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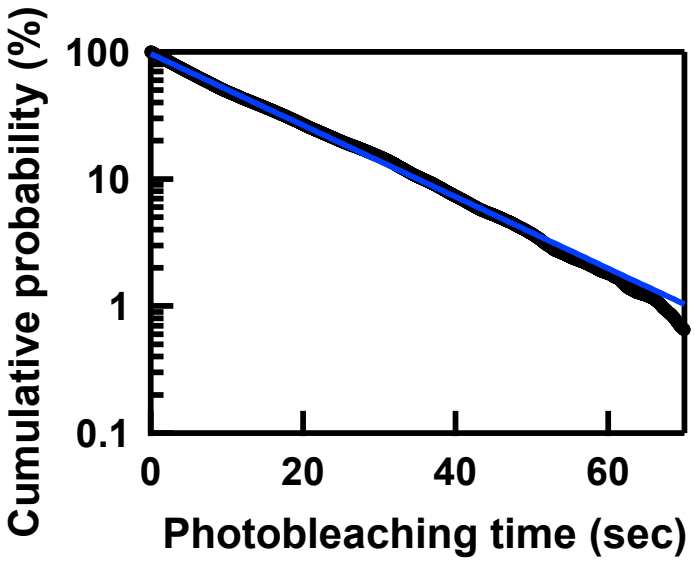
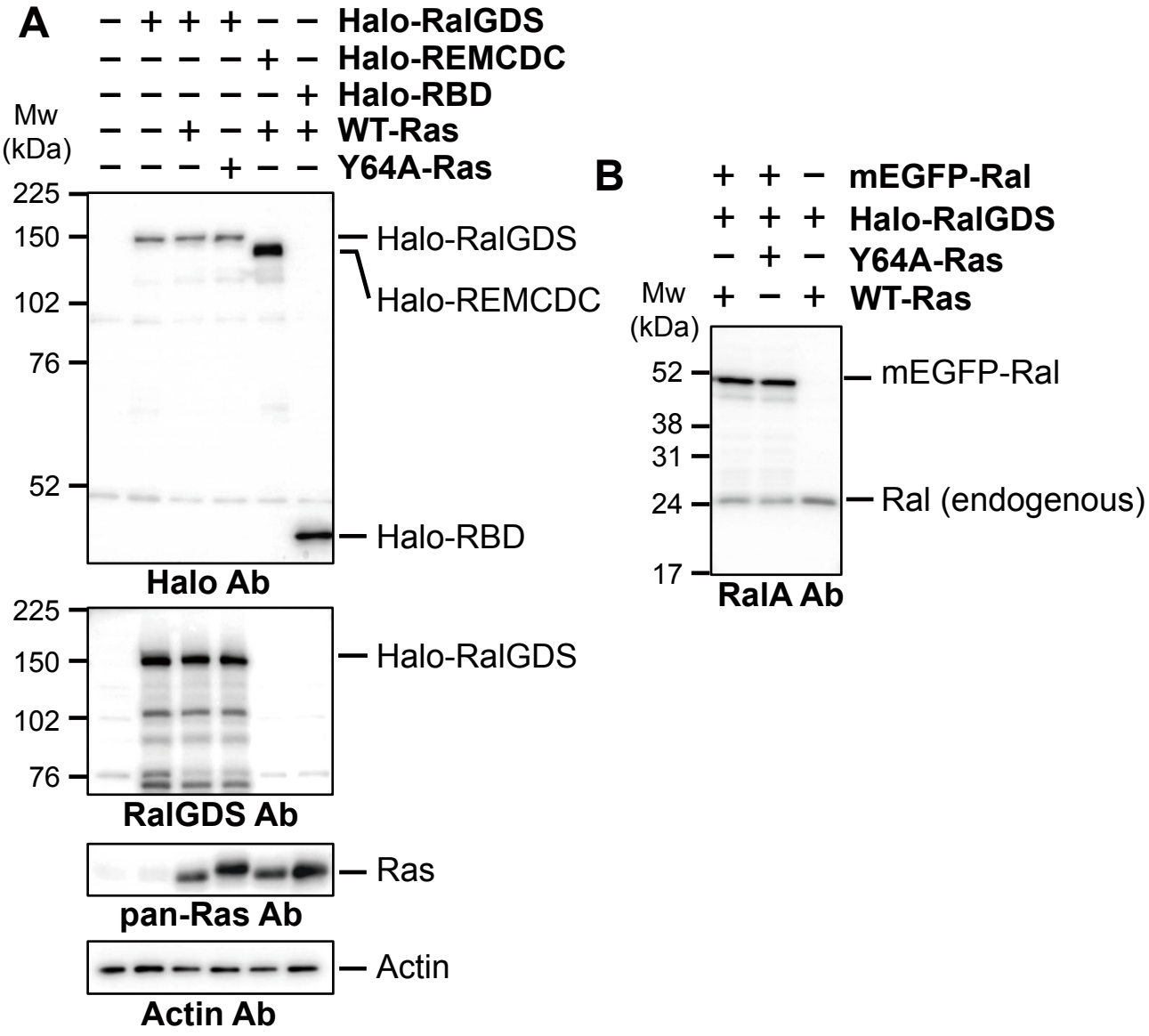
