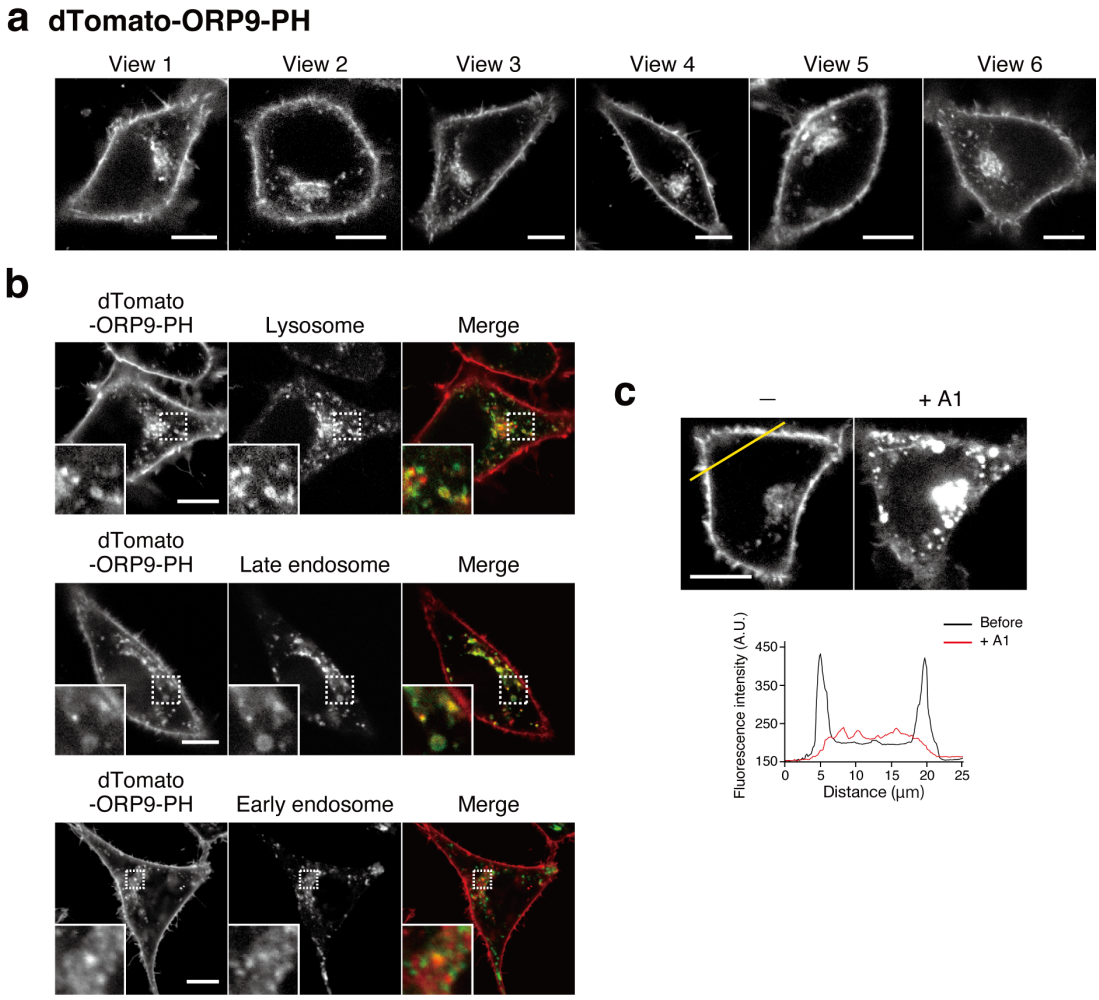

Figure S9. PI4P-binding properties of dTomato-ORP9-PH. (**a**) Confocal fluorescence images of HeLa cells expressing dTomato-ORP9-PH. Scale bars, 10 μm. (**b**) Confocal fluorescence images of HeLa cells coexpressing dTomato-ORP9-PH and organelle markers: lysosome, LAMP1-miRFP703; late endosome, iRFP713-Rab7; early endosome: iRFP713-Rab5. Insets show the regions indicated by white dashed boxes at higher magnification. Scale bars, 10 μm. (**c**) PM PI4P depletion experiment. Confocal fluorescence images of HeLa cells expressing dTomato-ORP9-PH were taken before (left) and 20 min after the addition of A1 (100 nM) (right). Fluorescence intensity profiles of dTomato-ORP9-PH across the yellow line before (black) and after the A1 addition (red) are shown below the image. Scale bar, 10 μm.

