Supporting Information

Materials and Methods

The phospholipids DOPC, DOPG, DPPC, and DPPG were purchased from Avanti Polar Lipids (Alabama, USA). Cholesterol and the other reagents were obtained from Merck (Darmstadt, Germany). BDP 650/665 X NHS ester was purchased from Lumiprobe (Hannover, Germany). ATTO-550 maleimide was obtained from ATTO-TEC (Siegen, Germany). All lipids and cholesterol were dissolved in chloroform to obtain stock solutions of 10 mg/mL. All buffers used were filtered using 0.02 µm pore size filters (Whatman, Dassel, Germany).

K-Ras4B protein synthesis and labeling

The synthesis of full-length K-Ras4B has been described in detail before (1-4). The plasmid of K-Ras4B-intein-CBD (obtained from Prof. Dr. Herbert Waldmann, MPI Dortmund) in pTWIN2-vector of the impact^{TM} system (NEB) was transformed into *E. coli* BL21 (DE3) cells. They were grown with 2 L of LB media at 37 °C and induced with 0.5 mM IPTG at 20 °C when the culture reached an OD_{600} of 0.6 for 16 h. Cultured cells were lysed by ultrasonification in lysis buffer (20 mM Tris, 500 mM NaCl, 2 mM $MgCl_2$, pH = 7.4). Protease inhibitor (SIGMA*FAST* Protease Inhibitor Tables) was added to the unclarified lysate. Centrifugation of lysate at 15,000 rpm for 30 mins at 4 °C clarified the lysate, which was loaded onto a chitin beads affinity column after equilibration with lysis buffer. The intein splicing was introduced by incubation of the column with intein splicing buffer (20 mM Tris, 500 mM NaCl, 2 mM $MgCl_2$, 50 MESNA, pH = 8.5) for 12 h. The final K-Ras4B (Δ15)-MESNA thioester generated was transferred to lysis buffer using a HiPrep 26/10 desalting column (GE Healthcare) and concentrated using size exclusion filtration (Amicon Ultra-15 MWCO 10 kD). K-Ras4B peptide containing an N-terminal cysteine was synthesized using solid phase peptide synthesis technology as decribed in detail in reference 1. K-Ras4B peptide was ligated to the C-terminal K-Ras4B thioester using the native chemical ligation strategy, resulting in the full-length K-Ras4B protein with a native amide bond at the ligation site. After ligation, the protein was purified using a Hi-Trap Sp-X1 column (GE Healthcare). GppNHp (the non-hydrolyzable analogue of GTP)-bound K-Ras4B was generated by nucleotide exchange from GDP to GppNHp. Alkaline phosphatase (Roche, Basel, Switzerland) at a concentration of 5 units/mg and a two-fold molar excess of GppNHp were added to the protein solution, and the completion of nucleotide exchange was confirmed by reverse-phase HPLC using the mobile phase (10 mM tetrabutylammonium bromide, 33 mM K_2HPO_4, 68.5 mM KH_2PO_4, 6% acetonitrile). The K-Ras4B protein solution obtained was quick-frozen and stored at -80 °C. Details on the synthesis of ATTO-550 labeled K-Ras4B are decribed in references 1 and 3.

LAF1 and EGFP-LAF1 protein expression and purification

The LAF1 and EGFP-tagged LAF1 genes were codon optimized and synthesized by GenScript (Netherlands) for *E. coli.* The LAF1 and EGFP-LAF1 genes were inserted in a pET28a back-bond with

a C-terminal 6×His tag and a thrombin recognition and cleavage site. The amino acid sequence of EGFP-LAF1 is listed below.

EGFP-LAF1 (1-951) amino acid sequence (green, EGFP; RGG domain, red; blue, helicase domain)

MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWPTLVTTLTYGVQCFSRYPD
HMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYI
MADKQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMVLLEFVTAA
GITLGMDELYKMESNQSNNGGSGNAALNRGGRYVPPHLRGGDGGAAAAASAGGDDRRGGAGGGGYRRGGG
NSGGGGGGGYDRGYNDNRDDRDNRGGSGGYGRDRNYEDRGYNGGGGGGGNRGYNNNRGGGGGGYNRQD
RGDGGSSNFSRGGGYNNRDEGSDNRGSGRSYNNDRRDNGGDGQNTRWNNLDAPPSRGTSKWENRGARDERIE
QELFSGQLSGINFDKYEEIPVEATGDDVPQPISLFSDLSLHEWIEENIKTAGYDRPTPVQKYSIPALQGGRDLMSCAQ
TGSGKTAAFLVPLVNAILQDGPDAVHRSVTSSGGRKKQYPSALVLSPTRELSLQIFNESRKFAYRTPITSALLYGGREN
YKDQIHKLRLGCHILIATPGRLIDVMDQGLIGMEGCRYLVLDEADRMLDMGFEPQIRQIVECNRMPSKEERITAMF
SATFPKEIQLLAQDFLKENYVFLAVGRVGSTSENIMQKIVWVEEDEKRSYLMDLLDATGDSSLTLVFVETKRGASDL
AYYLNRQNYEVVTIHGDLKQFEREKHLDLFRTGTAPILVATAVAARGLDIPNVKHVINYDLPSDVDEYVHRIGRTGR
VGNVGLATSFFNDKNRNIARELMDLIVEANQELPDWLEGMSGDMRSGGGYRGRGGRGNGQRFGGRDHRYQG
GSGNGGGGNGGGGGFGGGGQRSGGGGGFQSGGGGGRQQQQQQRAQPQQDWWSLVPR

