

Supplementary note**Development of Rho GTPase FRET sensors**

Our Rho activity sensor consists of two components: a Rho GTPase (RhoA or Cdc42) tagged with mEGFP (mEGFP-Rho GTPase) and its binding partner Rho GTPase binding domain (RBD) doubly tagged with mCherry (mCherry-RBD-mCherry) (**Supplementary Figs. 1, 2**). By measuring fluorescence lifetime of mEGFP-RhoGTPase, the binding fraction of mEGFP-Rho GTPase bound to mCherry-RBD-mCherry can be measured. The binding fraction is a function of the expression level of RBD and the dissociation constant between Rho GTPase and RBD^{1,2}. To optimize the sensitivity and kinetics of the sensors, we have chosen RBDs for the sensors based on three criteria: first, the dissociation constant between the RBD and the active, GTP-bound form of Rho GTPase is ~micromolars^{1,2}; second, the dissociation constant for the inactive, GDP-bound form is higher than ~tens of micromolars; and third, it binds selectively to the target Rho GTPase. We measured the affinity between Rho GTPases (RhoA, Cdc42 and Rac1) and several RBDs and their mutants in the presence of either GppNHP (non-hydrolyzable GTP analog) or GDP in a cuvette using fluorescence lifetime measurements (**Supplementary Fig. 1**). Through these experiments, we identified Rhotekin(8–89) for the RhoA sensor and Pak3(60–113)/S74A/F84A for the Cdc42 sensor (**Supplementary Fig. 1, Supplementary Table 1**).

Characterization of Rho GTPase sensors in HeLa cells and neurons.

To test the sensitivity and specificity of the Rho GTPase FRET sensors in cells, we transfected Rho GTPase FRET sensors with constitutively active (Q63L for RhoA, Q61L for Cdc42), or dominant negative mutations (T19N for RhoA, T17N for Cdc42) in HeLa cells, and imaged under 2-photon fluorescence lifetime imaging microscopy (2pFLIM). The fraction of mEGFP-Rho GTPase bound to mCherry-RBD-mCherry was calculated from the fluorescence lifetime decay (see Methods). Constitutively active and dominant negative mutants of Rho GTPase FRET sensors showed binding fractions of ~45% and ~15%, respectively (**Supplementary Fig. 2**), indicating high sensitivity and specificity of FRET signal. Cells expressing the wildtype sensors together with constitutively active Dbp (guanine exchange factor; activator of RhoA and Cdc42) and p50RhoGAP (GTPase activating protein; inactivator of RhoA and Cdc42) showed high (~45%) and low (~15%) binding fractions, respectively (**Supplementary Fig. 2**). Finally, we tested the specificity of the RBDs used for the sensors by swapping the RBDs for the RhoA and Cdc42 sensors: constitutively active RhoA was paired with Pak3(60–113)/S74A/F84A and constitutively active Cdc42 with Rhotekin(8–89). Under these conditions, the binding fractions were low (~10%), suggesting that the RBDs used for the sensors are specific to their target Rho GTPases (**Supplementary Fig. 2**). It should be noted that we measure fluorescence lifetime only from mEGFP-Rho GTPase, and thus non-specific binding of mCherry-RBD-mCherry should not affect our

FRET measurements. Overall, these results indicate that our RhoA and Cdc42 sensors are sensitive and specific to RhoA and Cdc42 activation, respectively.

Next, we sparsely transfected neurons with RhoA or Cdc42 sensors in cultured hippocampal slices using ballistic gene transfer, and imaged CA1 pyramidal neurons expressing these sensors (**Supplementary Fig. 11**). In response to bath application of NMDA (15 μM in zero Mg^{2+} for 2 min), the RhoA and Cdc42 sensors showed robust and reversible signal in spines and dendrites (**Supplementary Fig. 11a, b**)^{3,4}.

In order to test if changes in fluorescence lifetime of Rho GTPase sensors during spine structural plasticity (**Fig. 1 and 2**) is due to binding between Rho GTPase and RBD, we performed control experiments in which mEGFP-RhoA is paired with Cdc42 acceptor and mEGFP-Cdc42 with RhoA acceptor. These non-optimal sensors showed much smaller FRET changes during spine structural plasticity than the Rho GTPase sensors with the optimal combinations did (**Supplementary Fig. 12, 13**), suggesting that the FRET signal of our Rho GTPase sensors is caused by binding between Rho-GTPases and RBDs of the sensors.

Effects of overexpression of Rho GTPase sensors on the spatiotemporal dynamics of Rho GTPases and spine structural plasticity

When neurons in hippocampal cultured slices were ballistically⁵ transfected with RhoA or Cdc42 sensors, the expression level of mEGFP-Rho GTPase was measured to be 0.2–8 μM (**Supplementary Figs. 11, 14**), several folds higher than the level of endogenous proteins on average (0.25 μM for RhoA, 0.38 μM for Cdc42; **Supplementary Fig. 15**). The expression level of mCherry-RBD-mCherry was 1–250 μM (**Supplementary Figs. 11, 14**). To test the effects of the overexpression of Rho GTPase sensors on the spatiotemporal dynamics of Rho GTPases, we measured the time constant of FRET signal decay in neurons after bath-application of NMDA at the different expression levels of mEGFP-Rho GTPases and mCherry-RBD-mCherry (**Supplementary Fig. 11d, e**)¹. There was no correlation between the decay time constant and the concentration of mEGFP-Rho GTPase or mCherry-RBD-mCherry (**Supplementary Fig. 11d, e**), suggesting that overexpression of the sensors does not alter the decay kinetics of Rho GTPases. Furthermore, the length constant of the spreading of Rho GTPase activity during spine structural plasticity (**Fig. 3a–d**) was similar across the whole range of the sensor concentrations (**Supplementary Fig. 6, 14**), suggesting that the expression of the sensor does not affect the spatial profile of the Rho GTPase activation. Finally, the time constant and immobile fraction of the fluorescence decay after photoactivation of paGFP-Rho GTPase in spines (**Fig. 3e–g**) were independent of the basal fluorescence intensity of paGFP at the thick dendrite (**Supplementary Fig. 16**), implicating that overexpression of Rho GTPase does not affect the mobility of Rho GTPase.

The time courses of spine enlargement induced by 2-photon glutamate uncaging in neurons expressing Rho GTPase sensors were in general similar to that in neurons expressing

EGFP (**Figs 1d, 2d, 4a-h**), suggesting that overexpression of Rho GTPase sensors does not affect spine structural plasticity. One exception was that KN62 partially inhibited the transient phase of structural plasticity in neurons expressing RhoA sensor (**Fig. 1d**) but not in neurons expressing Cdc42 sensor (**Fig. 2d**). Because KN62 is known to inhibit the sustained phase, but has significantly less of an effect on the transient phase in neurons expressing only EGFP^{1,6,7}, this difference may be due to expression of RhoA sensor.

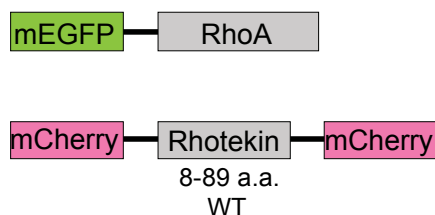
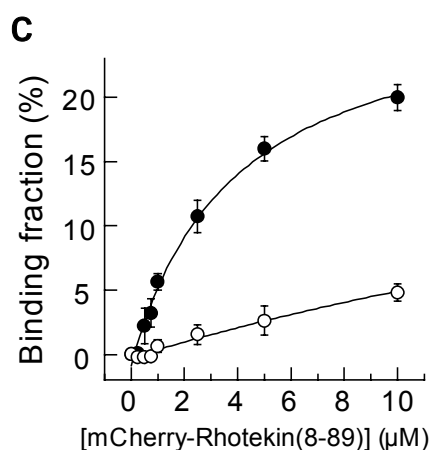
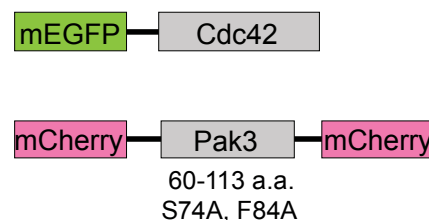
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4. Semenova, M. M. et al. Rho mediates calcium-dependent activation of p38alpha and subsequent excitotoxic cell death. *Nat Neurosci* **10**, 436-443 (2007).
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6. Matsuzaki, M., Honkura, N., Ellis-Davies, G. C. & Kasai, H. Structural basis of long-term potentiation in single dendritic spines. *Nature* **429**, 761-766 (2004).
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Supplementary Table 1. Dissociation constants between Rho GTPases and RBD mutants

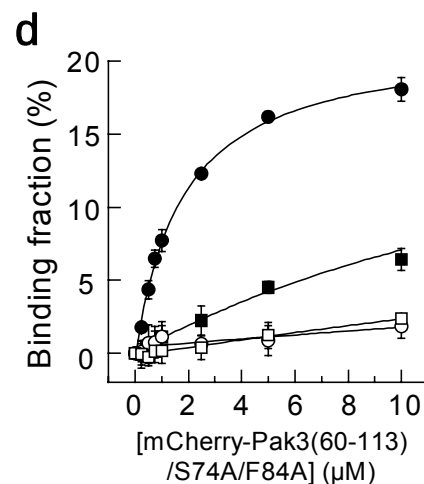
Dissociation constants (*Kd*) were measured as in **Supplementary Fig. 1**.