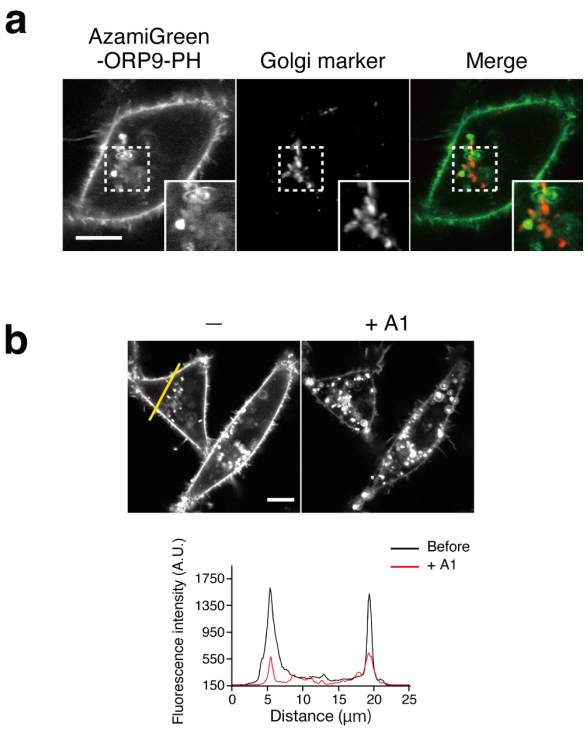

Figure S10. Intracellular properties of AzamiGreen-ORP9-PH. (**a**) Confocal fluorescence images of a HeLa cell coexpressing AzamiGreen-ORP9-PH and Golgi marker (GalT-mCherry). Insets show the regions indicated by white dashed boxes at higher magnification. Scale bar, 10 μm. (**b**) PM PI4P depletion experiment. Confocal fluorescence images of HeLa cells expressing AzamiGreen-ORP9-PH were taken before (left) and 20 min after the addition of A1 (100 nM) (right). Fluorescence intensity profiles of AzamiGreen-ORP9-PH across the yellow line before (black) and after the A1 addition (red) are shown below the image. Scale bar, 10 μm.

