PNAS

Supporting Information for

Optogenetic clustering and membrane translocation of the BcLOV4 photoreceptor

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Legends for Movies S1 to S5

Other supporting materials for this manuscript include the following:

Movies S1 to S5

Supplementary Figures

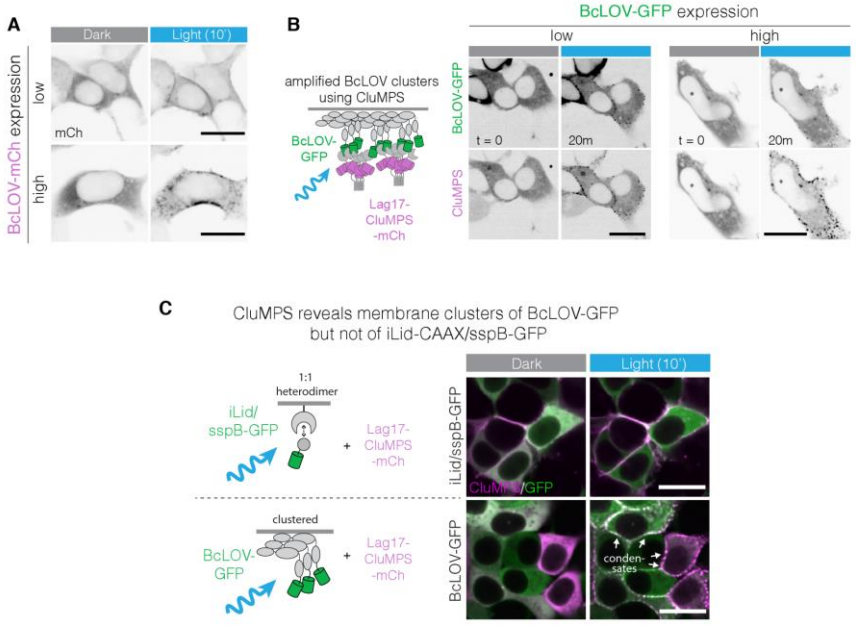

Figure S1: Light-induced clustering of BcLOV4 at the plasma membrane. A) Representative images of HEK 293T cells transiently transfected with BcLOV-mCherry under 40X confocal microscopy. In cells with high expression, light-induced aggregation at the plasma membrane could be observed. Scale bar = 20 µm. **B)** Representative images of cells co-expressing BcLOV-GFP with a CluMPS reporter of GFP clustering (LaG17-CluMPS). Light-activated BcLOV4 demonstrated both membrane-association and condensate formation even with low levels of BcLOV4 expression. Scale bar = 20 µm. **C)** Representative images of membrane association and clustering of GFP when recruited by either iLid/sspB (1:1 heterodimer) or BcLOV4 in the presence of the CluMPS reporter. Light-induced recruitment through BcLOV4 resulted in membrane-associated GFP condensates, whereas recruitment through heterodimerization did not, suggesting that clustering and CluMPS activation is not simply a result of membrane translocation. Scale bars = 20 µm.