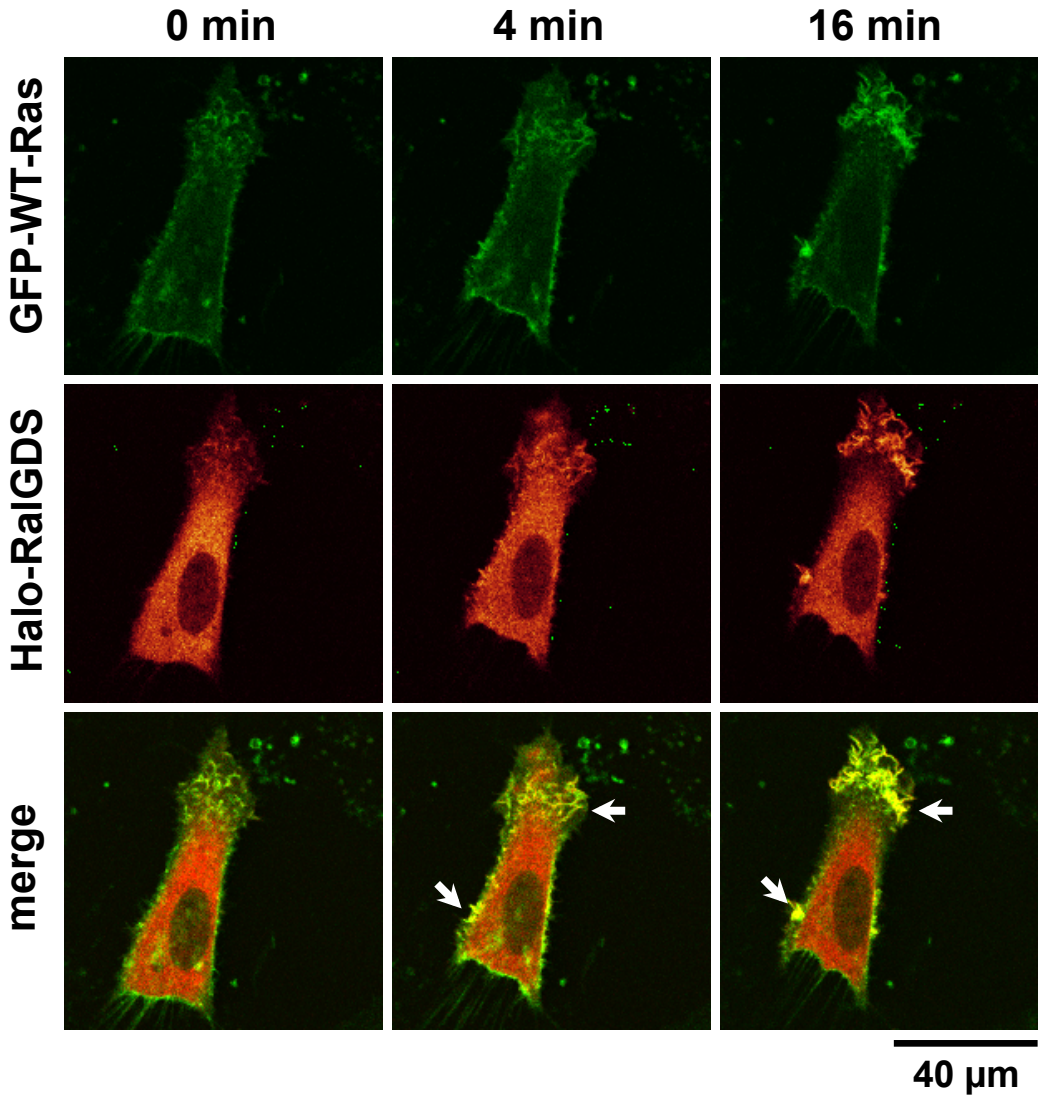
Supplementary Materials



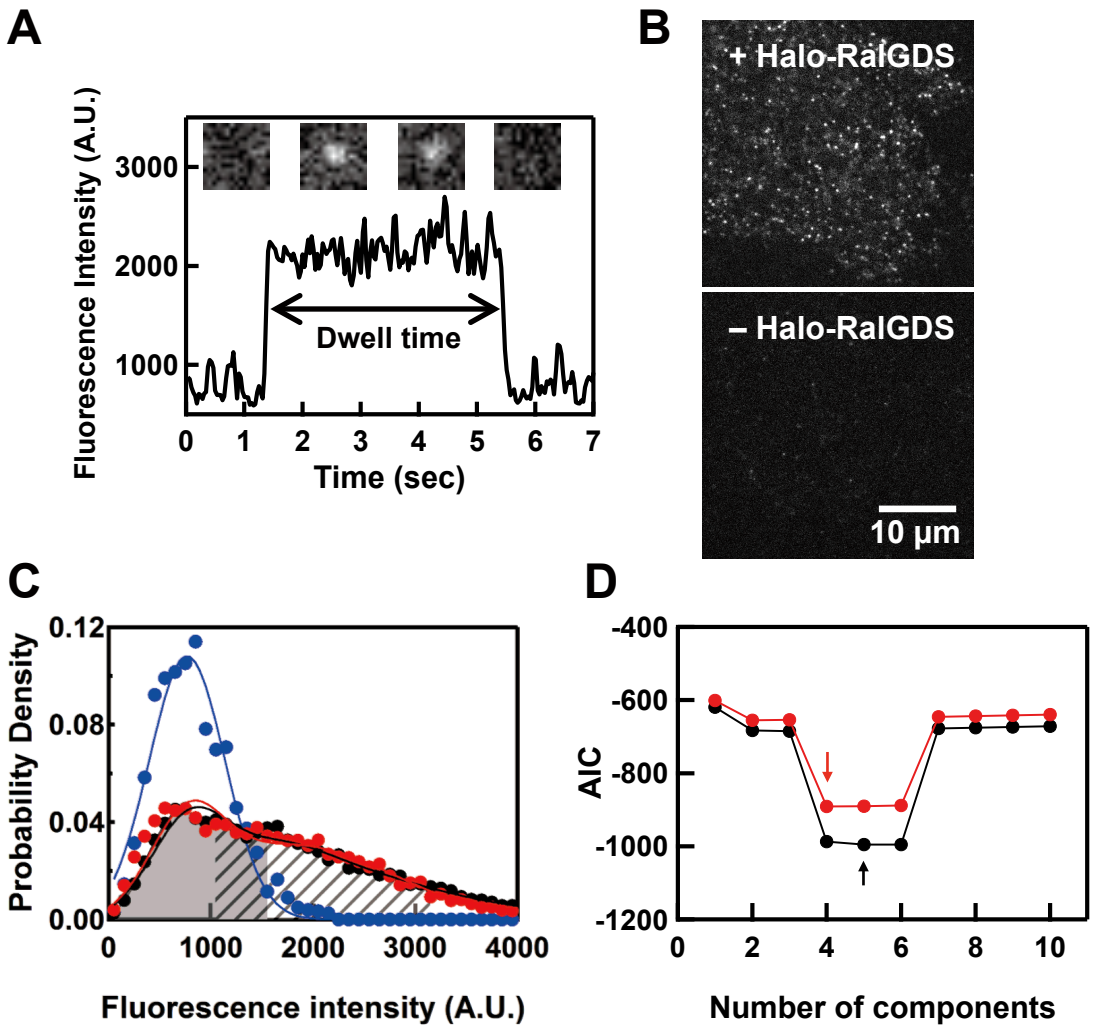
Supplementary Figure S1 Photobleaching time course of Halo–RalGDS on the fixed cell membrane. This distribution was fitted by a single exponential decay (*blue line*) with a photobleaching rate constant of 0.07 s^{-1}.