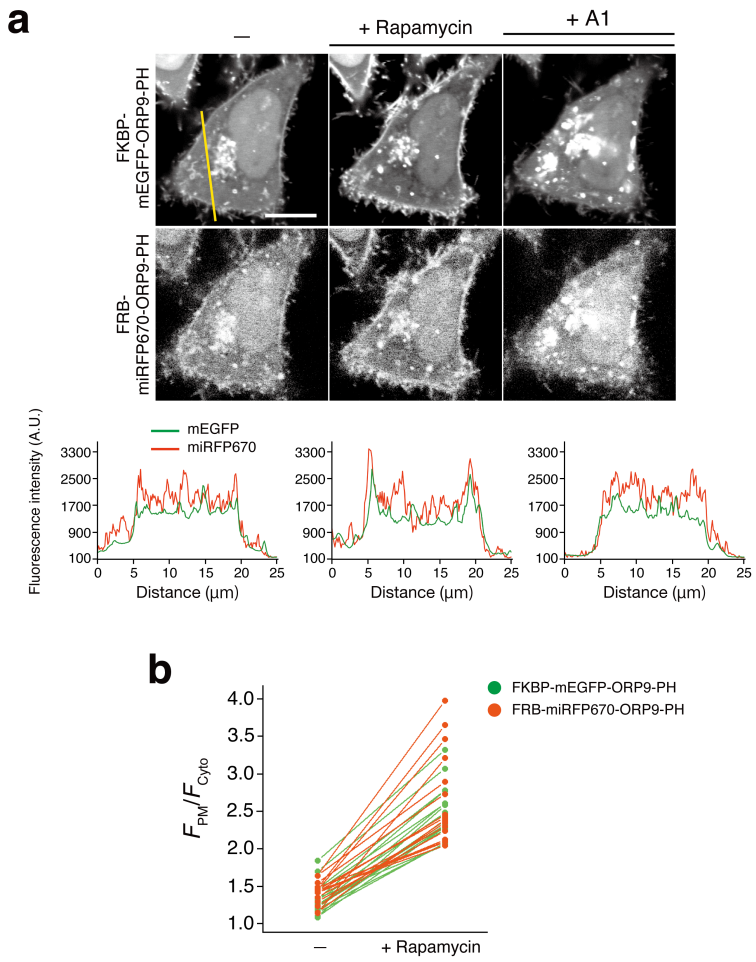

Figure S11. Dimerization of the ORP9-PH domain improves its PI4P detection ability. (**a**) Confocal fluorescence images of a HeLa cell coexpressing FKBP-mEGFP-ORP9-PH (top) and FRB-miRFP670-ORP9-PH (bottom). Images were taken before (left) and 20 min after the addition of rapamycin (200 nM) (center), and 20 min after the subsequent addition of A1 (100 nM). Fluorescence intensity profiles of FKBP-mEGFP-ORP9-PH (green) and FRB-miRFP670-ORP9-PH (red) across the yellow line are shown below the images. Scale bar, 10 μm. (**b**) Quantification of the PI4P detection sensitivity. The PI4P detection sensitivity was evaluated by quantifying the ratios of the PM to the cytosolic fluorescence intensity (F_{PM}/F_{cyto}) of FKBP-mEGFP-ORP9-PH (green) and FRB-miRFP670-ORP9-PH (red) before and after the addition of rapamycin.

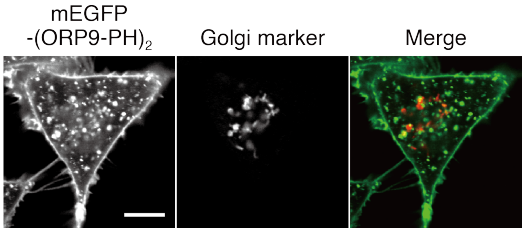

Figure S12. Intracellular properties of mEGFP-(ORP9-PH)$_2$. Confocal fluorescence images of a HeLa cell coexpressing mEGFP-(ORP9-PH)$_2$ and Golgi marker (GalT-mCherry). Scale bar, 10 μm.

