Table S1. The fractions of fluorescent analog lipids, 594neg-SM, 594neg-DOPC, and 594-DOPE, located in the PM inner leaflet (lipid analogs that flipped from the outer to inner leaflet) and those internalized into the cytoplasm in CHO-K1 and T24 cells 15 min after the addition of the lipid analogs at 37°C. Refer to Fig. 6 D for representative images and its caption for further explanations (as well as Materials and methods).

Lipid Analogs	Cells	% Molecular fractions		N‡
		Inner leaflet	Cytoplasm†	
594neg-SM	CHO-K1	0*	9.3 ± 0.7	10
	T24	0*	10.3 ± 1.4	15
594neg-DOPC	CHO-K1	0*	8.3 ± 1.6	10
	T24	0*	9.5 ± 1.1	15
594-DOPE	CHO-K1	0*	7.6 ± 1.3	13
	T24	0*	9.5 ± 1.6	11

*After the addition of the membrane-impermeable quencher CuTSP (without washing out the free lipid analogs in the observation medium), virtually no signal was detectable using TIRF illumination. This shows that all molecules in the PM are located in the outer leaflet, and, therefore, the %molecular fraction in the inner leaflet was determined to be 0%. Note that all incubations were conducted on the microscope stage, and the same cells were observed before and after CuTSP addition. Virtually no photobleaching was detectable under our observation conditions.

†When the cells were observed using oblique-angle illuminations, the lipid analog signals were detectable even after CuTSP addition, showing that these signals came from the cytoplasm. Since no signal was detectable deeper in the cytoplasm when the focus was shifted in the oblique-angle illumination at single-molecule sensitivities, we assumed that no 594neg-SM existed deeper in the cytoplasm. Therefore, the %molecular fraction in the cytoplasm was obtained by calculating the ratios of the analog signal intensities after vs. before the quencher addition, observed by the oblique-angle illumination with a focus near/at the PM (the total fluorescence intensity after background subtraction was measured, and the relative signal remaining after quencher addition, compared with the signal before the quencher addition, was obtained).

‡The number of cells examined.

Table S2. The lifetimes ($\tau_{Lifetime}$) of homo- and hetero-colocalizations of lipid analogs developed in the present research in the CHO-K1-cell PM at 37˚C, as well as in the Lα- and Lo-phase domains of planar lipid bilayers.

Molecules	Host membrane	Pre-treatment	Temp.	$\tau_{Lifetime} \pm SE^{\dagger}$	$N^{\ddagger}$
			°C	*ms*	
594neg-SM (homo)	PM (CHO-K1)	None (intact)	37	$48 \pm 3^{*1}$	260
		Chol. Depl. (Saponin)[§]		33 ± 3^{Y1}	223
		Chol. Depl. (MβCD)[§]		$35 \pm 3^{*2,Y1}$	237
		Chol Repl. after MβCD[§]		51 ± 4^{Y2}	220
594neg-DSPC (homo)		None (intact)		35 ± 2^{Y1}	243
594neg-DOPC (homo)		None (intact)		32 ± 2^{Y1}	206
488neg-SM/594neg-SM	PM (CHO-K1)	None (intact)	37	$46 \pm 3^{*1}$	214
488neg-SM/594neg-DOPC				30 ± 2^{Y1}	206
594neg-SM (homo)	DMPC (Lα)	—	30	$15 \pm 1^{*3}$	223
594neg-DOPC (homo)				14 ± 1^{N3}	225
594neg-SM (homo)	DMPC(65)/Chol(35) (Lo)	—	20	$33 \pm 2^{*4}$	206
594neg-DOPC (homo)				32 ± 2^{N4}	210
594neg-SM (homo)	DOPC (Lα)	—	25	$12 \pm 1^{*5}$	221
594neg-DOPC (homo)				11 ± 1^{N5}	243
594neg-SM (homo)	DPPC(65)/Chol(35) (Lo)	—	37	$34 \pm 2^{*6}$	227
594neg-DOPC (homo)				36 ± 2^{N6}	231

[†]Exponential decay lifetimes obtained by fitting the distributions of colocalization periods with single exponential functions (see Fig. 7 [B and C] and Fig. S3 B). Errors indicate the fitting errors for the 68.3% confidence limits. SE, standard error.