N.D.: not determined. All values are averages of three independent experiments.

<i>Kd</i> (μM)	Cdc42 GDP	Cdc42 Gpp(NH)p	Rac1 GDP	Rac1 Gpp(NH)p	RhoA GDP	RhoA Gpp(NH)p
Pak1 (wt)	7.7	0.15	17.4	0.36	N.D.	N.D.
Pak2 (wt)	10.2	0.18	22.1	0.51	N.D.	N.D.
Pak3 (wt)	57	0.34	68.87	1.8	N.D.	N.D.
Pak3 (F84A)	96	0.89	132	9.4	N.D.	N.D.
Pak3 (S74A, F84A)	149	1.77	77	22	N.D.	N.D.
Rhotekin (wt)	N.D.	N.D.	N.D.	N.D.	47	3.9

a RhoA FRET sensor**b** Cdc42 FRET sensor

○ RhoA-GDP: 47 μM
 ● RhoA-GppNHp: 3.9 μM



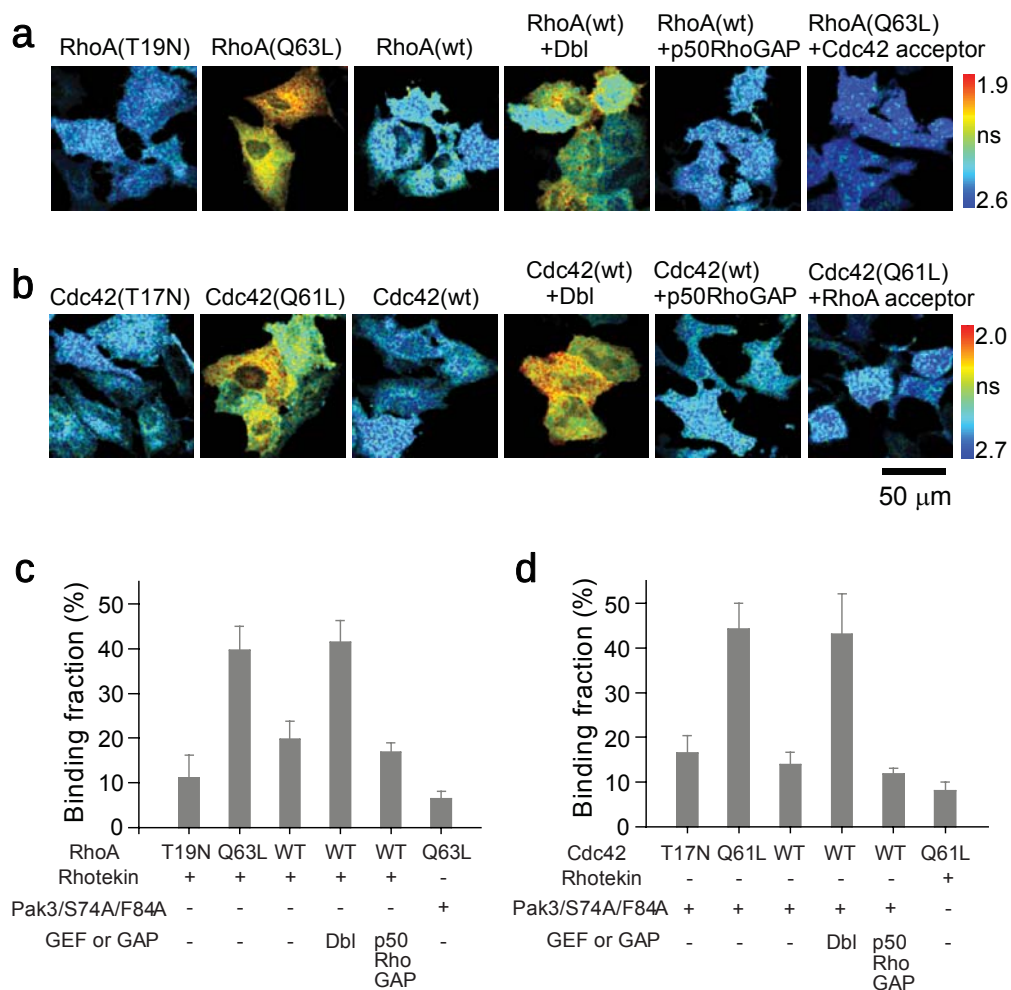
○ Cdc42-GDP: 149 μM
 ● Cdc42-GppNHp: 1.8 μM
 □ Rac1-GDP: 78 μM
 ■ Rac1-GppNHp: 22 μM

Supplementary Fig. 1. Design of Rho GTPase sensors

a, Schematics of RhoA FRET sensor. mEGFP was tagged to the N terminus of RhoA, and two mCherrys are attached to the N and C termini of Rhotekin(8–89), which binds to active Rho selectively. When RhoA is activated, Rhotekin(8–89) binds to RhoA, producing FRET between mEGFP and mCherry.

b, Schematics of Cdc42 FRET sensor. mEGFP was tagged to the N-terminus of Cdc42, and two mCherrys are attached to the N and C termini of Pak3(60–113)/ S74A/F84A which binds to activated Cdc42.

c-d, Measurements of the affinity between sfGFP-tagged RhoA (**c**) or Cdc42 (**d**) and their binding partners tagged with mCherry. The binding fraction was measured with 2pFLIM as a function of the RBD concentration. The dissociation constant was obtained by fitting the data with a Michaelis-Menten function (curves). Average of three independent experiments. The dissociation constants between Rho GTPases and various RBDs and their mutants are summarized in **Supplementary Table 1**.

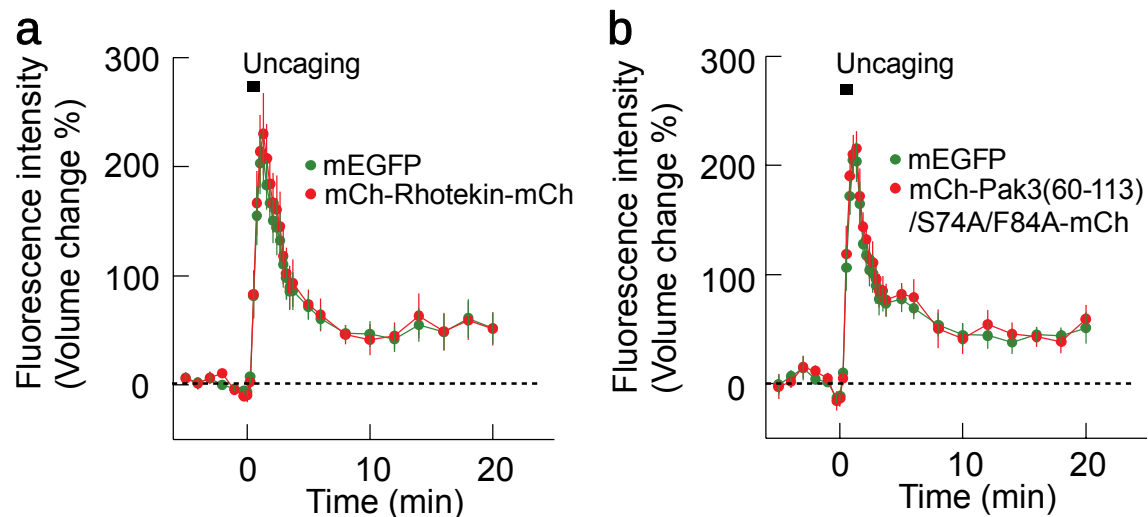


Supplementary Fig. 2. Characterization of FRET sensors in HeLa cells

a-b, Representative fluorescence lifetime images in HeLa cells transfected with Rho GTPase sensors with dominant negative (RhoA(T19N), Cdc42(T17N)) and constitutively active (RhoA(Q63L), Cdc42(Q61L)) mutations, wild-type sensors together with Rho GTPase activator (Dbl) and inactivator (p50 RhoGAP), constitutively active mEGFP-RhoA(Q63L) paired with Cdc42 acceptor (mCherry-Pak3(60-113)/S74A/F84A-mCherry) and mEGFP-Cdc42(Q61L) paired with RhoA acceptor (mCherry-Rhotekin(8-89)-mCherry).