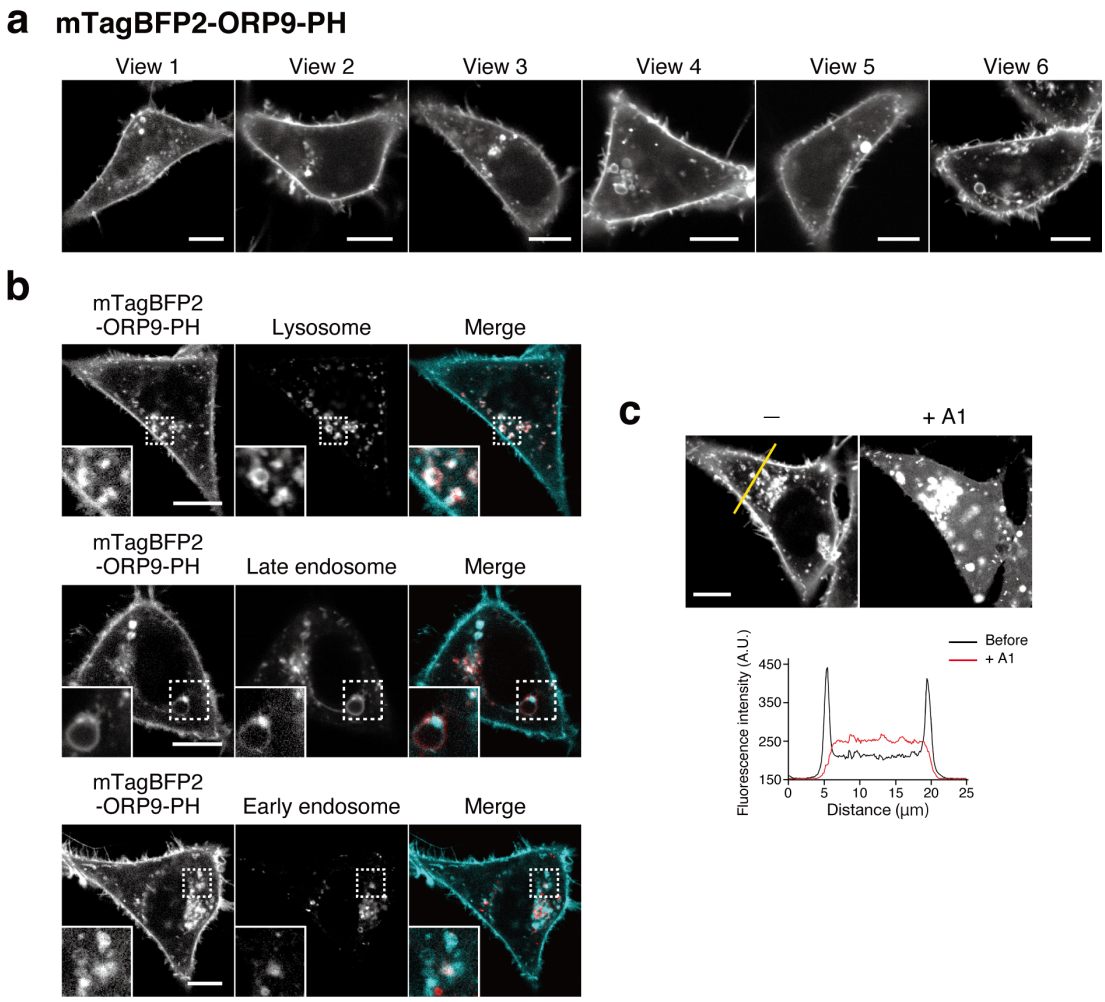

Figure S13. PI4P-binding properties of mTagBFP2-ORP9-PH. (**a**) Confocal fluorescence images of HeLa cells expressing mTagBFP2-ORP9-PH. Scale bars, 10 μm. (**b**) Confocal fluorescence images of HeLa cells coexpressing mTagBFP2-ORP9-PH and organelle markers: lysosome, LAMP1-mCherry; late endosome, iRFP713-Rab7; early endosome: iRFP713-Rab5. Insets show the regions indicated by white dashed boxes at higher magnification. Scale bars, 10 μm. (**c**) PM PI4P depletion experiment. Confocal fluorescence images of HeLa cells expressing mTagBFP2-ORP9-PH were taken before (left) and 20 min after the addition of A1 (100 nM) (right). Fluorescence intensity profiles of mTagBFP2-ORP9-PH across the yellow line before (black) and after the A1 addition (red) are shown below the image. Scale bar, 10 μm.