[‡]The number of examined colocalization events.

*, [Y], and [N]. The results of the statistical test. The distribution selected as the basis for the

comparison is shown by the superscript *. The numbers (1–6) indicate the different bases. The superscript ^{Y} or ^{N} indicates that the distribution is or is not significantly different, respectively, with t-test p values smaller or greater than 0.05, respectively.

[§]The effects of partial cholesterol depletion observed here require due caution in their interpretation. In addition to implicating that cholesterol is directly required as a component of the putative meso-scale raft domains, the effects of partial cholesterol depletion could include changes in membrane elasticity, mobility of proteins and lipids, and protein trafficking (Kusumi et al., 2004), as well as the reduction of the $PI(4,5)P_2$ levels, which could enhance the polymerization of subcortical actin (Kwik et al., 2003). To reduce the uncertainties in the interpretations of the effects of partial cholesterol depletion, we took the following precautions in the present research.

(1) We extensively employed typical non-raft control molecules, such as 594neg-DOPC (Figs. 7–10) and CD59TM (Fig. 9 C).

(2) We used milder conditions for partial cholesterol depletion, using MβCD (4 mM MβCD for 30 min at 37°C).

(3) We used a second method for partial cholesterol depletion, using saponin (200 μg/ml saponin on ice for 15 min).

(4) Kwik et al. (2003) showed that partial cholesterol depletion performed under harsher conditions induced the reduction of the $PI(4,5)P_2$ levels, which enhanced the polymerization of actin filaments. This paper further reported that the recovery from such changes did not occur within 12 h, even after the control cholesterol levels were restored by cholesterol replenishment. This means that if the results obtained before cholesterol depletion were recovered quickly after cholesterol replenishment (e.g., within an hour, as was done in this research), then the $PI(4,5)P_2$ reduction and the subsequent polymerization and stabilization of actin filaments upon cholesterol depletion (that Kwik et al. observed) would not have occurred or have occurred at much lower levels. This is the reason why we performed the cholesterol replenishment experiments after partial cholesterol depletion (this table and Fig. 7B). The 594neg-SM colocalization lifetime (48 ms) observed in the intact PM was reduced upon cholesterol depletion (using two different methods; to 33–35 ms), but the

colocalization lifetime quickly recovered after cholesterol replenishment (51 ms).

(5) Previously, we found that partial cholesterol depletion reduces CD59 homodimer lifetimes, without affecting CD59TM homodimer lifetimes (Suzuki et al., 2012). It would be quite difficult to explain this result by processes such as changes in membrane elasticity, mobility of proteins and lipids, protein trafficking, and subcortical actin polymerization, without considering the cholesterol interaction with CD59 homodimers (and the lack of it with CD59TM homodimers).

Note that, when we deal with PMs, we never directly relate our results to the concept of the "phase properties" of the membrane. However, we consider that "cooperative interactions" of molecules are important in the PM organization and function, and that, in GUVs, cooperative molecular interactions manifest themselves dramatically in the form of phase separation. Therefore, phase-separated GUVs have been extensively used in the present study for understanding how molecules of interest (various fluorescent analogues of SM(18:0), DSPC, and DOPC) behave in the presence (Lo-like domains) and absence (Lα-like domains) of cooperative molecular interactions that involve cholesterol. However, this does not mean that "real" phases appear in the PM.

Table S3. The time fractions of mobile, TALL, and immobile periods, as well as TALL durations (mean value, τ_{TALL}), for 594neg-SM, 594neg-DOPC, and 594neg-DSPC trajectories obtained at a time resolution of 0.5 ms (2,000 frames/s), in the intact PMs of the T24 and PtK2 cell lines. These results show that raft-associated SM and DSPC and non-raft DOPC rarely exhibited TALL in the intact PMs.