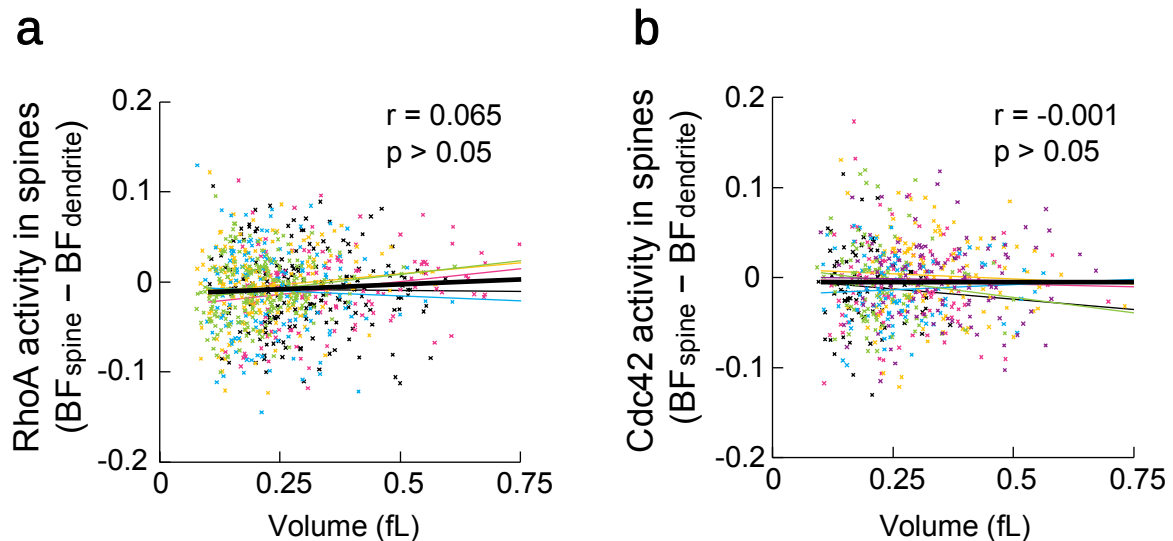
Warmer colors indicate shorter lifetimes and higher levels of Cdc42 or RhoA activity. Scale bar, 5 μ m. Fluorescence lifetime imaging was performed 18–24 hours after transfection in a solution containing 30 mM Na-HEPES(pH 7.3), 130 mM NaCl, 2.5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 2 mM NaHCO₃, 1.25 mM NaH₂PO₄ and 25 mM glucose.

c-d, The fraction of mEGFP-Rho GTPases bound to mCherry-RBD-mCherry calculated by fitting the fluorescence lifetime curve with a double exponential function (Eq. S2 in **Supplementary methods**). Because some fraction of mCherry is in a dark state (~50% in HeLa cells^{2,8}), the binding fraction is underestimated. Error bars indicate s.e.m. over 10 fields from 1–2 preparations.



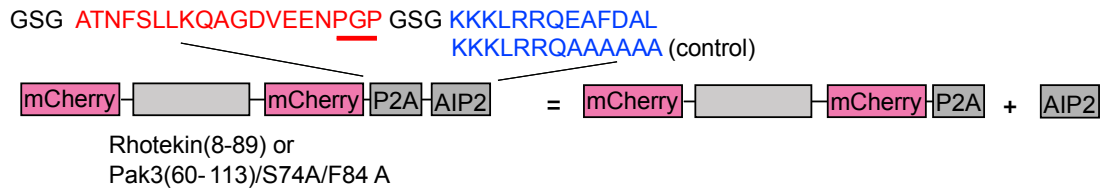
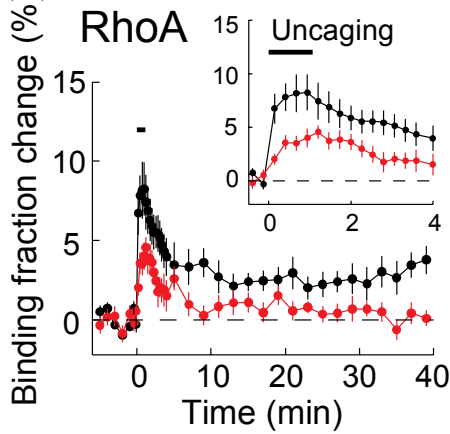
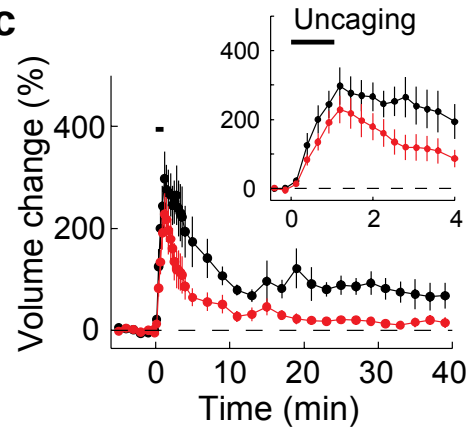
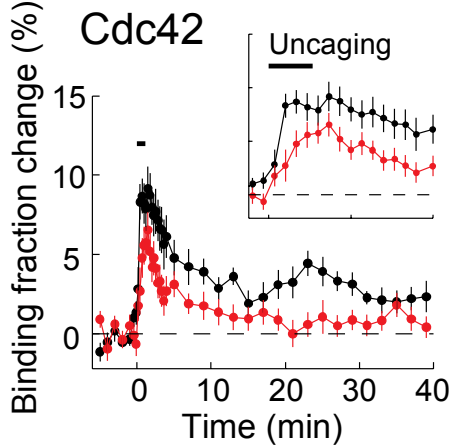
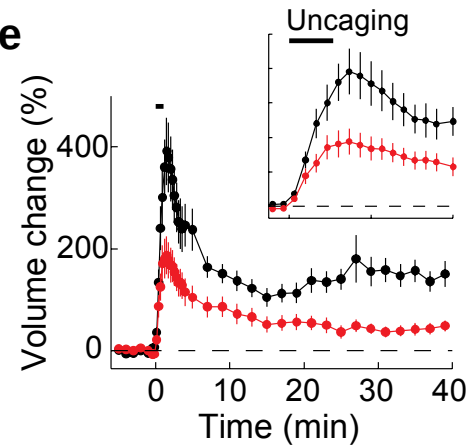
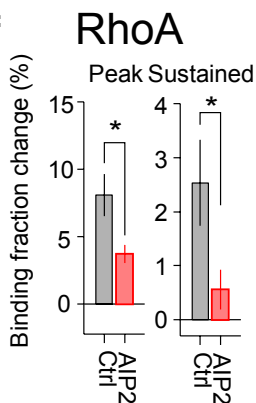
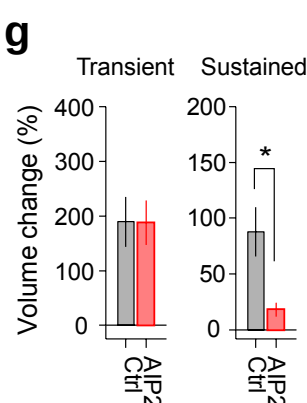
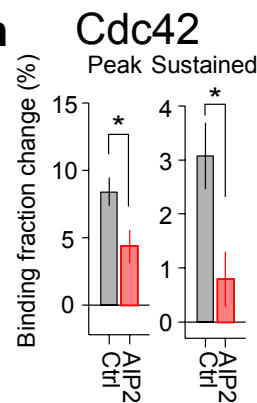
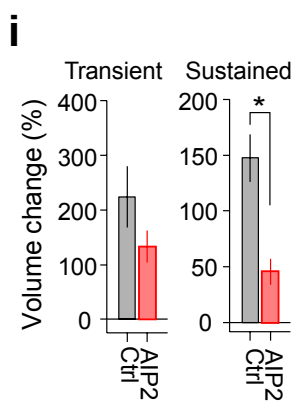
Supplementary Fig. 3. Fluorescence intensity of mCherry-RBD-mCherry reflects the spine volume change during spine enlargement induced by 2-photon glutamate uncaging.

mEGFP was co-transfected with mCherry-Rhotekin(8-89)-mCherry (**a**) or mCherry-Pak3 (60–113)/S74A/ F84A-mCherry (**b**), and fluorescence from mEGFP and mCherry was simultaneously imaged during spine structural plasticity. There was no detectable difference in the time course of fluorescence intensity change between mEGFP and mCherry-RBD-mCherry, indicating that mCherry-RBD-mCherry can be used as space filling marker. The numbers of samples (spines/neurons) are 10/5 for (**a**), and 7/4 for (**b**). Error bars are s.e.m..