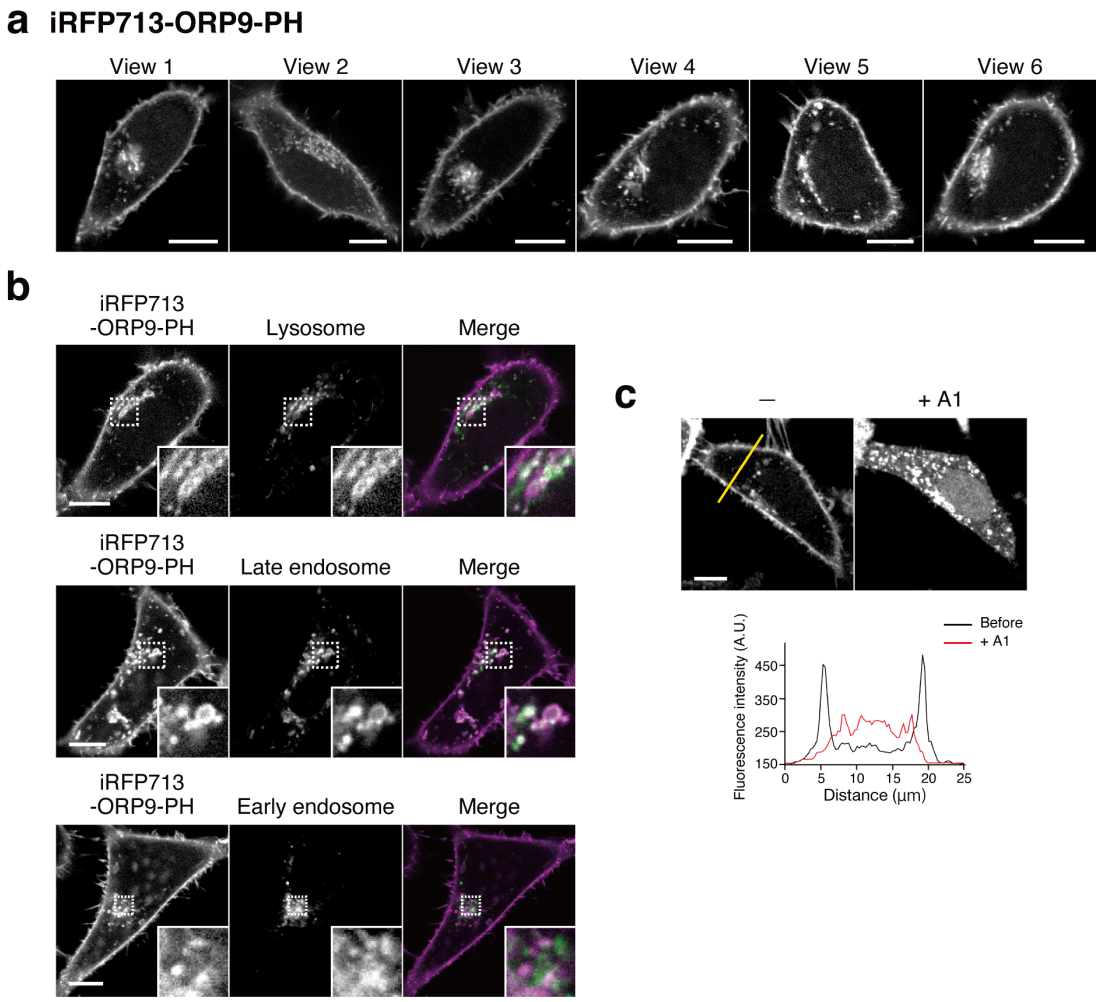

Figure S14. PI4P-binding properties of iRFP713-ORP9-PH. (**a**) Confocal fluorescence images of HeLa cells expressing iRFP713-ORP9-PH. Scale bars, 10 μm. (**b**) Confocal fluorescence images of HeLa cells coexpressing iRFP713-ORP9-PH and organelle markers: lysosome, LAMP1-mCherry; late endosome, mTagBFP2-Rab7; early endosome: mTagBFP2-Rab5. Insets show the regions indicated by white dashed boxes at higher magnification. Scale bars, 10 μm. (**c**) PM PI4P depletion experiment. Confocal fluorescence images of HeLa cells expressing iRFP713-ORP9-PH were taken before (left) and 20 min after the addition of A1 (100 nM) (right). Fluorescence intensity profiles of iRFP713-ORP9-PH across the yellow line before (black) and after the A1 addition (red) are shown below the image. Scale bar, 10 μm.

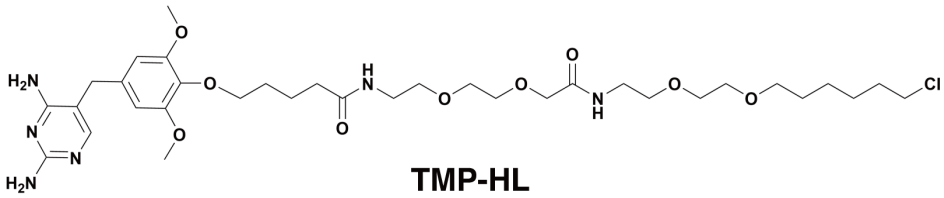

Figure S15. Chemical structure of TMP-HL.