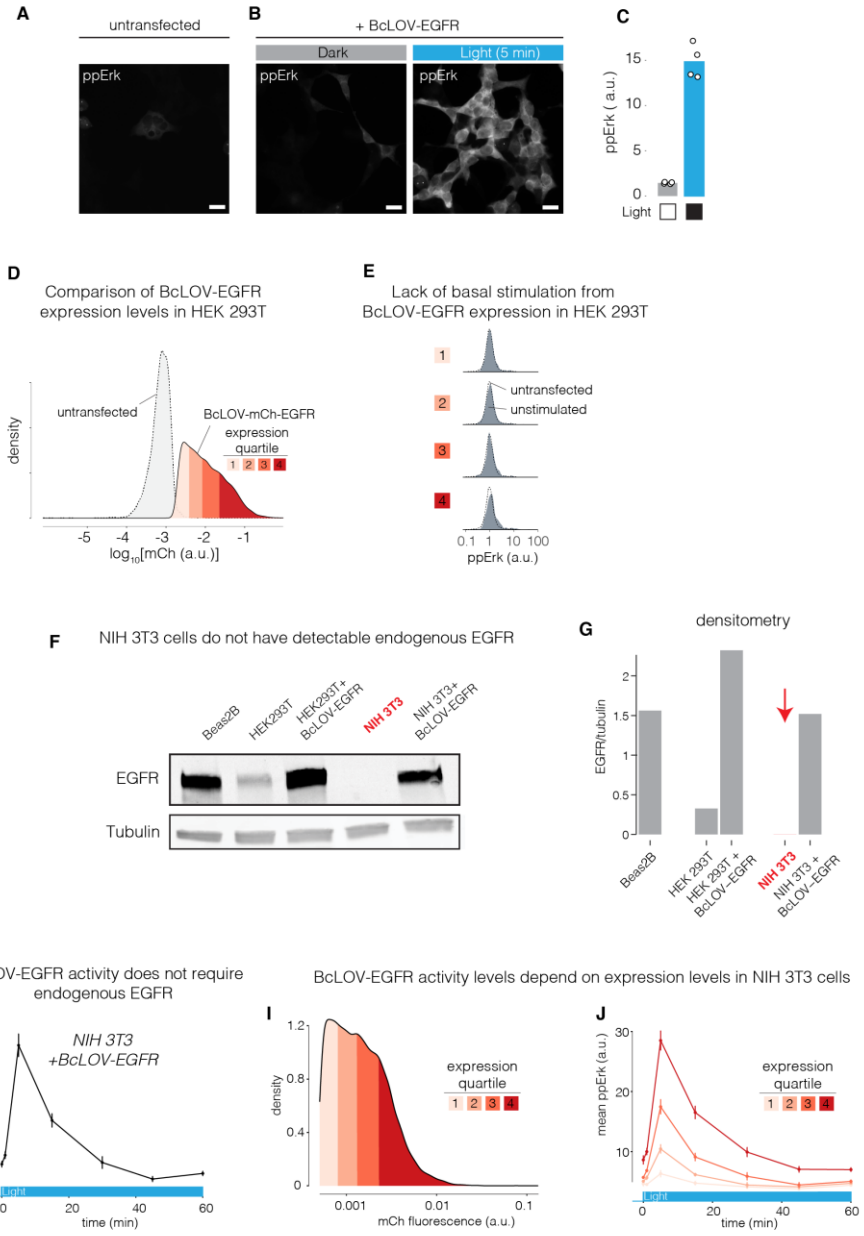
A
untransfected
ppErk
B
+ BcLOV-EGFR
Dark
Light (5 min)
ppErk
ppErk
C
ppErk (a.u.)
15
10
5
0
Light
D
Comparison of BcLOV-EGFR expression levels in HEK 293T
density
untransfected
BcLOV-mCh-EGFR
expression quartile
1 2 3 4
-5 -4 -3 -2 -1
log10[mCh (a.u.)]
E
Lack of basal stimulation from BcLOV-EGFR expression in HEK 293T
1
2
3
4
untransfected
unstimulated
0.1 1 10 100
ppErk (a.u.)
F
NIH 3T3 cells do not have detectable endogenous EGFR
Beas2B
HEK293T
HEK293T+ BcLOV-EGFR
NIH 3T3
NIH 3T3+ BcLOV-EGFR
EGFR
Tubulin
G
densitometry
EGFR/tubulin
2
1.5
1
0.5
0
Beas2B
HEK 293T
HEK 293T + BcLOV-EGFR
NIH 3T3
NIH 3T3 + BcLOV-EGFR
BcLOV-EGFR activity does not require endogenous EGFR
H
NIH 3T3 +BcLOV-EGFR
mean ppErk (a.u.)
16
12
8
Light
0 20 40 60
time (min)
BcLOV-EGFR activity levels depend on expression levels in NIH 3T3 cells
I
density
1.2
0.8
0.4
0
expression quartile
1 2 3 4
0.001 0.01 0.1
mCh fluorescence (a.u.)
J
mean ppErk (a.u.)
30
20
10
expression quartile
1 2 3 4
Light
0 20 40 60
time (min)