			Time fraction				
Molecules	Cell	Temp.	Mobile	TALL*	Immobile	N†	τ_{TALL} (mean)‡
		˚C	*%*	*%*	*%*		*ms*
594neg-SM			98.2	1.8	0	180	5.9 ± 0.2
594neg-D0PC	T24	23	98.5	1.5	0	173	5.8 ± 0.2
594neg-DSPC			98.5	1.5	0	178	6.0 ± 0.3
594neg-SM			99.3	0.7	0	189	5.5 ± 0.1
594neg-DOPC	T24	37	99.1	0.9	0	186	5.9 ± 0.2
594neg-DSPC			98.9	1.1	0	183	5.8 ± 0.2
594neg-SM			98.0	2.0	0	171	5.9 ± 0.3
594neg-DOPC	PtK2	23	96.4	3.3	0	163	7.6 ± 0.6
594neg-DSPC			98.0	2.0	0	167	5.9 ± 0.1
594neg-SM			99.6	0.3	0	179	5.5 ± 0.3
594neg-DOPC	PtK2	37	99.8	0.2	0	181	5.3 ± 0.3
594-neg-DSPC			99.8	0.2	0	173	5.6 ± 0.2
594	On glass		0	1.5	98.5	61	-

*The detection circle radius and the threshold trapped period used were 50 nm and 5 ms (10 frames), respectively. A decrease of the detection circle radius, down to 20 nm, reduced the TALL time fraction (0.1% or less with a detection circle radius of 20 nm for all molecules and all cell lines examined here).

†The number of examined trajectories.

‡The values of the mean ± standard error, for all of the observed TALL periods. Since very few TALL events were observed, exponential curve fitting to estimate the lifetimes was inappropriate.

Table S4. Diffusion coefficients on the time scale of 2.3 ms (D_{2ms}) for 594neg-SM, DOPC, and DSPC in the T24-cell PM and the PtK2-cell PM at 23 and 37˚C.

Molecules[†]	Cell	Temp.	D_{2ms} median (mean ± SE)	Diffusion coefficient estimated from ensemble averaged MSD-Δt plot	N[‡]
		℃	$\mu m^2/s$	$\mu m^2/s$	
594neg-SM			0.29 (0.31±0.02)^{*1}	0.24	180
594neg-DOPC	T24	23	0.28 (0.31±0.02)^{*2,N1}	0.20	173
594neg-DSPC			0.28 (0.29±0.01)^{*3,N1}	0.23	178
594neg-SM			0.82 (0.82±0.03)^{*4,Y1}	0.72	189
594neg-DOPC	T24	37	0.81 (0.81±0.03)^{Y2,N4}	0.72	186
594neg-DSPC			0.85 (0.84±0.04)^{Y3,N4}	0.73	183
594neg-SM			0.41 (0.42±0.02)^{*5}	0.41	171
594neg-DOPC	PtK2	23	0.42 (0.43±0.02)^{*6,N5}	0.40	163
594neg-DSPC			0.42 (0.44±0.02)^{*7,N5}	0.42	167
594neg-SMs			1.22 (1.23±0.04)^{*8,Y5}	1.12	179
594neg-DOPC	PtK2	37	1.20 (1.25±0.04)^{Y6,N8}	1.21	181
594neg-DSPC			1.29 (1.30±0.04)^{Y7,N8}	1.15	173

[‡]Number of examined molecules.

*, ^{Y}, and ^{N}. The results of the statistical test. The distribution selected as the basis for the comparison is shown by the superscript *. Different numbers (1–4) indicate different bases. The superscript ^{Y} or ^{N} indicates that the distribution is or is not significantly different, respectively, with p values of the U-test smaller or greater than 0.05, respectively.

Table S5. The colocalization lifetimes ($\tau_{Lifetime}$) of CD59 clusters with 594neg-SM, 594neg-DOPC, and 594neg-DSPC (T24 cells) and those of CD59 transient homodimer rafts and monomers with 594neg-SM and 594neg-DOPC (CHO-K1 cells) at 37˚C. The monoclonal anti CD59 IgG used for inducing CD59 clusters can only bind to human CD59, and thus human T24 cell line which expresses CD59 was employed. Meanwhile, to visualize CD59 monomers and transient homodimer rafts, ACP-CD59 was expressed in CHO-K1 cells, which do not express endogenous CD59.