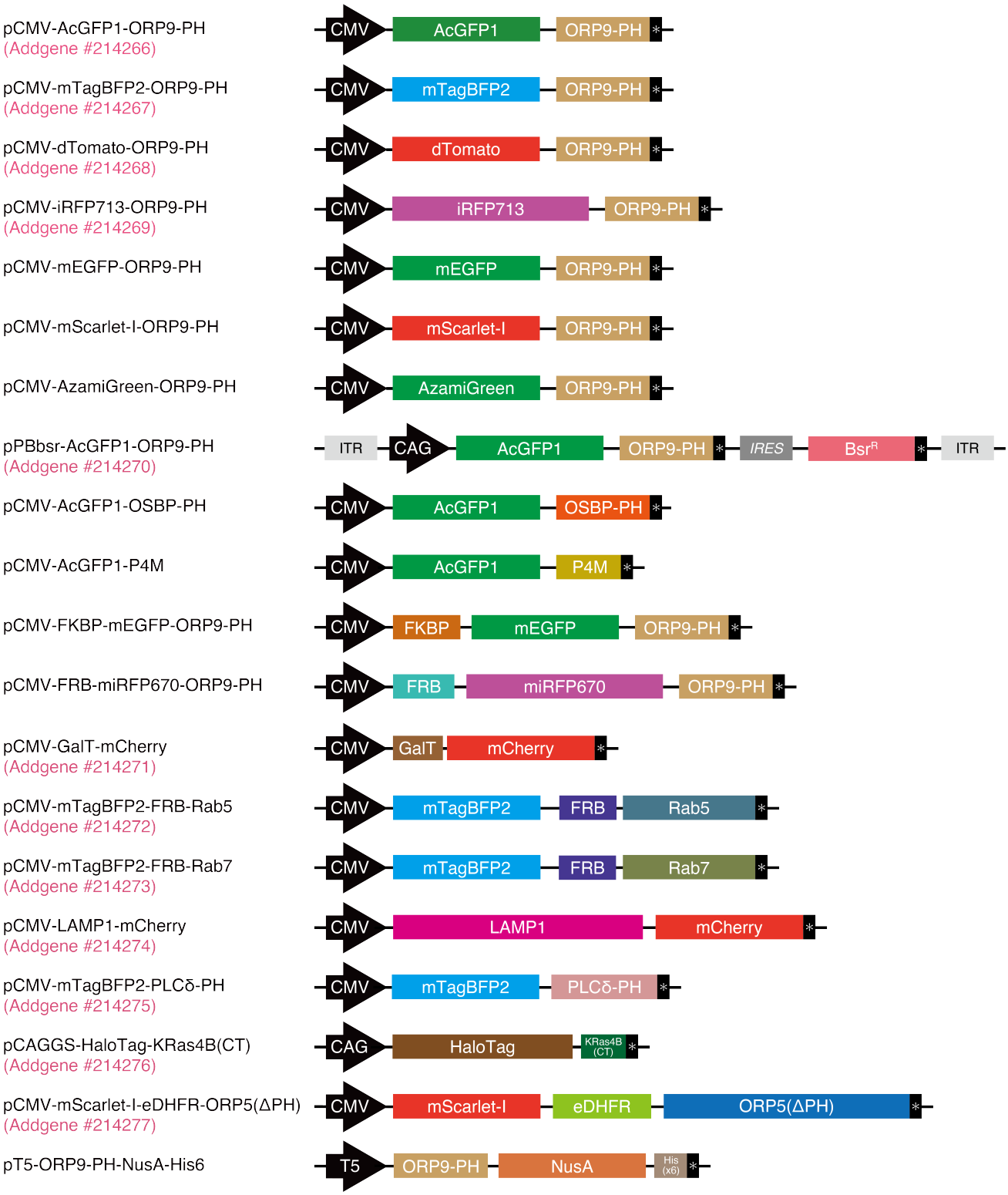

Figure S16. Schematic illustration of the domain structures of fusion proteins constructed in this study. DNA and amino acid sequences of the constructs are shown in the "**Supplementary Sequences**" section.

Supplementary Movies

Movie S1. Visualization of PI4P dynamics with AcGFP1-ORP9-PH upon synthetic ORP5-mediated ER–PM contact manipulation (time-lapse movie of **Figure 3b**).

Movie S2. Visualization of PM PI4P dynamics with AcGFP1-ORP9-PH upon M1R stimulation (time-lapse movie of **Figure 4a**).

For all movies, scale bars are 10 μm.

Supplementary Sequences

pCMV-AcGFP1-ORP9-PH (Addgene #214266)

>Amino acid sequence

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>DNA sequence

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>Components: AcGFP1 ORP9-PH

pCMV-mTagBFP2-ORP9-PH (Addgene #214267)

>Amino acid sequence

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>Components: mTagBFP2 ORP9-PH

pCMV-dTomato-ORP9-PH (Addgene #214268)

>Amino acid sequence

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>DNA sequence

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>Components: dTomato ORP9-PH

pCMV-iRFP713-ORP9-PH (Addgene #214269)
>Amino acid sequence
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DEDDSTFTITVDQKTFHFQARDADEREKWIHALEETILRHTLQLQGLDSG*
>DNA sequence
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>Components: iRFP713 ORP9-PH

pCMV-mEGFP-ORP9-PH
>Amino acid sequence
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DDEDDSTFTITVDQKTFHFQARDADEREKWIHALEETILRHTLQLQGLDSG*
>DNA sequence
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>Components: mEGFP ORP9-PH

pCMV-mScarlet-I-ORP9-PH
>Amino acid sequence
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>DNA sequence
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>Components: mScarlet-I ORP9-PH

pCMV-AzamiGreen-ORP9-PH
>Amino acid sequence
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GGSRAQASMASIVEGPLSKWTNVMKGWQYRWFVLDYNAGLLSYYTSKDKMMRGSRRGCVRLRGAVIGIDDEDDSTFTITVD
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>DNA sequence
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>Components: AzamiGreen ORP9-PH

pPBbsr-AcGFP1-ORP9-PH (Addgene #214270)
>Amino acid sequence
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-[*IRES*]-MLYEDNKHHVGAAIRTKTGEIISAVHIEAYIGRVTVCAEAIAIGSAVSNGQKDFDTIVAVRHPYSDEVDRSIR
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>DNA sequence
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>Components: AcGFP1 ORP9-PH *IRES* Bsr^{R}(blasticidin S-deaminase)

pCMV-AcGFP1-OSBP-PH

>Amino acid sequence
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>DNA sequence
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>Components: AcGFP1 OSBP-PH

pCMV-AcGFP1-P4M

>Amino acid sequence
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>DNA sequence
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>Components: AcGFP1 P4M

pCMV-FKBP-mEGFP-ORP9-PH

>Amino acid sequence
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LQGLDSG*
>DNA sequence
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>Components: FKBP12 mEGFP ORP9-PH

pCMV-FRB-miRFP670-ORP9-PH
>Amino acid sequence
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>DNA sequence
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TTAGAAGAAACTATTCTTCGCCATACTCTTCAGCTTCAAGGTTTGGATTCAGGATGA
>Components: FRB iRFP670 ORP9-PH