Figure S2: Optogenetic stimulation of Ras-Erk signaling using BcLOV-EGFR. Representative images of ppErk signal from untransfected HEK 293T cells (**A**) as well as from cells transfected with BcLOV-EGFR and kept in the dark or illuminated with 5 min of blue light (**B**)**.** Scale bars = 20 μm. **C)** Quantification of ppErk immunofluorescence resulting from 5 min of optogenetic stimulation of BcLOV-EGFR. Data represent mean ppErk intensity from four replicates. Each replicate represents the mean of ~100 cells. See **Table S1** for details of optogenetic stimulation. **D)** Visualization of expression level bins of BcLOV-EGFR vs untransfected HEK 293Ts. **E)** Single cell distributions of Erk activity in expressing (dark-state) vs non-expressing HEK 293T cells. Plots show no elevation of basal signaling resulting from probe expression. **F)** Western blot for EGFR in various cell lines shows low EGFR in HEK 293T cells and undetectable levels of EGFR in NIH 3T3 cells. Cell lines in lanes 3 and 5 were engineered to stably express BcLOV-EGFR. Beas2B is a lung epithelial cell line with strong native EGFR expression. **G)** Densitometry of Western blot in **(F)**. **H)** Activation of stably-expressed BcLOV-EGFR in NIH 3T3 cells, as assessed by single-cell immunofluorescence staining for ppErk. Data show that BcLOV-EGFR can be activated in the absence of endogenous EGFR. Data represents the mean ± SEM of four replicates, with each replicate representing the mean of ~600-1200 cells. **I)** Single-cell mean expression levels of BcLOV-EGFR for cells used in **(H).** Colors represent expression quartiles. **J)** Immunofluorescence data from **(H)** visualized by expression level demonstrate expression-dependent activation amplitude, as seen for transiently-transfected HEK 293T cells (**Figure S3E**). Data represents the mean ± SEM of four replicates, with each replicate representing the mean of ~150-300 cells per expression quartile. See **Table S1** for details of optogenetic stimulation parameters.

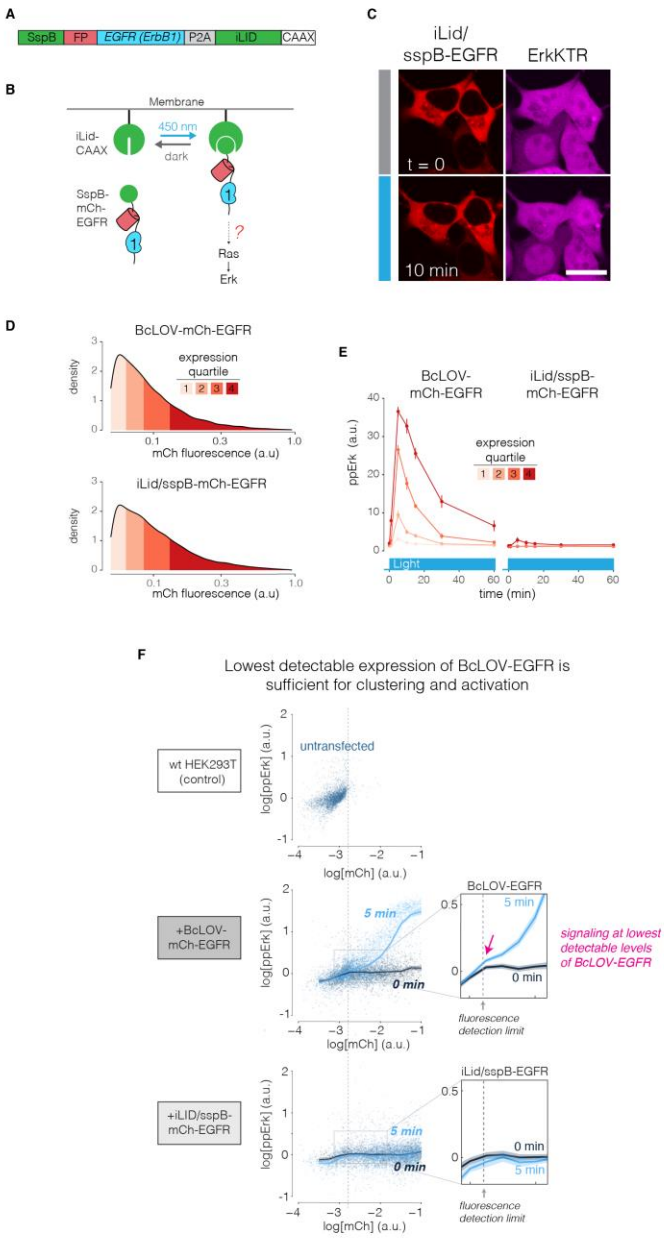
A
SspB
FP
EGFR (ErbB1)
P2A
iLID
CAAX
B
Membrane
iLid-CAAX
450 nm
dark
SspB-mCh-EGFR
Ras
Erk
C
iLid/ sspB-EGFR
ErkKTR
t = 0
10 min
D
BcLOV-mCh-EGFR
expression quartile
density
mCh fluorescence (a.u)
iLid/sspB-mCh-EGFR
E
BcLOV-mCh-EGFR
iLid/sspB-mCh-EGFR
ppErk (a.u.)
Light
time (min)
F
Lowest detectable expression of BcLOV-EGFR is sufficient for clustering and activation
wt HEK293T (control)
untransfected
log[ppErk] (a.u.)
log[mCh] (a.u.)
+BcLOV-mCh-EGFR
5 min
0 min
BcLOV-EGFR
signaling at lowest detectable levels of BcLOV-EGFR
fluorescence detection limit
+iLID/sspB-mCh-EGFR
iLid/sspB-EGFR