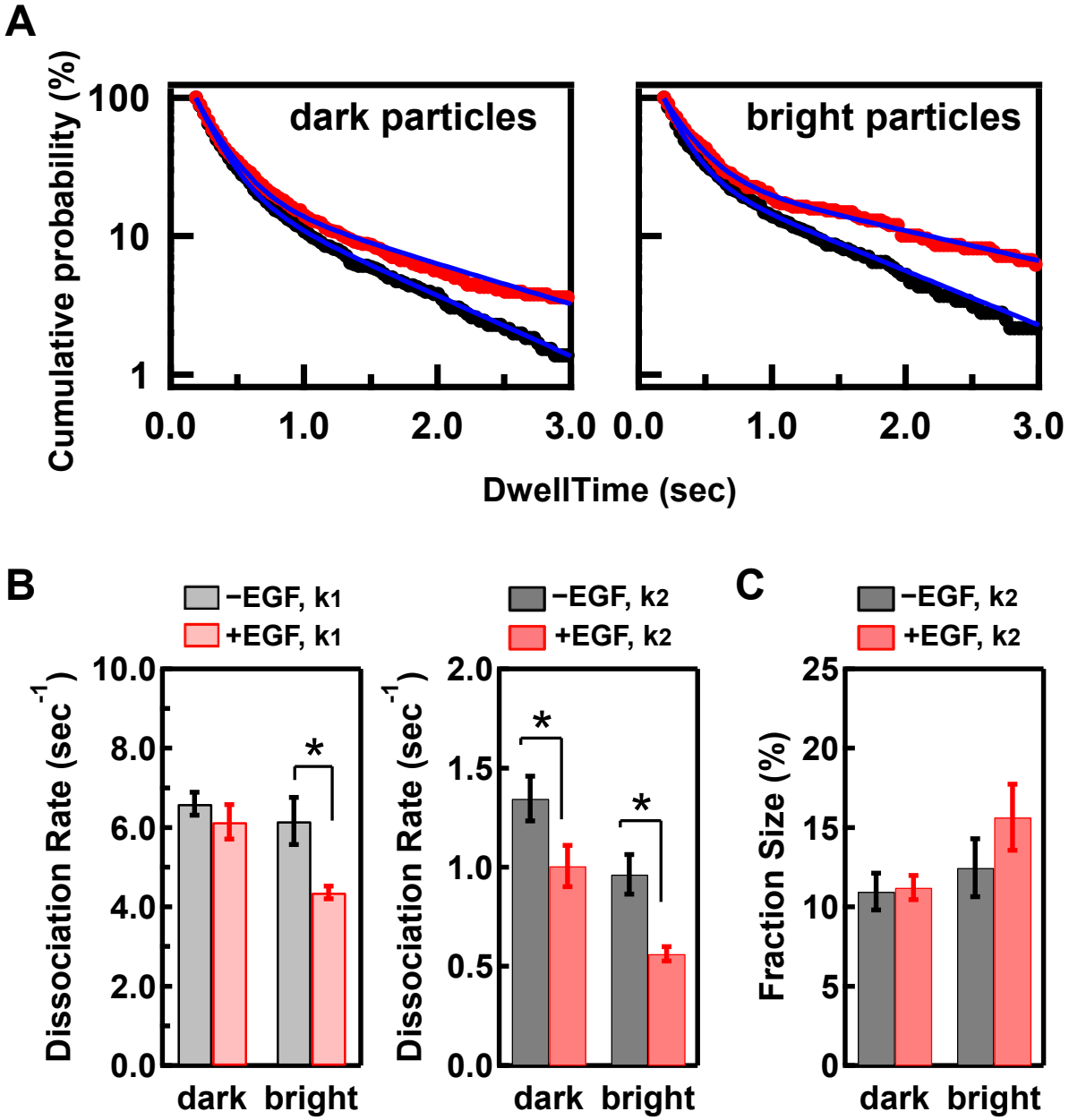
Supplementary Figure S2 Expression levels of RalGDS and Ras in HeLa cells. (A) Expression of Halo–RalGDS and Ras, assessed with a Western blotting analysis of cell lysates with the indicated antibodies (Ab). The left-most lane shows the control HeLa cell lysate (without transfection). Note that endogenous Ras (about 20 kDa) and endogenous RalGDS (about 100 kDa) were not detected because their concentrations in the cell lysates were low. Because the epitope of the anti-RalGDS antibody is located in the C-terminal region of RalGDS, Halo–REMCDC was not detected. (B) Comparison of the expression levels of exogenous and endogenous Ral in HeLa cells. Mw, molecular weight markers.



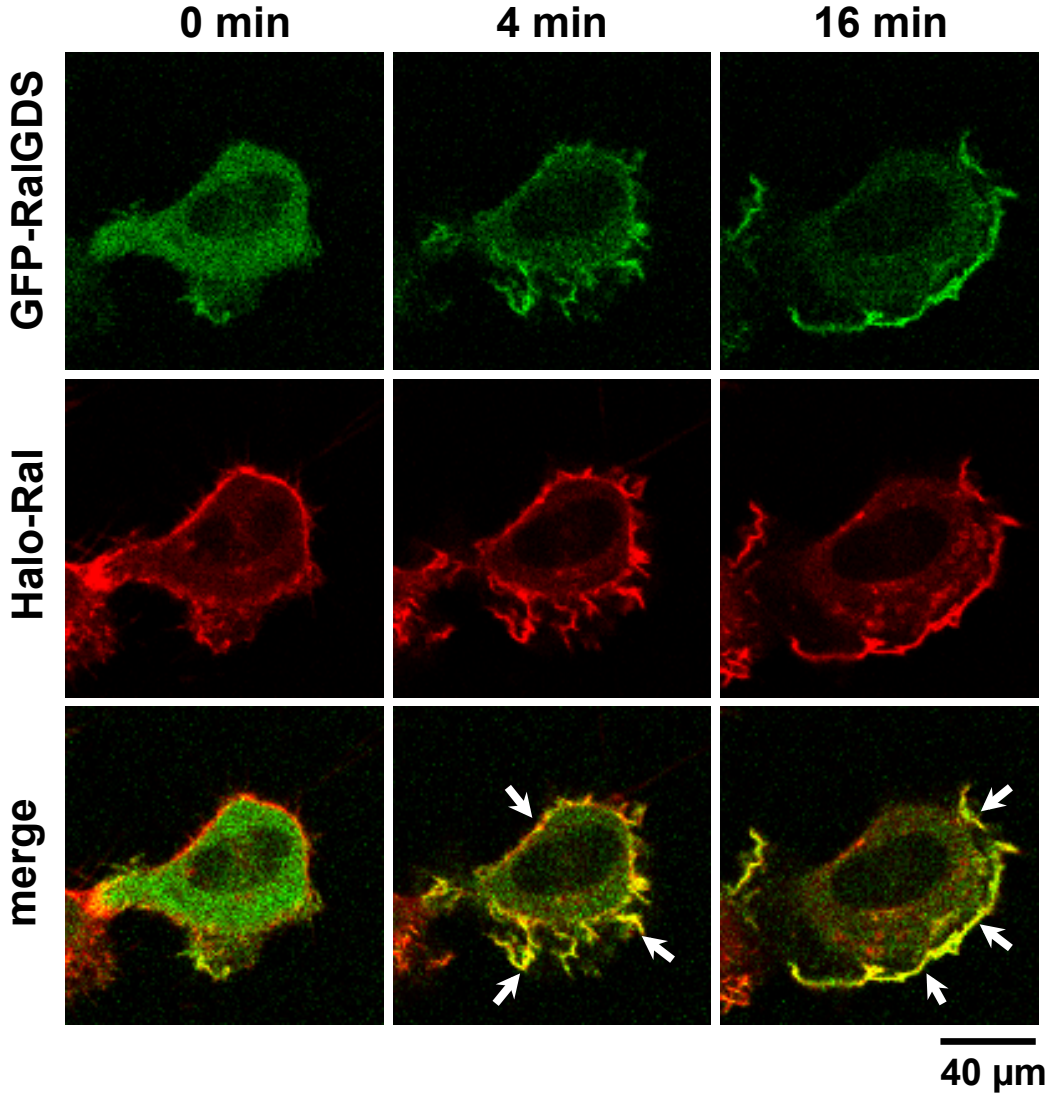
Supplementary Figure S3 Colocalization of Halo–RalGDS and GFP–Ras in living HeLa cells. Halo–RalGDS (*middle*) was coexpressed with GFP-tagged Ras (*top*). After serum starvation (0 min), EGF was added (final 100 ng/mL) at 25 °C. Fluorescent images were taken with CLS microscopy at 0, 4, and 16 min after the application of EGF. Arrows show the colocalization of RalGDS and Ras on the plasma membrane.