Molecules	CD59 assembly state	$\tau_{Lifetime}$ ± SE[†]	N[‡]
		ms	
594neg-SM[¶]	CD59 clusters	108 ± 7^{*1,}	233
	CD59 clusters after chol. depl.[§]	64 ± 2^{Y1}	232
	CD59TM clusters[♯]	57 ± 2^{Y1}	226
	CD59 homodimer rafts[∞]	72 ± 2^{*2}	203
	CD59 monomers	51 ± 2^{*3,Y2}	313
594neg-DOPC[¶]	CD59 clusters	58 ± 2^{*4,Y1}	212
	CD59 monomers	39 ± 1^{*5Y3}	234
594neg-DSPC[¶]	CD59 clusters	93 ± 2^{Y1,Y4}	214
	CD59 homodimer rafts[∞]	66 ± 2^{*6,N2}	191
	CD59 monomers	47 ± 1^{N3,Y5,Y6}	288

[†]Exponential decay lifetimes obtained by fitting the distributions of colocalization periods with single exponential functions (see Fig. 8 C and Fig. 9 C). Errors indicate the fitting errors for the 68.3% confidence limits.

[‡]The number of examined colocalizations.

*, ^{Y}, and ^{N}. The results of the statistical test. The distribution selected as the basis for the comparison is shown by the superscript *. The numbers (1–6) indicate the different bases. The superscript ^{Y} or ^{N} indicates that the distribution is or is not significantly different, respectively, with t-test p values smaller or greater than 0.05, respectively.

[§]See the Note [§] in Table S2.

[♯]T24 cells are human cells that express CD59 molecules, and therefore we were able to induce clusters of endogenous CD59 molecules by the addition of 50-nm latex beads coated with anti-human CD59 monoclonal antibody IgG. However, to induce CD59TM clustering, this method would not work due to the much higher expression levels of endogenous CD59. Therefore, we expressed myc-CD59TM in T24 cells and induced myc-CD59TM clusters by the addition of 50-nm latex beads coated with anti-myc monoclonal antibody IgG. Since we used antibody-coated beads, which are multivalent, to cross-link CD59, the affinity difference of the antibodies used would not strongly affect the cluster sizes of CD59 and CD59TM, because the avidity effect (multivalent binding) would win out. Indeed, the diffusion coefficients of CD59TM clusters and CD59 clusters were both ~10x smaller than those of their respective monomers (Suzuki et al., 2007a,b).

[¶]Recently, Sevcsik et al. (2015) reported that, when mGFP-GPI was concentrated on 3-μm-sized spots of anti-GFP antibodies at densities between 500 – 10,000 molecules/μm^2, which occupied 0.5 – 11% of the cross-section area in the spots (formed by micropatterning), CD59, another GPI-anchored protein (GPI-AP), did not become concentrated in the mGFP-GPI spots. However, more recently, Komura et al. (2016) crosslinked CD59 by the addition of primary and secondary antibodies, to induce CD59 clusters with CD59's spatial occupancies of 20 or 7.7% (in the cross section), although the overall sizes of the crosslinked clusters were mostly smaller than 3 μm in diameter (the spatial occupancy calculation is based on the assumptions in which the average distance between CD59 molecules is 63 or 9.6 nm [Werner et al., 1972] and the CD59 radius is 1.5 nm^2). Komura et al. (2016) found that CD59 clusters formed by its primary and secondary antibodies induced the concentration of the gangliosides (fluorescent ganglioside analogs that behave very much like the native gangliosides) at the CD59 clusters. In fact, this was consistent with the expected steep decrease in the diffusion coefficient of the probe molecule when the nearest-neighbor distance of the steric obstacles was less than 10 nm (Fig. 3 [d and e] in Sevcsik et al. [2015]). These results of Sevcsik et al. (2015) together with those of Komura et al. (2016) indicate that the lack of recruitment of another GPI-AP to the mGFP-GPI clusters (that they produced by micropatterning) was due to the insufficient

concentrations of mGFP-GPI in the micropatterned spots. This strongly suggests that the density of the saturated acyl chains in the cluster (in the cross section plane), rather than the size of the entire cluster, is a key factor for inducing the second raft-associated molecules.