pCMV-GalT-mCherry (Addgene #214271)
>Amino acid sequence
MRLREPLLSGSAAMPGASLQRACRLLVAVCALHLGVTLVYYLAGRDLSRLPQLVGVSTPLQGGSNSAAAIGQSSGELRTGG
AKDPPVMVSKGEEDNMAIIKEFMRFKVHMEGSVNGHEFEIEGEGEGRPYEGTQTAKLKVTKGGPLPFAWDILSPQFMYGSK
AYVKHPADIPDYLKLSFPEGFKWERVMNFEDGGVVTVTQDSSLQDGEFIYKVKLRGTNFPSDGPVMQKKTMGWEASSERMY
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>DNA sequence

ATGAGGCTTCGGGAGCCGCTCCTGAGCGGCAGCGCCGCGATGCCAGGCGCGTCCCTACAGCGGGCCTGCCGCCTGCTCGTG
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GGAGTCTCCACACCGCTGCAGGGCGGCTCGAACAGTGCCGCCGCCATCGGGCAGTCCTCCGGGGAGCTCCGGACCGGAGGG
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GCCTACGTGAAGCACCCCGCCGACATCCCCGACTACTTGAAGCTGTCCTTCCCCGAGGGCTTCAAGTGGGAGCGCGTGATG
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CCCGAGGACGGCGCCCTGAAGGGCGAGATCAAGCAGAGGCTGAAGCTGAAGGACGGCGGCCACTACGACGCTGAGGTCAAG
ACCACCTACAAGGCCAAGAAGCCCGTGCAGCTGCCCGGCGCCTACAACGTCAACATCAAGTTGGACATCACCTCCCACAAC
GAGGACTACACCATCGTGGAACAGTACGAACGCGCCGAGGGCCGCCACTCCACCGGCGGCATGGACGAGCTGTACAAGTGA
>Components:
GalT (N-terminal 81 amino acids of human beta-1,4-galactosyltransferase) mCherry

pCMV-mTagBFP2-FRB-Rab5 (Addgene #214272)

>Amino acid sequence
MVSKGEELIKENMHMKLYMEGTVDNHHFKCTSEGEGKPYEGTQTMRIKVVEGGPLPFAFDILATSFLYGSKTFINHTQGIP
DFFKQSFPEGFTWERVTTYEDGGVLTATQDTSLQDGCLIYNVKIRGVNFTSNGPVMQKKTLGWEAFTETLYPADGGLEGRN
DMALKLVGGSHLIANAKTTYRSKKPAKNLKMPGVYYVDYRLERIKEANNETYVEQHEVAVARYCDLPSKLGHKLNSGLRSG
GGGSGGGGSGGGGSRAGGAGAILSRILWHEMWHEGLEEASRLYFGERNVKGMFEVLEPLHAMMERGPQTLKETSFNQAYGR
DLMEAQEWCRKYMKSGNVKDLTQAWDLYYHVFRRISKGGSAGGSAQASNSAVDGTMANRGATRPNGPNTGNKICQFKLVLL
GESAVGKSSLVLRFVKGQFHEFQESTIGAAFLTQTVCLDDTTVKFEIWDTAGQERYHSLAPMYYRGAQAAIVVYDITNEES
FARAKNWVKELQRQASPNIVIALSGNKADLANKRAVDFQEAQSYADDNSLLFMETSAKTSMNVNEIFMAIAKKLPKNEPQN
PGANSARGRGVDLTEPTQPTRSQCCSN*
>DNA sequence
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TAA
>Components: mTagBFP2 FRB Rab5

pCMV-mTagBFP2-FRB-Rab7 (Addgene #214273)

>Amino acid sequence
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DLMEAQEWCRKYMKSGNVKDLTQAWDLYYHVFRRISKGGSAGGSAQASMTSRKKVLLKVIILGDSGVGKTSLMNQYVNKKF
SNQYKATIGADFLTKEVMVDDRLVTMQIWDTAGQERFQSLGVAFYRGADCCVLVFDVTAPNTFKTLDSWRDEFLIQASPRD
PENFPFVVLGNKIDLENRQVATKRAQAWCYSKNNIPYFETSAKEAINVEQAFQTIARNALKQETEVELYNEFPEPIKLDKN
DRAKTSAESCSC*
>DNA sequence
ATGGTGTCTAAGGGCGAAGAGCTGATTAAGGAGAACATGCACATGAAGCTGTACATGGAGGGCACCGTGGACAACCATCAC
TTCAAGTGCACATCCGAGGGCGAAGGCAAGCCCTACGAGGGCACCCAGACCATGAGAATCAAGGTGGTCGAGGGCGGCCCT
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GTGATGCAGAAGAAAACACTCGGCTGGGAGGCCTTCACCGAGACGCTGTACCCCGCTGACGGCGGCCTGGAAGGCAGAAAC
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GACCGGGCCAAGACCTCAGCGGAAAGCTGCAGTTGCTGA
>Components: mTagBFP2 FRB Rab7