Supplementary Fig. 4. Relationship between spine volume and Rho GTPase activity

The binding fraction in spines (BF_{spine}) is subtracted by the binding fraction in their parent dendrites (BF_{dendrite}). The numbers of samples (spines/neurons) are 837/5 for RhoA (**a**), 610/6 for Cdc42 (**b**). Thick black and thin colored lines show the correlation between spine volume and activity from all cells and each cells, respectively. Different colors represent the data from different cells. No significant correlations ($p > 0.05$) were found.

a**b****c****d****e****f****g****h****i**

Supplementary Fig. 5. The effect of Autocamtide-2 Related Inhibitory Peptide 2 (AIP2) expression on RhoA and Cdc42 activity during structural plasticity induced by 2-photon glutamate uncaging.

a, Schematic drawing of the constructs used to express AIP2 or control peptide together with mCherry-RBD-mCherry. DNA of FRET acceptor and AIP2 are linked together via P2A element so that they are translated into different polypeptides in cells⁹⁻¹². The concentration of AIP2 should be the same as FRET acceptor, and thus can be measured (5–50 μ M).

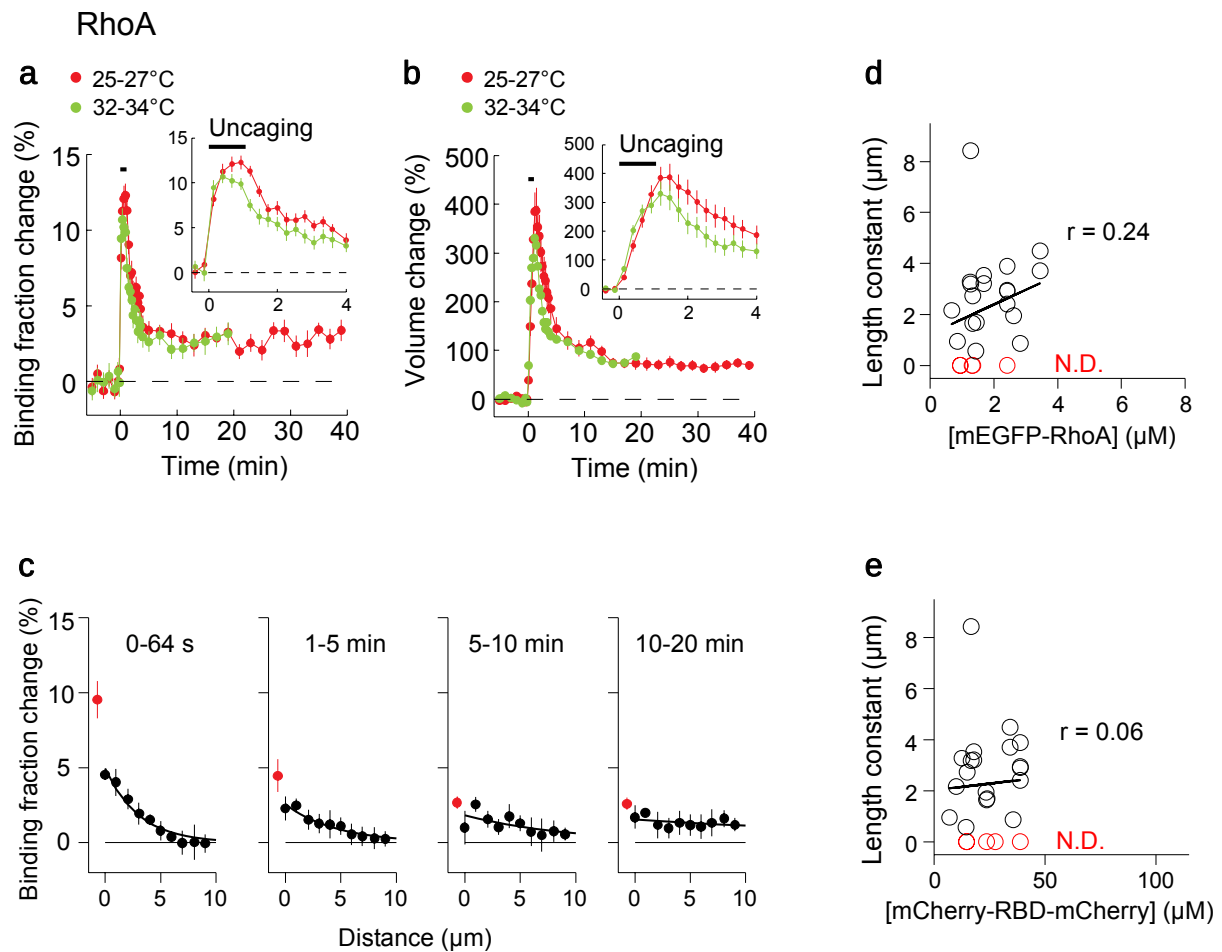
b, Time course of RhoA activation in response to 2-photon glutamate uncaging. AIP2 expression suppressed the activity of RhoA during structural plasticity (Ctrl, Black; AIP2, Red). Inset: closer view. The numbers of samples (spines/neurons) are 11/9 for stimulated spines and 13/9 for AIP2 expression. Error bars are s.e.m.

c, Averaged time course of spine volume change in the same experiments in **b**.

f, Transient (averaged over 16–64 s) and sustained (averaged over 20–38 min) RhoA activation. Stars denote statistically significant difference (< 0.05 , t-test) from the value in the stimulated spine under control condition.

g, Transient (volume change averaged over 1.5–2 min subtracted by that over 20–38 min) and sustained volume change (volume change averaged over 20–38 min). Stars denote statistically significant difference ($p < 0.05$, t-test) from the value in the stimulated spine under control condition.

d,e,h,i, The same experiments and analyses as in **b,c,f**, and **g** except for measuring Cdc42 activity instead of RhoA activity. The numbers of samples (spines/neurons) are 14/11 for stimulated spines and 19/13 for AIP2 expression. Error bars are s.e.m.



Supplementary Fig. 6. Spatial profile of RhoA activity at near physiological temperature during spine structural plasticity

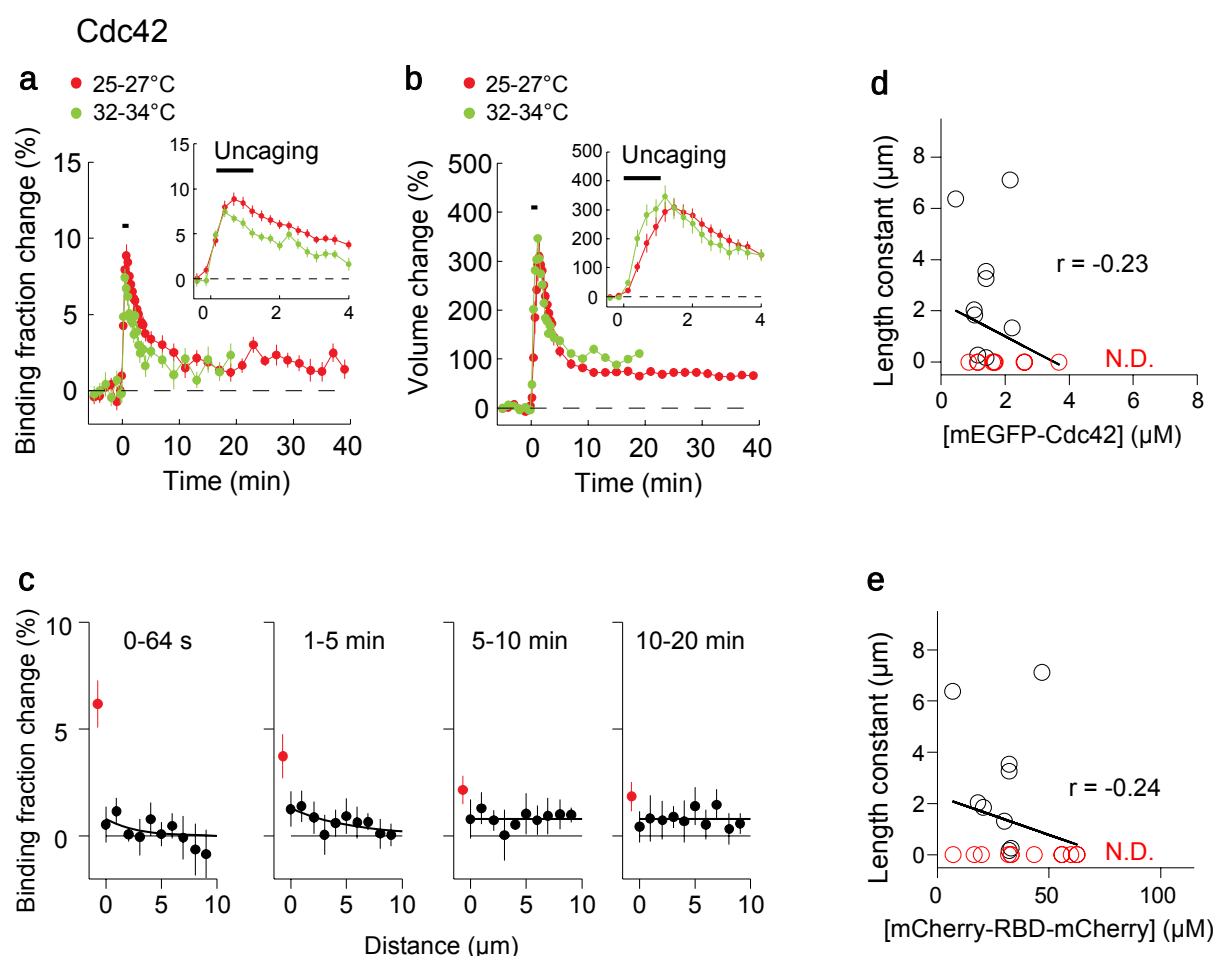
a, Time course of RhoA activation in stimulated spines at near physiological temperature (32–34°C, green), compared with that at room temperature (red, data from **Fig. 1b**). Inset: closer view. Error bars are s.e.m.

b, Averaged time course of spine volume change in the same experiments in **b**.

c, Averaged spatial profile of RhoA activation. Red circles indicate the activity in the stimulated spine, and black circles indicate the activity in dendrite, plotted as a function of the distance along the dendrite from the stimulated spine.