Figure S3: Membrane recruitment of the EGFR intracellular domain is not sufficient to activate downstream signaling. A) Schematic of iLID-CAAX/sspB-EGFR fusion construct. **B)** We asked whether light-induced recruitment of the EGFR intracellular domain to the plasma membrane was sufficient to activate downstream Ras-Erk signaling. **C)** Representative images of sspB-EGFR and ErkKTR-miRFP in HEK 293T cells in the presence and absence of light. Erk activation was not observed upon light stimulation. Scale bar = 20 μm. **D)** Single-cell mean expression levels of BcLOV- or iLid/sspB-based EGFR tools. Colors represent expression quartiles. **E)** Immunofluorescence of ppErk levels downstream of either BcLOV-EGFR or iLid/sspB-EGFR after light stimulation, separated by expression level. Expression-level-dependent stimulation was observed for BcLOV-EGFR. By comparison, no signaling was observed for iLid/sspB-EGFR except for a small amount at the very highest expression levels. Data represents the mean ± SEM of four replicates, with each replicate representing the mean of ~10-120 cells per expression quartile. See **Supplementary Table 1** for details of optogenetic stimulation parameters. (**F**) Visualization of ppErk activation as a function of probe expression in single cells. Top plot shows mCh autofluorescence in untransfected HEK 293T cells which was used to set the minimum threshold for mCh-expressing cells (dashed line). Middle and bottom panels show the same for HEK 293T cells that transiently expressed BcLOV-EGFR and iLID/sspB-EGFR. Data shows unstimulated cells (black) and cells stimulated with 5 minutes of blue light (blue). Solid lines represent a rolling average of ppErk across construct expression levels. Ribbons represent the 95% confidence interval. Data show elevated ppErk, and thus stimulated and clustered BcLOV-EGFR, at all expression levels, including at the mCherry detection limit. No activation was observed in cells expressing iLID/sspB-EGFR.