pCMV-LAMP1-mCherry (Addgene #214274)
>Amino acid sequence
MAAPGARRPLLLLLLAGLAHSAPALFEVKDNNGTACIMASFSASFLTTYEAGHVSKVSNMTLPASAEVLKNSSSCGEKNAS
EPTLAITFGEGYLLKLTFTKNTTRYSVQHMYFTYNLSDTQFFPNASSKGPDTVDSTTDIKADINKTYRCVSDIRVYMKNVT
IVLWDATIQAYLPSSNFSKEETRCPQDQPSPTTGPPSPSPPLVPTNPSVSKYNVTGDNGTCLLASMALQLNITYMKKDNTT
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AEGRHSTGGMDELYK*
>DNA sequence
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GCCGAGGGCCGCCACTCCACCGGCGGCATGGACGAGCTGTACAAGTAA
>Components: LAMP1 mCherry

pCMV-mTagBFP2-PLCδ-PH (Addgene #214275)
>Amino acid sequence
MVSKGEELIKENMHMKLYMEGTVDNHHFKCTSEGEGKPYEGTQTMRIKVVEGGPLPFAFDILATSFLYGSKTFINHTQGIP
DFFKQSFPEGFTWERVTTYEDGGVLTATQDTSLQDGCLIYNVKIRGVNFTSNGPVMQKKTLGWEAFTETLYPADGGLEGRN
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SCLRKADKNKDNKMSFKELQNFLKELNIQ*
>DNA sequence

ATGGTGTCTAAGGGCGAAGAGCTGATTAAGGAGAACATGCACATGAAGCTGTACATGGAGGGCACCGTGGACAACCATCAC
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ATCCAGTAA
>Components: mTagBFP2 PLCδ-PH

pCAGGS-HaloTag-KRas4B(CT) (Addgene #214276)
>Amino acid sequence
MAEIGTGFPFDPHYVEVLGERMHYVDVGPRDGTPVLFLHGNPTSSYVWRNIIPHVAPTHRCIAPDLIGMGKSDKPDLGYFF
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IDQNVFIEGTLPMGVVRPLTEVEMDHYREPFLNPVDREPLWRFPNELPIAGEPANIVALVEEYMDWLHQSPVPKLLFWGTP
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VIM*
>DNA sequence
ATGGCAGAAATCGGTACTGGCTTTCCATTCGACCCCCATTATGTGGAAGTCCTGGGCGAGCGCATGCACTACGTCGATGTT
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GTGATTATGTAG
>Components: HaloTag KRas4B(CT)

pCMV-mScarlet-I-eDHFR-ORP5(ΔPH) (Addgene #214277)
>Amino acid sequence
MVSKGEAVIKEFMRFKVHMEGSMNGHEFEIEGEGEGRPYEGTQTAKLKVTKGGPLPFSWDILSPQFMYGSRAFTKHPADIP
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NHILK*
>DNA sequence
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>Components: mScarlet-I eDHFR ORP5(ΔPH)

pT5-ORP9-PH-NusA-His6

>Amino acid sequence
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ALATATKKKYEQEIDVRVQIDRKSGDFDTFRRWLVVDEVTQPTKEITLEAARYEDESLNLGDYVEDQIESVTFDRITTQTA
KQVIVQKVREAERAMVVDQFREHEGEIITGVVKKVNRDNISLDLGNNAEAVILREDMLPRENFRPGDRVRGVLYSVRPEAR
GAQLFVTRSKPEMLIELFRIEVPEIGEEVIEIKAAARDPGSRAKIAVKTNDKRIDPVGACVGMRGARVQAVSTELGGERID
IVLWDDNPAQFVINAMAPADVASIVVDEDKHTMDIAVEAGNLAQAIGRNGQNVRLASQLSGWELNVMTVDDLQAKHQAEAH
AAIDTFTKYLDIDEDFATVLVEEGFSTLEELAYVPMKELLEIEGLDEPTVEALRERAKNALATIAQAQEESLGDNKPADDL
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HHH*

>DNA sequence
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CACCACCACTAA
>Components: ORP9-PH TEV protease cleavage sequence NusA His6