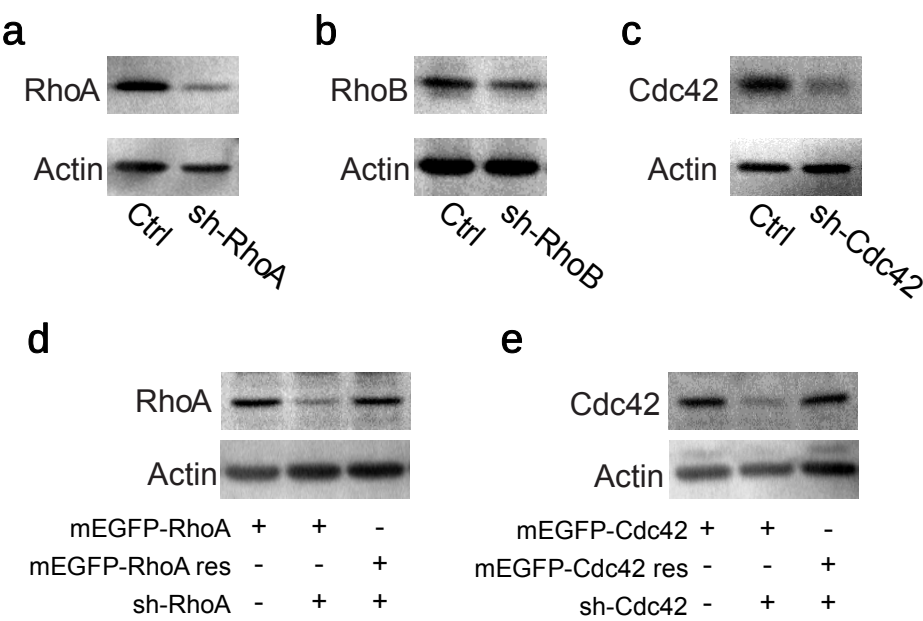
d-e, The length constants of activated RhoA spreading along dendrites following single spine activation at different concentrations of mEGFP-Rho GTPase (**d**) and mCherry-RBD-mCherry (**e**). The length constant during uncaging was measured by fitting the spatial profile of the activity between 0–9 μm with a single exponential function. In some experiments, there was no significant activity spreading from the stimulated spine to dendrites, and thus the length constant was not determined (red).

The number of samples (spines/neurons) is 24/13.



Supplementary Fig. 7. Spatial profile of Cdc42 activity at near physiological temperature during spine structural plasticity.

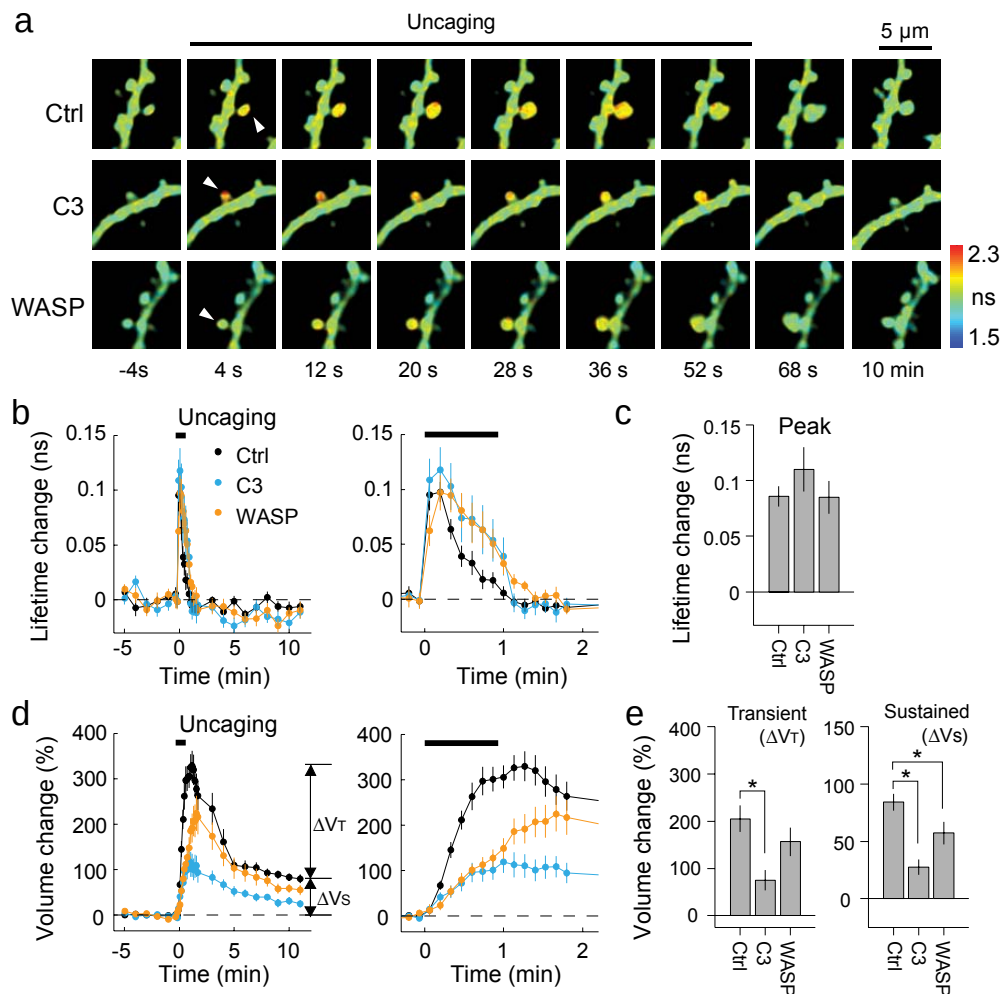
The same experiments and analyses as in **Supplementary Fig. 6** except for measuring Cdc42 activity instead of RhoA activity. Data at room temperature (25-27 °C) is from **Fig. 2**. The number of samples (spines/neurons) is 21/9.



Supplementary Fig. 8. Verification of shRNA and shRNA-resistant RhoA and Cdc42

a-c, Verification of shRNA. Rat1 cells were transfected with scramble shRNA (Ctrl) or shRNA for RhoA (**a**), RhoB (**b**) and Cdc42 (**c**). Cell extracts were prepared 72–96 hrs after transfection and analyzed by Western blotting.

d-e, Verification of shRNA-resistant RhoA and Cdc42. HeLa cells were transfected with scramble shRNA or shRNA against RhoA (**d**) and Cdc42 (**e**), co-expressed with wildtype mEGFP-Rho GTPases or their shRNA resistant mutants, respectively.