Supplementary Figure S4 Single-molecule imaging of RalGDS on the plasma membranes of living HeLa cells. (A) Typical time course of the change in fluorescence intensity of a RalGDS particle on the basal plasma membrane, observed with TIRF microscopy. When RalGDS associated with its counterparts on the plasma membrane, a fluorescent spot appeared and the fluorescence intensity increased at an individual binding site. The disappearance of the fluorescent spot reflects the dissociation (or photobleach) of RalGDS at the binding site. The period of time between the appearance and disappearance of the fluorescent particle on the plasma membrane was measured as the dwell time (*bidirectional arrow*). A.U., arbitrary units. (B) TIRF images of HeLa cells with (*upper*) and without (*lower*) the expression of Halo–RalGDS molecules. Both cells were stained with TMR. (C) Distribution of the fluorescence intensities of Halo–RalGDS particles on the plasma membrane in living HeLa cells before (*black circles*) and after (*red circles*) EGF stimulation. Here, typical distributions of the fluorescence intensities of Halo–RalGDS particles in a single cell are shown. The distributions were fitted with five (*black*) and four (*red*) components of the Gaussian functions described in the METHODS (*lines*), respectively. The quality of the fitting model was evaluated by AIC as shown in (D). Gray ($\mu \pm 2\sigma$) and hatched ($2\mu \pm 2\sigma$) regions indicate the regions of dark and bright particles, respectively, analyzed in Supplementary Fig. S5 (μ and σ are mean and standard deviation of the single-molecule fluorescence intensities, respectively). Blue circles show the distribution of the photobleaching step size of the Halo–RalGDS molecules on the plasma membranes of fixed cells. A.U., arbitrary units. (D) AIC Scores. Black and red arrows indicate the lowest scores for before (*black*) and after (*red*) EGF stimulation, respectively. The most probable maximal size of Halo-RalGDS oligomers were five (*black*) and four (*red*), respectively.