In the present research (this Table S5), we further found that SM and DSPC were recruited to CD59-cluster signaling rafts. Furthermore, they were also recruited to CD59 homodimer rafts, although their lifetimes at CD59 homodimer rafts are substantially reduced from those at CD59-cluster rafts. Following the discussion described in the previous paragraph, these results are consistent with the previous results reported by Sevcsik et al. (2015) and Komura et al. (2016) in a very fundamental sense.

$^{\infty}$Homodimer rafts of GPI-anchored proteins (GPI-APs), including CD59, with lifetimes less than several hundred milliseconds have previously been identified and characterized by Suzuki et al. (2012). The properties of transient CD59 homodimer rafts can be summarized as follows.

1) CD59 molecules often become associated and diffuse together.

2) When two CD59 molecules co-diffuse, FRET between the two fluorescently-labeled CD59 molecules occurs. Therefore, we call this complex a CD59 homodimer.

3) CD59 molecules encounter (collide) with other GPI-AP molecules in the PM, but their colocalization lifetimes are only slightly longer than (or virtually the same as) those of non-interacting molecules. This result suggests that the “so-called” raft-lipid interaction alone cannot induce CD59 homodimerization (colocalization with significant durations), but that a specific protein-protein interaction is required for the formation of CD59 homodimers.

4) CD59TM forms homodimers, clearly showing that protein-protein interactions are responsible for the formation of CD59TM homodimers. However, their dimer lifetime is significantly shorter than (about half of) that of CD59 homodimers. Furthermore, the CD59TM dimer lifetime is about the same as that of CD59 homodimers after partial cholesterol depletion, performed by two different methods. These results suggest that, in the presence of a specific protein-protein interaction, the raft-lipid interaction stabilizes CD59 homodimers.

5) GFP-GPI behaves very similarly to CD59. mGFP-GPI is capable of forming homodimers,

but the lifetime was much shorter than that of GFP-GPI, again showing the importance of protein-protein interactions in the formation of GPI-AP homodimers.

6) CD59 homodimers coalesce to form hetero– and homo–GPI-AP tetramer rafts through raft-based lipid interactions (we could not detect the tetramers after partial cholesterol depletion). Importantly, the CD59 homotetramer lifetimes are about half of those of CD59 homodimers, suggesting that the CD59 homotetramers are not non-specifically formed aggregates of CD59.

7) When CD59 is ligated, it forms stable oligomer rafts containing an average of four CD59 molecules in a manner dependent on GPI anchorage and cholesterol (this CD59-cluster raft elicits the IP_3-Ca^{2+} signal by recruiting PLCγ), suggesting the involvement of transient CD59 homodimer rafts in the formation of CD59-cluster signaling rafts.

Based on these observations, we defined the CD59 homodimer raft in the following manner. It is a transient homodimer of CD59 that is formed by ectodomain protein interactions and stabilized by the presence of the GPI-anchoring chain and cholesterol.

In resting cells, virtually all of the GPI-APs we examined were mobile and continually formed transient (~200 ms) homodimers (termed homodimer rafts) through ectodomain protein interactions, stabilized by the presence of the GPI-anchoring chain and cholesterol. Heterodimers do not form, suggesting the fundamental role of the specific ectodomain protein interaction in forming the transient homodimer rafts of GPI-APs.

Therefore, we believe that transient homodimer rafts are most likely one of the basic units for the organization and function of raft domains containing GPI-APs, and thus we observed the recruitment of the fluorescent analogs of SM, DSPC, and DOPC developed in this study to CD59 homodimer rafts as well as monomers and clusters.