Supplementary Fig. 9. CaMKII activation under Rho GTPase inhibition

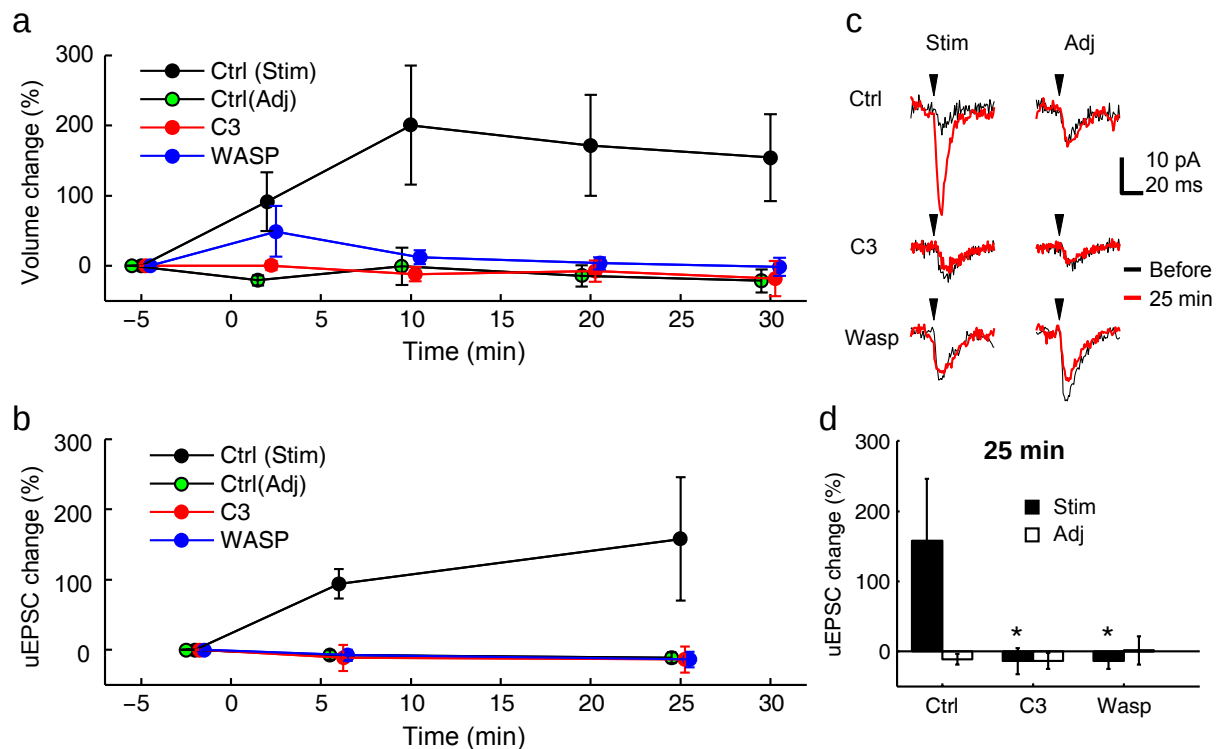
a, Fluorescence lifetime images of Green-Camuia, the CaMKII α activity sensor⁷, during the induction of spine enlargement by 2-photon glutamate uncaging in neurons expressing Green-Camuia together with mCherry-C3 transferase (C3; Rho inhibitor) or mCherry-Wasp (210-321) (Wasp; Cdc42 inhibitor). Arrow heads indicate the stimulated spine.

b, Left: Time course of CaMKII activation measured as a change in the fluorescence lifetime of Green-Camuia in the stimulated spine⁷ in neurons expressing mCherry (control), C3 or Wasp. Right: closer view. The numbers of samples (spines/neurons) are 20/5 for ctrl, 14/5 for C3, 15/6 for Wasp. Error bars are s.e.m.

c, Peak Camuia activation (averaged over 0–24 s). No statistical significance among control, C3 and Wasp ($p < 0.05$, ANOVA followed by post-hoc tests).

d, Averaged time course of spine volume change in the same experiments in **b**.

e, Transient (volume change averaged over 1.5–2 min subtracted by that over 9–11 min) and sustained volume change (volume change averaged over 9–11 min). Stars denote statistical significance ($p < 0.05$, ANOVA followed by post-hoc tests).



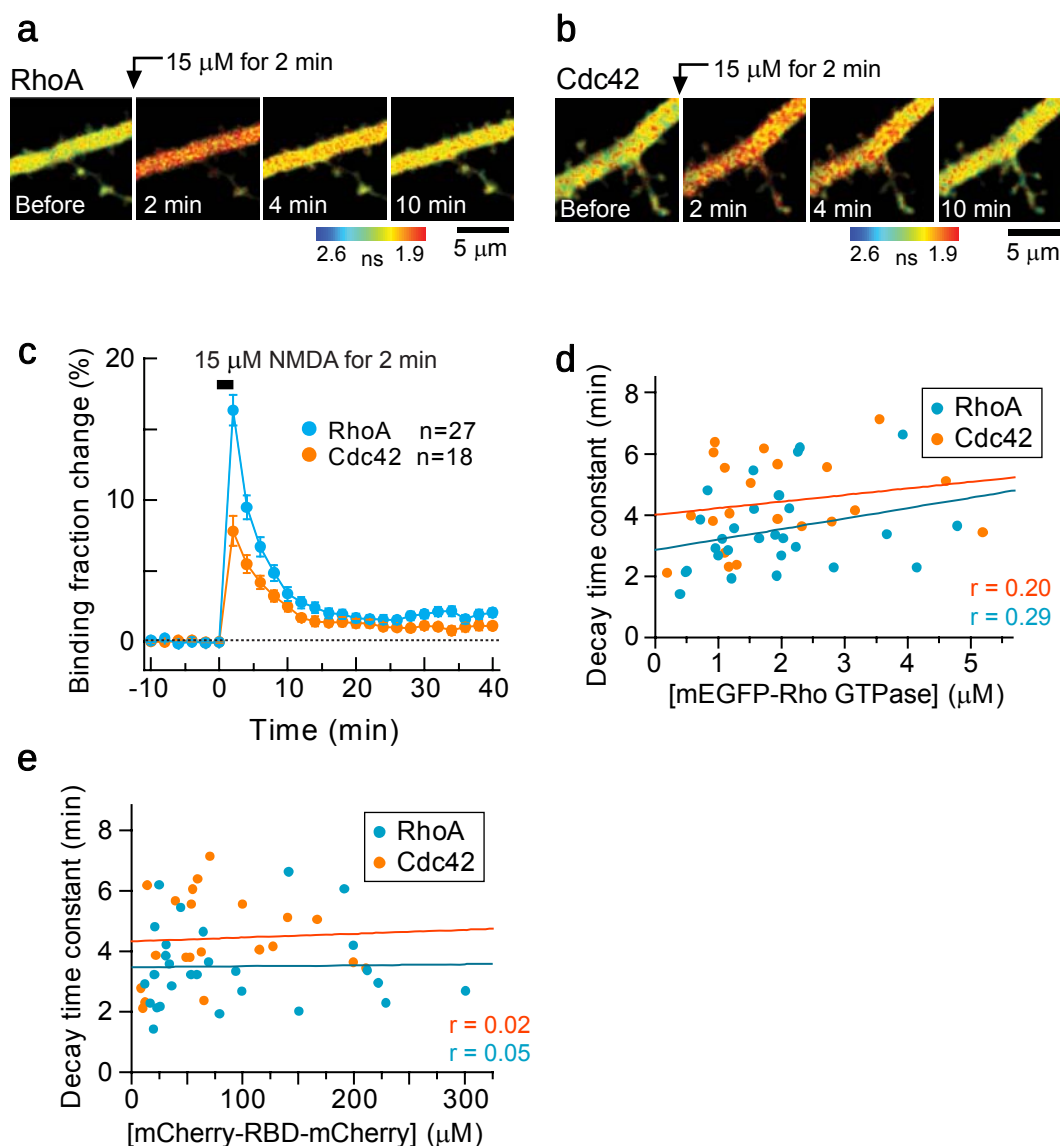
Supplementary Fig. 10. Inhibition of Rho and Cdc42 inhibits both spine enlargement and LTP induced by pairing protocol

a, Spine volume change during LTP induced by pairing 2-photon glutamate uncaging at a single synapse with depolarization of cell to 0 mV in neurons expressing mEGFP together with mCherry-C3 transferase (C3) or mCherry-Wasp(220-321) (Wasp)⁷. The numbers of samples (spines/cells) are 6/6 for control, 6/6 for Wasp, and 6/6 for C3.

b, Changes in excitatory postsynaptic current evoked by 2-photon glutamate uncaging (uEPSC) before and after LTP induction (same spines with **a**).

c, Representative traces of uEPSC in stimulated spines and their adjacent spines (averaged over 2-6 trials measured with 10 s intervals).

d, Potentiation of uEPSC in stimulated and adjacent spines at 25 min after stimulation. Stars, significant difference from control (ANOVA followed by post-hoc tests, $p < 0.05$).

