More examples of progressing rupture formation in isolated patches (islands) of the distal (upper) phospholipid bilayer membrane

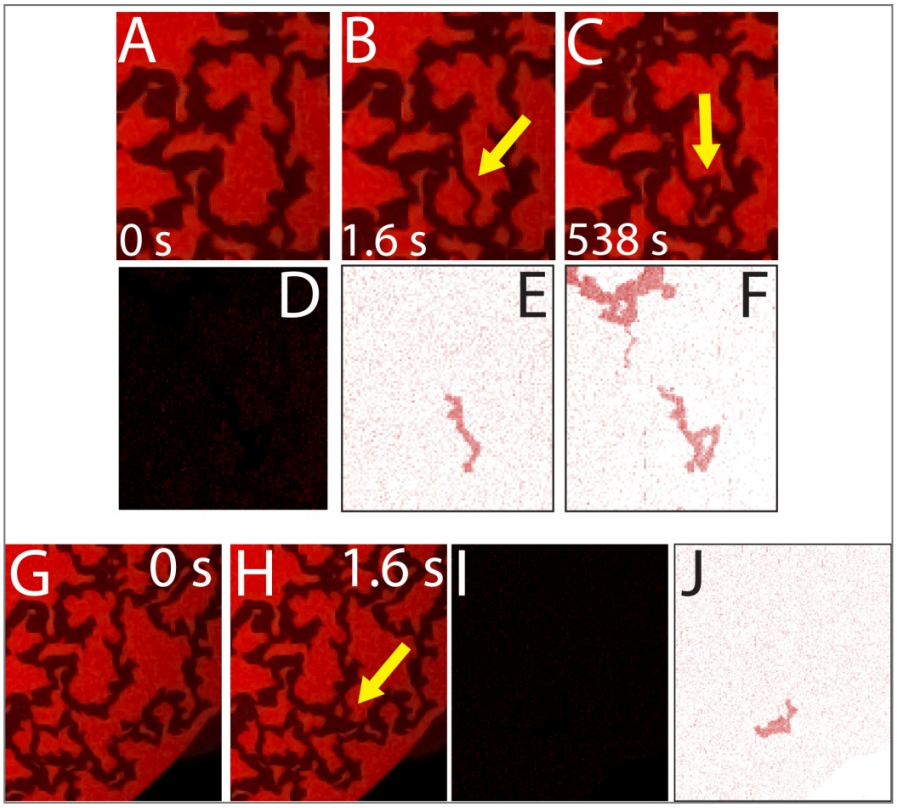

Figure S1. **Progressing rupture formation in isolated patches (islands) of the distal (upper) phospholipid bilayer membrane. (A-C), (G-H)** Laser scanning confocal micrographs of the isolated islands #2, #3 of Figure 2, respectively displaying avalanche ruptures. **(A-C)** Laser scanning confocal time series of the island #2 in Figure 2, displaying two sequential avalanche ruptures (yellow arrows). **(D)** The result of image subtraction of **(A)** from **(C)**. **(E)** The result of image subtraction of **(B)** from **(A)**. **(F)** The result of image subtraction of **(C)** from **(B)**. The distinct patterns in **(E)** and **(F)** correspond to the area of the ruptures appearing in **(B)** and **(C)**, respectively. **(G-H)** Laser scanning confocal time series of the island #3 in Figure 2, displaying an avalanche rupture (yellow arrow). **(I)** The result of image subtraction of **(G)** from **(H)**. **(J)** The result of image subtraction of **(H)** from **(G)**. The distinct pattern in **(J)** corresponds to the area of the rupture appearing in **(H)**.

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Viscous dissipation due to membrane flow towards a single pore:

Assuming radially symmetric membrane flow, $v = \frac{r_p}{r} v_p$, around a pore gives:

$$T\dot{S} = 2\mu \int dS \left(\frac{\partial v_i}{\partial x_k} + \frac{\partial v_k}{\partial x_i}\right)^2 = 2\mu \int dr 2\pi r \left[\left(\frac{\partial v}{\partial r}\right)^2 + \left(\frac{v}{r}\right)^2\right] = 8\pi\mu v_p^2 r_p^2 \int_{r_p}^{R} \frac{dr}{r^3} \approx 4\pi\mu v_p^2$$

The approximation is valid when the pore radius is small compared to the patch size. Taking into account dissipation on both sides of the pore gives: $T\dot{S} = 8\pi\mu v_p^2$.