Table S6. The colocalization lifetimes ($\tau_{Lifetime}$) of FcεRI clusters with 594neg-SM and 594neg-DOPC (RBL-2H3 cells) at 37˚C.

Molecules	FcεRI assembly state	$\tau_{Lifetime}$ ± SE[†]	N[‡]
		ms	
594neg-SM	FcεRI clusters	104 ± 7^{*1}	241
	FcεRI monomers	54 ± 2^{*2,Y1}	212
594neg-DOPC	FcεRI clusters	66 ± 2^{Y1}	230
	FcεRI monomers	52 ± 2^{Y1,N2}	224

[†]Exponential decay lifetimes obtained by fitting the distributions of colocalization periods with single exponential functions (see Fig. 10 C). Errors indicate the fitting errors for the 68.3% confidence limits.

[‡]The number of examined colocalizations.

*, ^{Y}, and ^{N}. The results of the statistical test. The distribution selected as the basis for the comparison is shown by the superscript *. The numbers (1 and 2) indicate the different bases. The superscript ^{Y} or ^{N} indicates that the distribution is or is not significantly different, respectively, with t-test p values smaller or greater than 0.05, respectively.

References

Komura, N., K.G.N Suzuki, H. Ando, M. Konishi, M. Koikeda, A. Imamura, R. Chadda, T.K. Fujiwara, H. Tsuboi, R. Sheng, W. Cho, K. Furukawa, K. Furukawa, Y. Yamauchi, H. Ishida, A. Kusumi, and M. Kiso. 2016. *Nat. Chem. Biol.* 12: 402-410. http://dx.doi.org/10.1038/nchembio.2059

Kusumi, A., I. Koyama-Honda, and K. G. N. Suzuki. 2004. Molecular dynamics and interactions for creation of stimulation-induced stabilized rafts from small unstable steady-state rafts. *Traffic* 5:213-30. http://dx.doi.org/10.1111/j.1600-0854.2004.0178.x

Kwik J., S. Boyle, D. Fooksman, L. Margolis, M. P. Sheetz, and M. Edidin. 2003. Membrane cholesterol, lateral mobility, and the phosphatidylinositol 4,5-bisphosphate-dependent organization of cell actin. *Proc. Natl. Acad. Sci. U.S.A.* 100:13964-13969. http://dx.doi.org/10.1073/pnas.2336102100

Sevcsik, E., M. Brameshuber, M. Fölser, J. Weghuber, A. Honigmann, and G. J. Schütz. 2015. GPI-anchored proteins do not reside in ordered domains in the live cell plasma membrane. *Nat. Commun.* 6:6969. http://dx.doi.org/10.1038/ncomms7969

Suzuki, K.G.N., T.K. Fujiwara, F. Sanematsu, R. Iino, M. Edidin, and A. Kusumi. 2007a. GPI-anchored receptor clusters transiently recruit Lyn and Gα for temporary cluster immobilization and Lyn activation: single-molecule tracking study 1. *J. Cell Biol.* 177: 717-730. http://dx.doi.org/10.1083/jcb.200609174

Suzuki, K.G.N., T.K. Fujiwara, M. Edidin, and A. Kusumi. 2007b. Dynamic recruitment of phospholipase Cγ at transiently immobilized GPI-anchored receptor clusters induces IP3–Ca2+ signaling: single-molecule tracking study 2. *J. Cell Biol*. 177: 731-742. http://dx.doi.org/10.1083/jcb.200609175

Suzuki, K.G.N., R.S. Kasai, K.M. Hirosawa, Y.L. Nemoto, M. Ishibashi, Y. Miwa, T.K. Fujiwara, and A. Kusumi. 2012. Transient GPI-anchored protein homodimers are units for raft organization and function. *Nat. Chem. Biol*. 8: 774-783. http://dx.doi.org/10.1038/nchembio.1028

Werner, T. C., Bunting, J. R. & Cathou, R. T. 1972. The shape of immunoglobulin G molecules in solution. *Proc. Natl. Acad. Sci. U.S.A.* 69: 795-799. http://dx.doi.org/10.1073/pnas.69.4.795