Supplementary Figure S5 Dependence of the dissociation kinetics of RalGDS on the fluorescence intensity. The dissociation rate constant was estimated for the dark (mainly monomers) and bright (mainly oligomeric) particles of RalGDS, as indicated in Supplementary Fig. S4C. (A) Dwell time distributions of the dark (*left*) and bright (*right*) particles of Halo–RalGDS on the basal plasma membrane before (*black*) and 3 min after (*red*) EGF stimulation. The blue lines are the best-fit curves of the two-component exponential function described in the METHODS. (B) Dissociation rate constants of the fast (k_1) and slow (k_2) components before (*black*) and 3 min after (*red*) EGF stimulation. dark, dark particles of RalGDS molecules; bright, bright particles of RalGDS molecules. Averages and SE of 5 cells are shown. Asterisks denote statistical significance ($p < 0.05$ using the *t* test). (C) Fractions of the slow component before (*black*) and 3 min after (*red*) EGF stimulation.



Supplementary Figure S6 Colocalization of RalGDS and Ral on the plasma membrane in living HeLa cells. GFP–RalGDS (*green images*) and Halo–Ral conjugated with TMR (*red images*) were observed on the same cell after serum starvation (0 min) and at 4 and 16 min after stimulation with EGF (final 100 ng/mL) at 25 °C. The fluorescent images were taken with CLS microscopy. Arrows show the colocalization of RalGDS and Ral on the plasma membrane.

Supplementary Table S1 Oligomer size of RalGDS and its fraction size on the plasma membrane in living HeLa cells before and 3 min after EGF stimulation. The distribution of the fluorescence intensities of Halo-RalGDS particles on the plasma membrane in each cell was fitted with a sum of four to six Gaussian functions. The averages of each fraction size for 5 cells are shown with SE.

Oligomer size	EGF −	EGF +
monomer	0.379 ± 0.014	0.423 ± 0.016
dimer	0.333 ± 0.018	0.330 ± 0.041
trimer	0.176 ± 0.026	0.159 ± 0.060
tetramer	0.067 ± 0.014	0.083 ± 0.016
pentamer	0.039 ± 0.010	< 0.001
hexamer	0.006 ± 0.006	0.005 ± 0.005

Supplementary Table S2 Dissociation rate constants (k_1 and k_2) for RalGDS and their fraction sizes before and 3 min after EGF stimulation. Averages of 5–8 cells are shown along with the SE.

	EGF	−		+	
	fraction	k_1	k_2	k_1	k_2
RalGDS + WT-Ras		6.05 ± 0.32[a]	0.93 ± 0.06	5.23 ± 0.20	0.66 ± 0.03
		(89.5 ± 1.1)[b]	(10.6 ± 1.1)	(87.8 ± 1.1)	(12.2 ± 1.1)
RBD + WT-Ras		5.19 ± 0.19	0.99 ± 0.06	5.64 ± 0.22	1.07 ± 0.13
		(91.3 ± 0.7)	(8.7 ± 0.7)	(91.1 ± 1.4)	(8.9 ± 1.4)
REMCDC + WT-Ras		5.81 ± 0.16	0.68 ± 0.02	6.00 ± 0.24	0.73 ± 0.05
		(91.4 ± 1.0)	(8.6 ± 1.0)	(90.6 ± 0.9)	(9.4 ± 0.9)
RalGDS + Y64A-Ras		5.28 ± 0.16	0.85 ± 0.04	5.70 ± 0.33	0.84 ± 0.06
		(86.5 ± 1.1)	(13.5 ± 1.1)	(86.7 ± 1.9)	(13.3 ± 1.9)
RalGDS + WT-Ras + Ral		4.16 ± 0.12	0.65 ± 0.03	4.44 ± 0.18	0.63 ± 0.03
		(86.3 ± 0.9)	(13.7 ± 0.9)	(85.6 ± 0.7)	(14.4 ± 0.7)
RalGDS + Y64A-Ras + Ral		4.87 ± 0.18	0.80 ± 0.04	4.84 ± 0.16	0.74 ± 0.04
		(85.7 ± 0.8)	(14.3 ± 0.8)	(86.3 ± 0.9)	(13.7 ± 0.9)

[a] The mean values (+/- SE) of the dissociation rate constant (s^{-1}).

[b] Fraction size (%).