Dissipation in case of N number of pores:

For 1 pore : T$\dot{S}$ = $8\pi\mu v_p^2$

J=$v_p 2\pi r_p$ => T$\dot{S}$ = $8\pi\mu\left(\frac{J}{2\pi r_p}\right)^2 = \frac{2\mu J^2}{\pi r_p^2}$

For N pores:

J=$N.\, v_p 2\pi r_p$

T$\dot{S}$ =$N\ 8\pi\mu v_p^2 = \ N\ 8\pi\mu\left(\frac{J}{\mathrm{N}2\pi r_p}\right)^2 = \frac{1}{N}\frac{2\mu J^2}{\pi r_p^2}$

J=Total membrane area flow (area/time) going through pores from upper to lower patch.

$T\dot{S}$ =Viscous dissipation rate for 2D membrane incompressible flow

μ = Membrane 2D viscosity

v_p = Membrane velocity in the fusion pore

r_p = Pore radius

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Analysis of possible direct lipid loss from isolated islands

Figure S2. depicts a scenario where lipids migrate from the distal to the proximal bilayer in a direct lipid transfer process. Such a unidirectional transfer mechanism is conceivable if the proximal membrane is under tension. Before lipid transfer, the (fluorescently labeled) membrane is unstreched (Fig. S2 (A)). Lipid molecules leave the membrane and cause the membrane to stretch, since the area of the island remains unchanged (Fig. S2 (B)). We have already shown in Fig. S1 above and in Figure 2 of the main article that the contour of the islands do not change during rupturing. As a consequence, the tension in the distal membrane will increase, which will eventually cause a rupture(Fig. S2(C)). During rupturing, lipids migrate from the rupture region into the main area of the distal membrane. The lipid density increases, the fluorescent intensity per area should also increase. We note that this mechanism will be valid regardless whether more fluorophore-conjugated or more non-conjugated lipids leave the distal membrane in the transfer process. Only the magnitude of fluorescence intensity change will be different. However, a comparison of the fluorescence intensities of rupturing and non-rupturing islands before the rupture event gave no evidence that fluorescently labeled lipids were exclusively transported out of the distal membrane (data not shown).

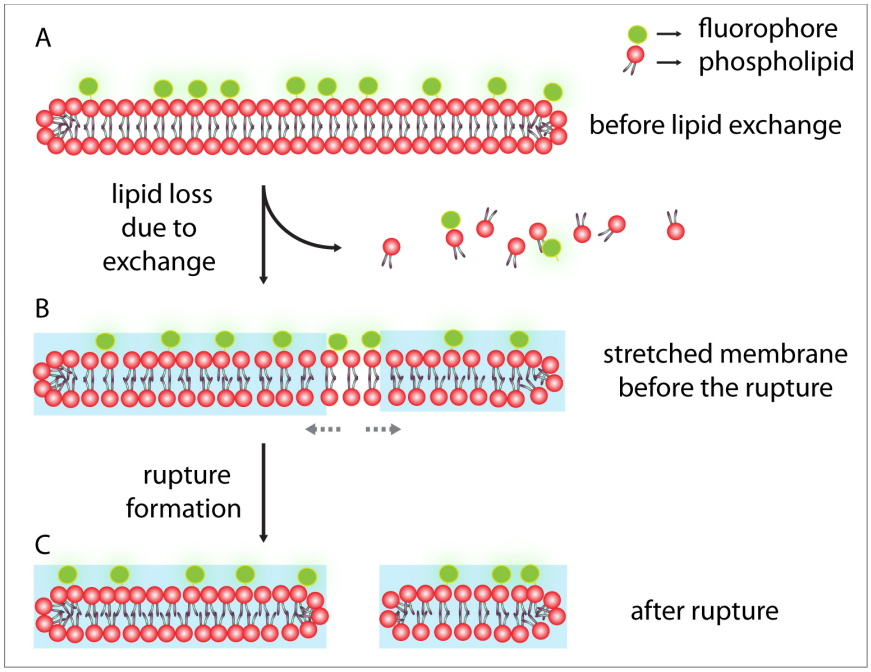

Figure S2. Mechanism of fluorescence intensity increase over unit area in the membrane of an isolated island upon rupturing. For simplicity, fluorophores are shown only on the upper leaflet of the bilayer. **(A)** Unstreched membrane doped with fluorophores. Due to lipid exchange through the solution, the membrane looses both fluorescently labeled and unlabeled lipids. **(B)** Stretched membrane due to direct lipid loss. The two light blue regions illustrate regions of interest (ROIs) of the membrane. **(C)** Tension built up in the membrane due to stretching causes a rupture. The membrane has relaxed and lipid area density increases due to rupture formation. The number of fluorophores per unit area of membrane in the ROIs has increased due to the flow of lipids out of the rupture region. The number of lipid molecules and fluorophores on the island remain unchanged**,** but they are now concentrated in a relatively smaller area, leading to an increase in fluorescence intensity.