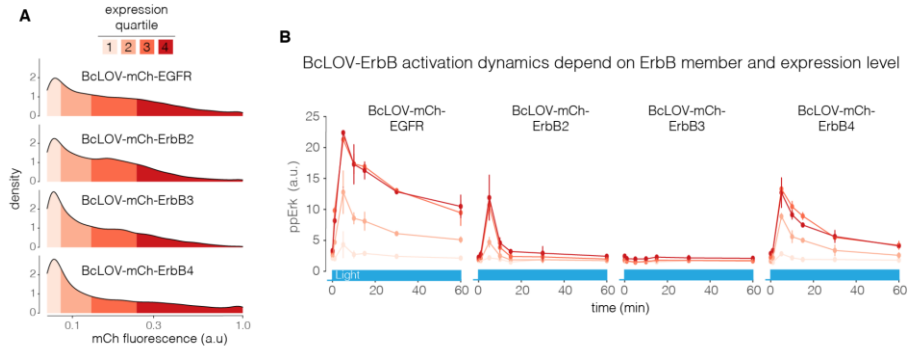

Figure S4: ppErk dynamics downstream of BcLOV-ErbB stimulation depend on the ErbB family member and on the expression level of the probe. A) Single-cell mean expression levels of BcLOV-ErbB tools. Colors represent expression quartiles. **B)** Comparison of differential magnitude, dynamics, and duration of ppErk signaling downstream of each ErbB member, visualized as a function of expression level. Data represent the mean ± SEM of two replicates, with each replicate representing the mean of ~200-800 cells per expression quartile. See **Table S1** for optogenetic stimulation parameters.

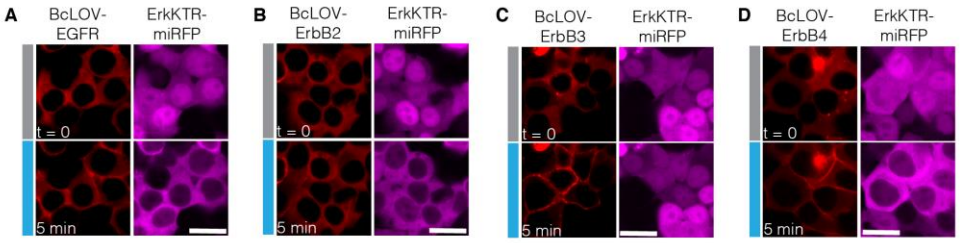

Figure S5: ErkKTR stimulation by BcLOV-ErbB1-4. Representative images of the ErkKTR-miRFP reporter in HEK 293T cells that were transiently transfected with BcLOV-ErbB1-4. Scale bars = 20 µm. See **Table S1** for optogenetic stimulation parameters.