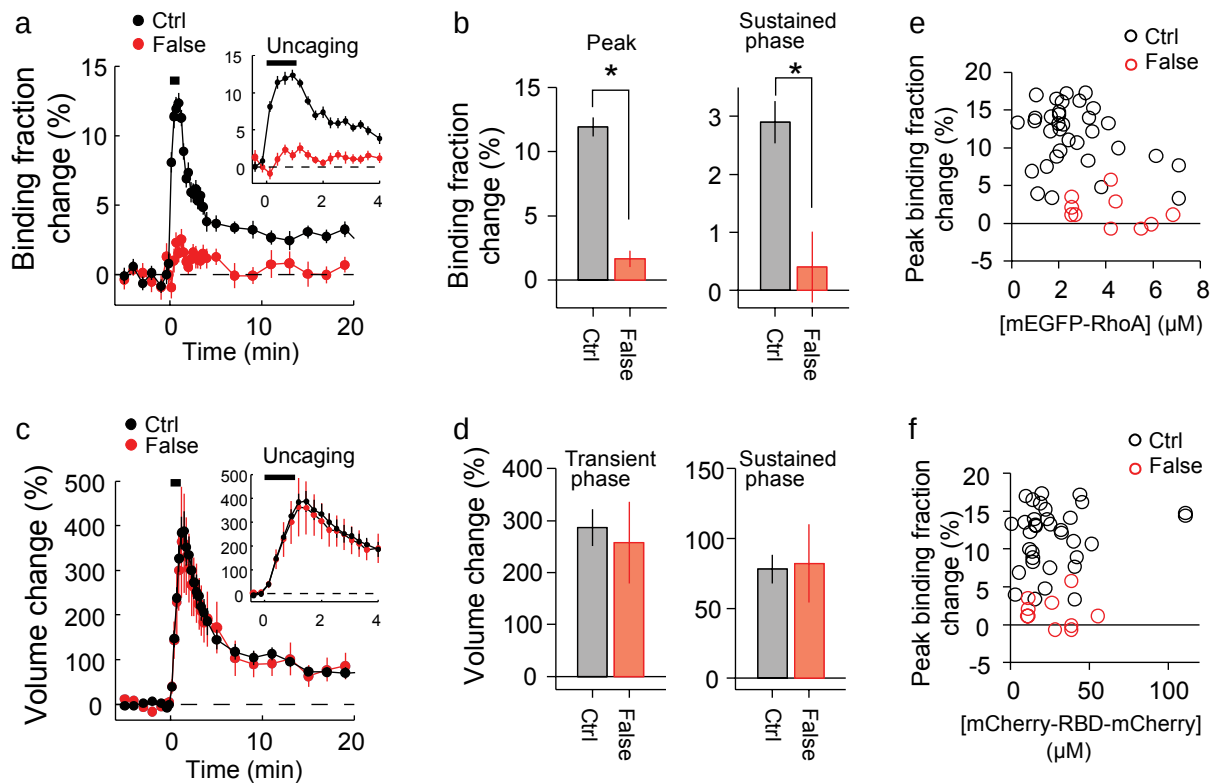


Supplementary Fig. 11. Characterization of Rho GTPase FRET sensors in neurons

a-b, Fluorescence lifetime images of RhoA (**a**) and Cdc42 (**b**) sensors in the primary dendrite of CA1 pyramidal neurons in response to bath-applied NMDA. Warmer colors indicate shorter lifetimes and higher activity of Rho GTPase. Scale bar, 5 μm . Hippocampal slice cultures (DIV 12–18) in ACSF plus 1 μM TTX was stimulated by replacing the solution with 15 μM NMDA in Mg^{2+} -free ACSF containing 1 μM TTX for 2 min. Subsequently, the solution was washed with ACSF with Mg^{2+} .

c, Time courses of the RhoA ($n = 27$) and Cdc42 ($n = 18$) activity after NMDA application. Error bars indicate s.e.m.

d-e, The time constants of the signal decay plotted as a function of the concentrations of mEGFP-Rho GTPase (**d**) or mCherry-RBD-mCherry (**e**). The time constant was obtained by fitting the decay of the binding fraction (**c**) with a single-exponential function.



Supplementary Fig. 12. Fluorescence lifetime change of mEGFP- RhoA paired with false acceptor during spine structural plasticity

a, Time course of changes in the fraction of mEGFP-RhoA bound to mCherry-RBD-mCherry in spines stimulated with 2-photon glutamate uncaging. When “false” sensor (mEGFP-RhoA paired with Cdc42 acceptor, mCherry-Pak3(60-113)/S74A/F84A-mCherry) is used (red), only small FRET changes occurred during spine structural plasticity. The numbers of samples (spines/neurons) is 10/6. For comparison, experiments using the right RhoA sensor (black, data from **Fig. 1b**) are also plotted. Inset: closer view. Error bars are s.e.m.

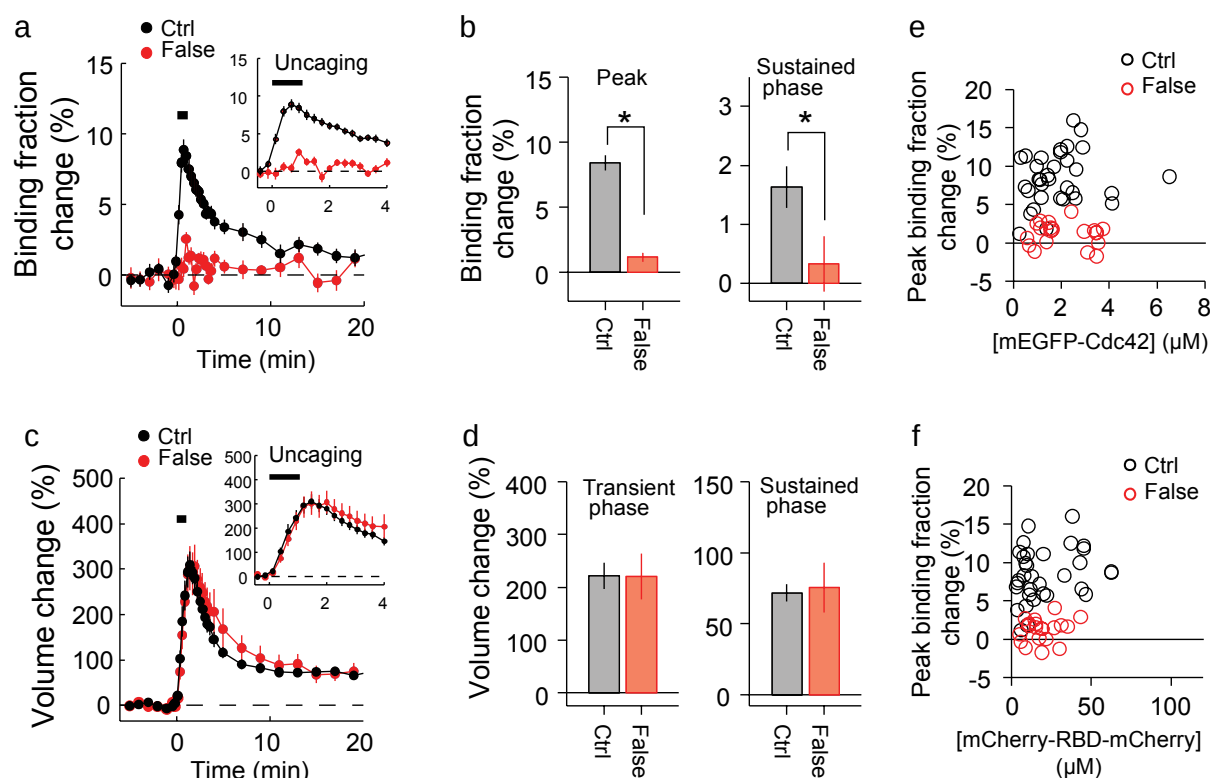
b, Transient (averaged over 16–64 s) and sustained (averaged over 12–20 min) activation in the same experiments in **a**.

c, Averaged time course of spine volume change in the same experiments in **a**.

d, Transient (volume change averaged over 1.5–2 min subtracted by that over 12–20 min) and sustained volume change (volume change averaged over 12–20 min) in the same experiments in **a**.

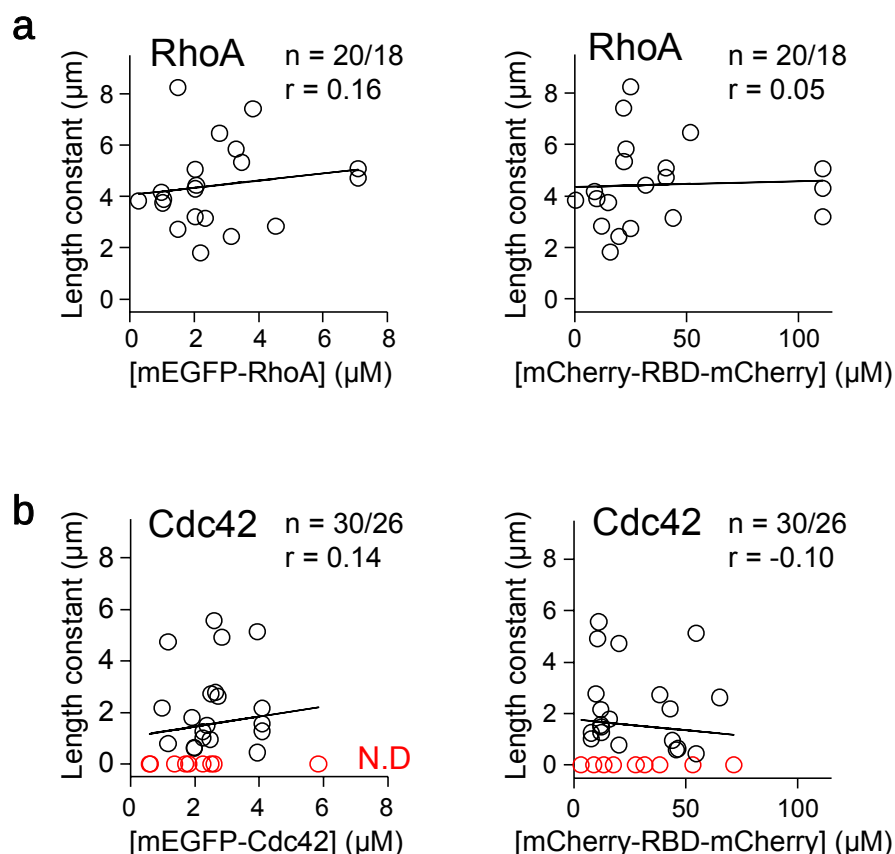
e,f, Relationship between the binding fraction change in spine at the different concentrations of mEGFP-RhoA (**e**) and mCherry-RBD-mCherry (**f**).