Quantitatively, the expected increase in fluorescence intensity per unit area of the isolated island is 6.4 % for a 0.06 rupture area fraction (island #1 in Fig. 2 or Fig. S3), and 9.3% for an rupture area fraction of 0.085 (island #2 in Fig. 2 or island #1 in Fig. S4), respectively. Using the free software package ImageJ, we performed a fluorescence intensity analysis for several regions of interest (ROI) on two islands which exhibit avalanche rupturing, and compared the results to some non-rupturing islands (Figure S2 and S3). Fig. S3 below shows the analysis for the island discussed in the main article, and Fig S4 for an additional island.

Island #1 in Fig. S3 displays avalanche ruptures over 3.2 seconds. We selected regions of interest on the patch shown in Fig. S3(A-C), distributed over 6 islands. The stepwise area loss of the island is 3.16% and 3.13%; relative to the respective prior states.

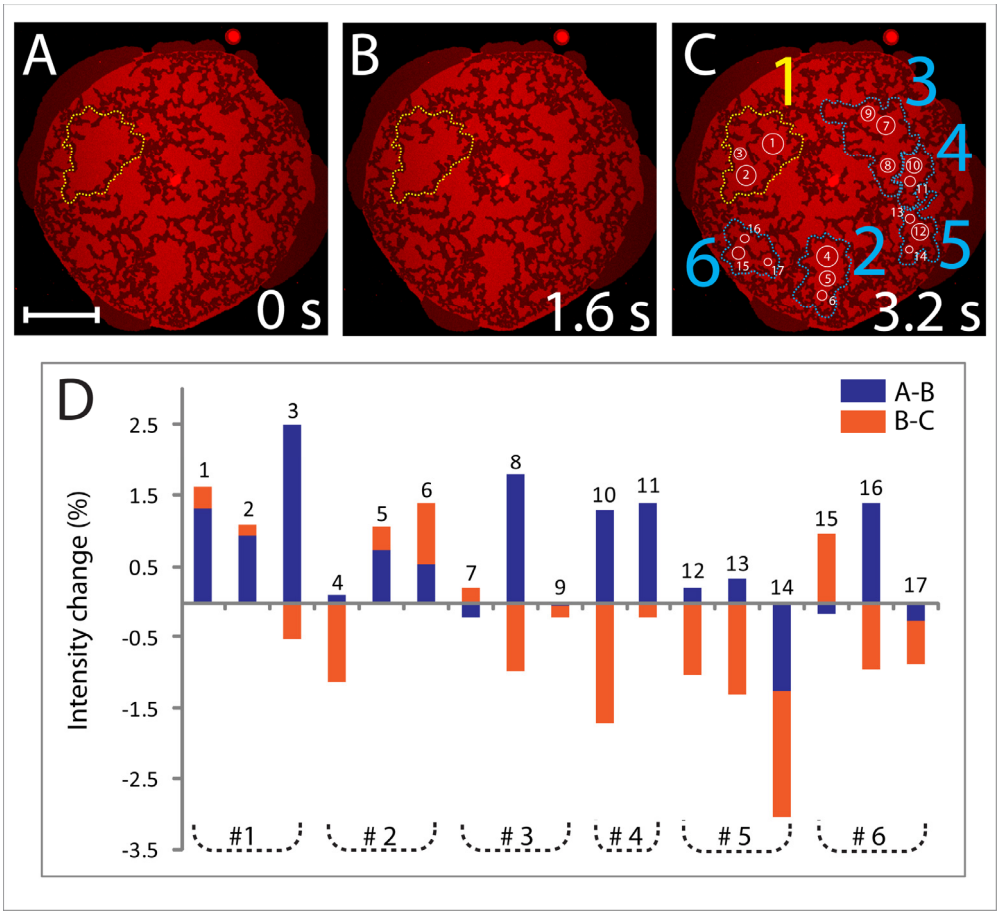

Figure S3. **Analysis of fluorescence intensity change during rupturing. (A-C)** Fluorescent micrographs showing the rupturing lipid patch of the main article, with the investigated island highlighted in yellow. **(C)** 17 circular regions of interests (ROIs) which were selected in 6 isolated islands on the lipid patch. Each ROI is marked with white a circle and numbered in white. Contours of the reference islands are represented by blue dashed lines and numbered in blue color. Island #1 displays an avalanche rupture, reference islands #2-6 do not exhibit any rupturing. **(D)** Histograms showing the intensity changes in percent for each ROI during **(A-C)**. The intensity change between **(A-B)** is shown in blue, between **(B-C)** in orange color. The island identifiers are displayed at the bottom of **(D)**. Scale bar: 60 µm.