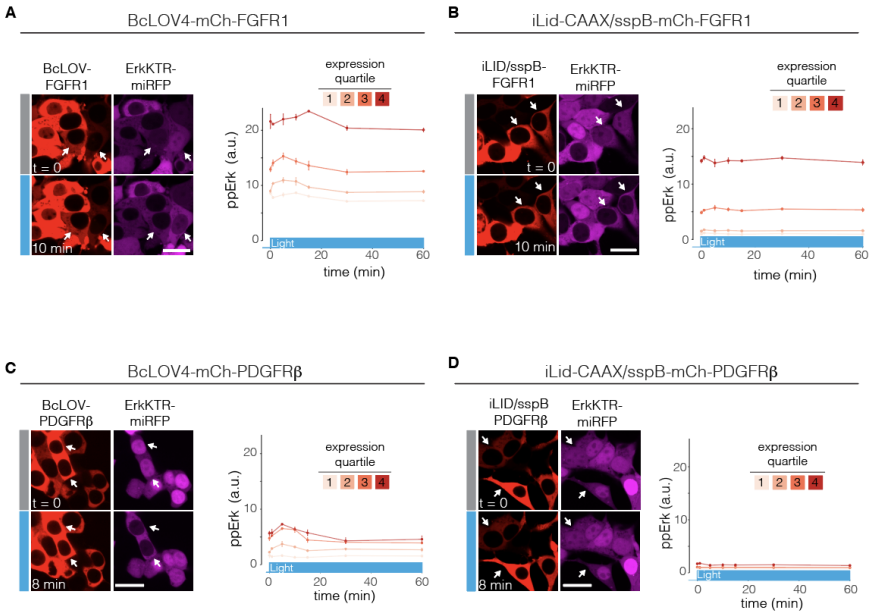

Figure S6: Stimulation of FGFR1 and PDGFRβ with BcLOV4 and comparison with stimulation through 1:1 heterodimeric recruitment to the plasma membrane. A) Stimulation of FGFR1 signaling with BcLOV4. Representative images (left) of the ErkKTR-miRFP reporter in HEK 293T cells that were transiently transfected with BcLOV-FGFR1. Light-induced ErkKTR stimulation can be observed only in low-expressing cells (white arrows). High-expressing cells show constitutively high Erk activity even in the absence of light. Immunofluorescence for ppErk (right) confirms expression-level dependence of basal levels and light-induced signaling. **B)** Stimulation of FGFR signaling through membrane recruitment with 1:1 heterodimerization of iLid/sspB. Representative images (left) of ErkKTR activity in HEK 293T cells that express iLid-CAAX/sspB-FGFR1. Here, membrane recruitment can stimulate the pathway at intermediate-high expression levels (white arrows). As in (**A**), high-expressing cells show constitutive activity even in the dark (t = 0). Immunofluorescence (right) confirms basal Erk activity for cells at high expression levels. **C)** Stimulation of PDGFRβ signaling with BcLOV4. Representative images (left) of the ErkKTR-miRFP reporter in HEK 293T cells that were transiently transfected with BcLOV-PDGFRβ. Light-induced ErkKTR stimulation can be observed only in mid-high-expressing cells (white arrows). High-expressing cells show constitutively high Erk activity even in the absence of light. Immunofluorescence for ppErk (right) shows expression-level dependence on Erk phosphorylation. **D)** Stimulation of PDGFRβ signaling through membrane recruitment with 1:1 heterodimerization of iLid/sspB. Representative images (left) of ErkKTR activity in HEK 293T cells that express iLid-CAAX/sspB-PDGFRβ. Unlike for FGFR1, membrane recruitment does not stimulate PDGFRβ and downstream Erk activity (white arrows). Scale bars = 20 μm. Immunofluorescence (right) confirms no Erk activation across all expression levels. For immunofluorescence, data represents the mean ± SEM of two replicates, with each replicate representing the mean of ~100-600 cells per expression quartile. See **Table S1** for details of optogenetic illumination parameters.