Stars denote statistically significant difference (< 0.05).



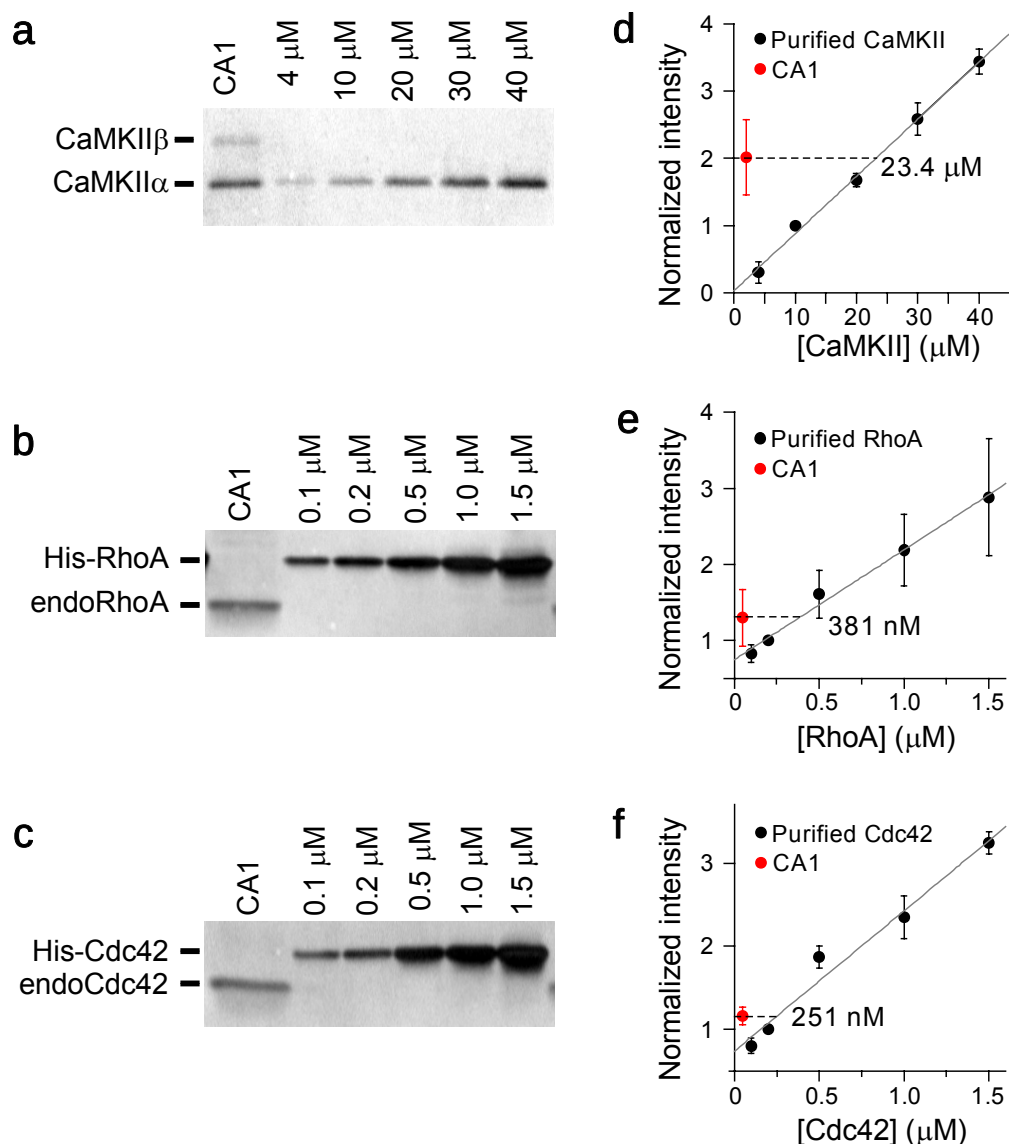
Supplementary Fig. 13. Fluorescence lifetime change of mEGFP-Cdc42 paired with false acceptor during spine structural plasticity

The same experiments and analyses as in **Supplementary Fig. 12** except for using Cdc42 sensor instead of RhoA sensor. In the “false” sensor, mEGFP-Cdc42 is paired with RhoA acceptor (mCherry-Rhotekin (8-89)-mCherry (red)). For comparison, experiments using the right RhoA sensor (black, data from **Fig. 2b**). The number of samples (spines/neurons) is 20/13.



Supplementary Fig. 14. Effects of overexpression of Rho GTPase sensors on the length constant of Rho GTPase activity spreading in dendrites.

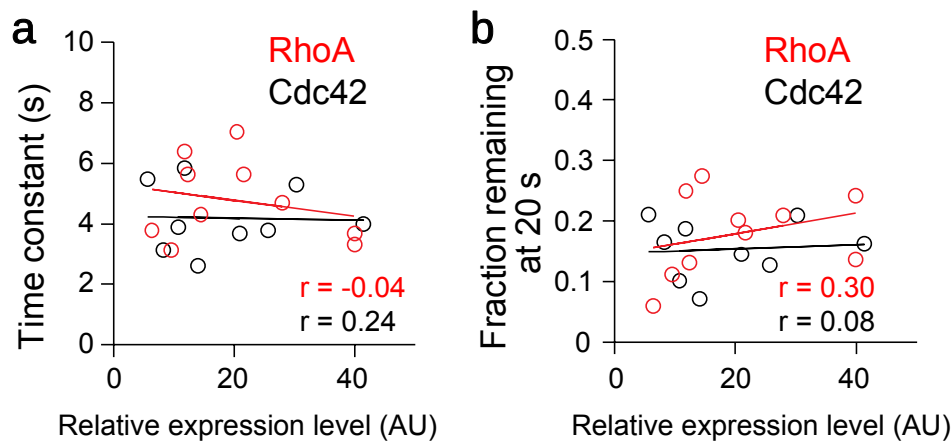
The length constants of activated RhoA (**a**) and Cdc42 (**b**) spreading along dendrites following single spine activation (**Fig. 3a-d**) at the different concentrations of mEGFP-Rho GTPase (left) and mCherry-RBD-mCherry (right). The length constant during uncaging was measured by fitting the spatial profile of the activity between 0–9 μm with a single exponential function (**Fig. 3a-d**). In some experiments for Cdc42, there was no significant activity spreading from the stimulated spine to dendrites, and thus the length constant was not determined (red). The numbers of samples (n = spines/neurons) are indicated in the figures.



Supplementary Fig. 15. Estimation of endogenous Rho GTPase expression level

a-c, Western blots of CA1 region of slice culture and known concentrations of purified CaMKIIα (**a**) polyhistidine-tagged RhoA (His-RhoA) (**b**) and poly-histidine-tagged Cdc42 (His-Cdc42) (**c**).

d-f, Quantification of protein expression level for CaMKIIα (**d**) polyhistidine-tagged RhoA (**e**) and polyhistidine-Cdc42 (**f**) from Western blots shown in **a-c**. The band intensities of purified proteins, CaMKIIα, RhoA, and Cdc42 were normalized at 10 μM, 0.2 μM, and 0.2 μM and averaged over 3 experiments. The protein concentration in CA1 was estimated by measuring the intersection of calibration curve and the intensity of protein from CA1 region.



Supplementary Fig. 16. Effects of overexpression of Rho GTPases on their mobility

a, The decay time constant of the fluorescence decay after photoactivation of paGFP-RhoA/Q63L and paGFP-Cdc42/Q61L in spines (**Fig. 3g**) at different levels of paGFP-RhoA and Cdc42 expression measured as the paGFP fluorescence at the primary dendrites. Each point represents individual cells (average of 6-12 spines). $n=10$ for RhoA; $n=9$ for Cdc42.

b, The fraction of fluorescence remaining at 20 s after photoactivation of paGFP-RhoA/Q63L and paGFP-Cdc42/Q61L at the different levels of paGFP-Rho GTPase expression (**Fig. 3h**).