The intensity changes are within the range of the fluctuations displayed by the other, non-rupturing islands. A second analysis was performed on island #1 in Figure S4. It displays an avalanche rupture over 1.6 seconds. The percentage of the area loss relative to the area of the island before the rupture is with 8.5 % even greater. It does not show a distinct increase in fluorescence intensity, either.

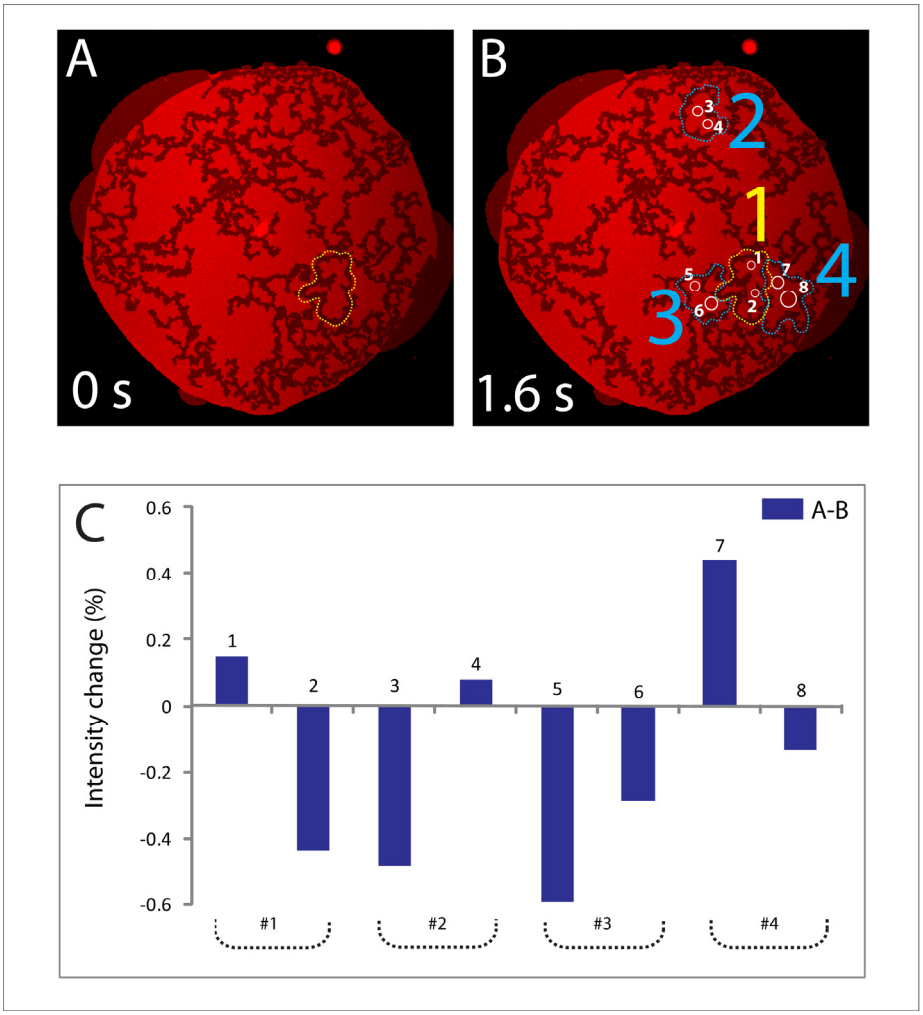

Figure S4. **Analysis of fluorescence intensity change during rupturing (additional island from the supporting figure S1 above).** (A-B) Fluorescent micrographs showing the rupturing lipid patch, with the investigated island highlighted in yellow. (C) 8 circular regions of interests (ROIs) which were selected in 4 isolated islands on the lipid patch. Each ROI is marked with a white circle and numbered in white. Contours of the reference islands are represented by blue dashed lines and numbered in blue color. Island #1 displays an avalanche rupture, reference islands #2-4 do not exhibit any rupturing. (C) Histograms showing the intensity changes in percent for each ROI during (A-B). The island identifiers are displayed at the bottom of (C). Further rupturing (S1 B-C) is not considered because of the formation of isolated islands in S1 (A-B).

The analysis on the fluorescence intensity change vs. area decrease of the isolated islands in both Figure S3 and S4 reveal that the fluorescent intensity does not increase according to the values predicted from the area loss. In addition, the intensity does not consistently increase in all ROIs of the rupturing islands, but rather shows fluctuation behaviour(Fig. S3(D), Fig. S4(C)). The intensity changes are of the same magnitute as the intensity fluctuations within the other islands over the same period of time. In conclusion, this analysis provides no evidence for a direct lipid loss mechanism.