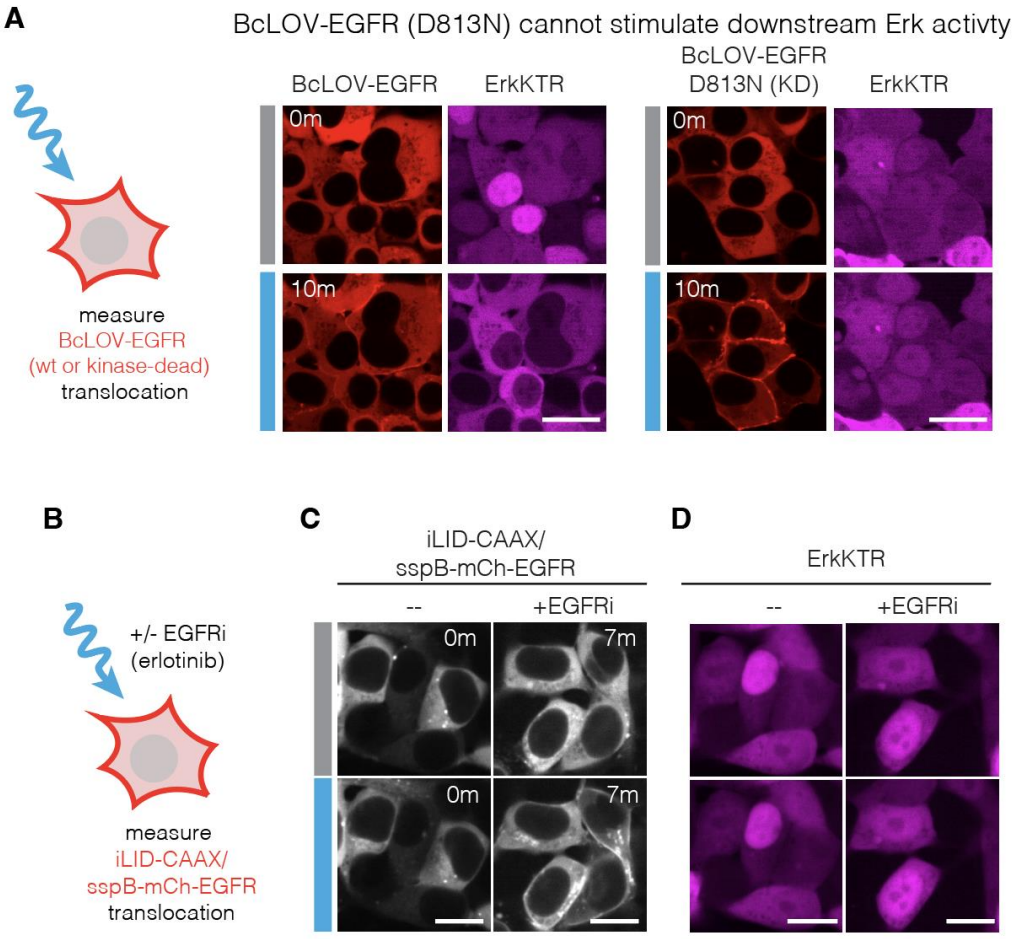

Figure S7: Inhibition of EGFR kinase activity enhances translocation of the EGFR domain. A) The ErkKTR reporter confirms that light-induced translocation of EGFR(D813N) cannot activate Ras-Erk signaling. Scale bar = 20 μm. **B**) HEK 293T cells expressing iLID-CAAX/sspB-mCh-EGFR were treated with 1 μM erlotinib (EGFR inhibitor) prior to blue-light stimulation, and membrane translocation and ErkKTR activity was observed in untreated and drug-treated conditions. **C)** In untreated cells, minimal membrane translocation could be observed. However, in cells treated with erlotinib, clear translocation was observed in response to light stimulation. **C)** Corresponding ErkKTR-miRFP images for cells in (**B**). ErkKTR activity could not be observed even with observable sspB-EGFR recruitment due to inhibition of the EGFR kinase.

Supplementary Movie Captions

Movie S1. CluMPS amplifies and visualizes membrane-associated BcLOV4 condensates. Confocal imaging of BcLOV-GFP translocation with or without CluMPS in HEK 293T cells upon stimulation with 488 nm light. Time is in minutes:seconds. Blue square indicates light stimulation. Scale bar = 10 µm.

Movie S2. The Ras-Erk pathway was activated in cells by BcLOV-EGFR. Confocal imaging of BcLOV-EGFR translocation and activation of ErkKTR-miRFP in HEK 293T cells upon stimulation with 488 nm light. Blue square indicates light stimulation.

Movie S3. Magnitude of membrane translocation differs between fusions of ErbB family members with BcLOV4. Confocal imaging of BcLOV-mCh-ErbB1-4 translocation to the membrane upon blue light stimulation with 488 nm light. Blue square indicates light stimulation.

Movie S4. ErbB activity correlates with membrane ruffling. Confocal imaging of BcLOV-mCh-ErbB1-4 translocation and induced membrane ruffling upon stimulation with 488 nm light. Blue square indicates light stimulation.

Movie S5. Disordered domains increase light-induced membrane translocation of BcLOV4. Confocal imaging of IDR-fused variants of BcLOV-mCh in HEK 293T cells upon stimulation with 488 nm light. Blue square indicates light stimulation.

Supplementary Table S1

Illumination and culture conditions for all experiments							
Figure	Temp (°C)	Duty Cycle	Intensity	ON time	OFF time	Humidified	CO_2
1B	37	3.3%	1.45W/cm^2	1s	30s	Yes	5%
1D	37	3.3%	1.45W/cm^2	1s	30s	Yes	5%
2C	37	3.3%	1.45W/cm^2	1s	30s	Yes	5%
2D	37	3.3%	1.45W/cm^2	1s	30s	Yes	5%
2E	25,37	10%	160mW/cm^2	0.5s	5s	Yes	5%
2F-G	37	Variable	160mW/cm^2	0.5s	Variable	Yes	5%
3B	37	3.3%	1.45W/cm^2	1s	30s	Yes	5%
3C	37	2.5%	160mW/cm^2	0.5s	20s	Yes	5%
3D	37	2.5%	160mW/cm^2	0.5s	20s	Yes	5%
3E	37	3.3%	1.45W/cm^2	1s	30s	Yes	5%
3F	37	3.3%	1.45W/cm^2	1s	30s	Yes	5%
4B	37	3.3%	1.45W/cm^2	1s	30s	Yes	5%
4C	37	3.3%	1.45W/cm^2	1s	30s	Yes	5%
5B	37	3.3%	1.45W/cm^2	1s	30s	Yes	5%
5C	37	100%	Variable	const.	----	Yes	5%
5D-E	37	Variable	160mW/cm^2	0.5s	Variable	Yes	5%
5F	37	2.5%	160mW/cm^2	0.5s	20s	Yes	5%
6B	37	3.3%	1.45W/cm^2	1s	30s	Yes	5%
6C	37	3.3%	1.45W/cm^2	1s	30s	Yes	5%
6D	37	100%	Variable	const.	----	Yes	5%
6E	37	100%	Variable	const.	----	Yes	5%
6F	37	20%	160mW/cm^2	0.5s	2.5s	Yes	5%
S1	37	3.3%	1.45W/cm^2	1s	30s	Yes	5%
S2A-E	37	5%	160mW/cm^2	0.5s	10s	Yes	5%
S2H,J	37	5%	160mW/cm^2	0.5s	10s	Yes	5%

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S3C	37	3.3%	1.45W/cm^2	1s	30s	Yes	5%
S3E,F	37	5%	160mW/cm^2	0.5s	10s	Yes	5%
S4-B	37	2.5%	160mW/cm^2	0.5s	20s	Yes	5%
S5	37	3.3%	1.45W/cm^2	1s	30s	Yes	5%
S6 (Live~~-cell Imaging~~)	37	3.3%	1.45W/cm^2	1s	30s	Yes	5%
S6 (IF)	37	2.5%	160mW/cm^2	0.5s	20s	Yes	5%
S7	37	3.3%	1.45W/cm^2	1s	30s	Yes	5%
SM1	37	3.3%	1.45W/cm^2	1s	30s	Yes	5%
SM2	37	3.3%	1.45W/cm^2	1s	30s	Yes	5%
SM3	37	3.3%	1.45W/cm^2	1s	30s	Yes	5%
SM4	37	3.3%	1.45W/cm^2	1s	30s	Yes	5%
SM5	37	3.3%	1.45W/cm^2	1s	30s	Yes	5%

Supplementary Table S2. Constructs used in this study

Construct	Backbone + Promoter
BcLOV-GFP	pHR CMV
SspB-GFP-2A-iLID-CAAX	pHR CMV
LaG17-mCh-HOTag3	pHR PGK
ErkKTR-miRFP670	pHR SFFV
BcLOV-mCherry-EGFR/Erbb1	pHR CMV
BcLOV-mCherry-ErbB2	pHR CMV
BcLOV-mCherry-ErbB3	pHR CMV
BcLOV-mCherry-ErbB4	pHR CMV
BcLOV-mCherry-EGFR D813N (KD)	pHR CMV
SspB-mCherry-EGFR-2A-iLID-CAAX	pHR CMV
BcLOV-mCherry-FGFR	pHR CMV
BcLOV-mCherry-PDGFRβ	pHR CMV
SspB-mCherry-FGFR-2A-iLID-CAAX	pHR CMV
SspB-mCherry-PDGFR-2A-iLID-CAAX	pHR CMV
BcLOV-mCherry	pcDNA3.1 CMV
FUS(LC)-BcLOV-mCherry	pcDNA3.1 CMV
RGG-BcLOV-mCherry	pcDNA3.1 CMV
FUS(LC)-BcLOV-mCherry-EGFR	pHR CMV
RGG-BcLOV-mCherry-EGFR	pHR CMV
BcLOV-mCherry-SOS_{cat}	pHR SFFV
FUS(LC)-BcLOV-mCherry-SOS_{cat}	pHR SFFV
RGG-BcLOV-mCherry-SOS_{cat}	pHR SFFV
GFP-CAAX	pHR CMV