The LAF1 and EGFP-LAF1 plasmids were transformed into BL21 (DE3) cells and grew until reach of an OD_{600} of 0.5. IPTG (0.5 mM) was added to induce protein expression at 20 °C. Cultured cells were lysed by ultrasonification in lysis buffer (20 mM Tris, 1 M NaCl, 20 mM imidazole, 10% (vol/vol) glycerol, 5 mM TCEP, pH = 7.4). Protease inhibitor (SIGMA*FAST* Protease Inhibitor Tables) was added to the unclarified lysate. Centrifugation of lysate at 15,000 rpm for 30 min at room temperature clarified the lysate, which was loaded onto a HisTrap (GE Healthcare) affinity column after equilibration with lysis buffer. Washing buffer (20 mM Tris, 1 M NaCl, 25 mM imidazole, 10% (vol/vol) glycerol, 5 mM TCEP, pH = 7.4) was used to wash the protein loaded column. Ni-Elution buffer (20 mM Tris, 1 M NaCl, 250 mM imidazole, 10% (vol/vol) glycerol, 5 mM TCEP, pH = 7.4) was used to elute the target protein. The eluate was loaded onto a HiTrap Benzamidine FF (GE Healthcare) column to remove the thrombin enzyme. Fractions were collected and analyzed by SDS-PAGE and Commassie-staining. Aliquots were flash-frozen in liquid nitrogen and stored at -80 °C.

Liquid-liquid phase separation of LAF1 and EGFP-LAF1

For in vitro liquid droplet formation, LAF1 or EGFP-LAF1 frozen aliquots were thawed at room themperature, centrifuged at 12, 000 rpm for 1 min to remove any aggregated protein and buffer exchanged with working buffer (20 mM Tris, 1 M NaCl, 1 mM TCEP, pH = 7.4). The protein concentration was determined using a Eppendorf BioPhotometer plus (Eppendorf AG, Hamburg, Germany). Protein solutions were subsequently diluted with varying volumes of no salt containing buffer (20 mM Tris, 1 mM TCEP, pH = 7.4) to obtain the desired protein and salt concentrations.

B-Raf-RBD and C-Raf-RBD protein expression, purification and labeling

B-Raf-RBD and C-Raf-RBD plasmid construction, protein expression and purification were described in detail in the reference 5. B-Raf-RBD and C-Raf-RBD were labeled using BDP 650/665 X NHS ester (Lumiprobe, Hannover, Germany) following standard labeling protocols. The labeling products were confirmed by ESI-MS and SDS-PAGE.

Confocal microscopy experiments

All the confocal experiments were conducted on a confocal laser scanning microscopy (Biorad MRC 1024, Germany) coupled via a side port to an inverted microscopy (Nikon, Eclipse TE-300DV) enabling fluorescence excitation in the focal plane of an objective lens (Nikon Plan Appochromat VC 60 × A WI, NA = 1.2). Signals were detected in three PMT channels (emission band pass filters at 522 nm/FWHM (full width at half-maximum) of 35 nm, 580 nm/FWTH of 40 nm, and 680 nm/FWTH of 32 nm. Images were taken at 1280 × 1024 pixels, and saved as 8-bit image files. Image acquisition was controlled by the software LaserSharp2000 (Biorad). The analysis of the images was performed using the software ImageJ.

Atomic force microscopy experiments

The preparation of supported lipid bilayers (SLBs) has been described in detail in references 2-5. AFM measurements were carried out on a MultiMode scanning probe microscope with a Nano-Scope IIIa controller (Digital Instruments, Santa Barbara, CA) and a J-Scanner (maximum scan size 125 μm). Images were obtained using the tapping-mode in Tris buffer (20 mM Tris, 5 mM $MgCl_2$, pH = 7.4) with sharp nitride lever (SNL) probes mounted in a fluid cell (MTFML, Bruker, Karlsruhe, Germany). Tips with nominal force constants of 0.24 Nm^{-1} were used at driving frequencies of 8.0-9.5 kHz and drive amplitudes between 200 and 600 mV. The scan frequency was 1.49 Hz. Images were taken with a resolution of 512 × 512 pixels. All AFM experiments were carried out at room temperature and analysed using NanoScope version 5. The height difference was calculated using section plot in the NanoScope version 5 software.

Additional Data and Results

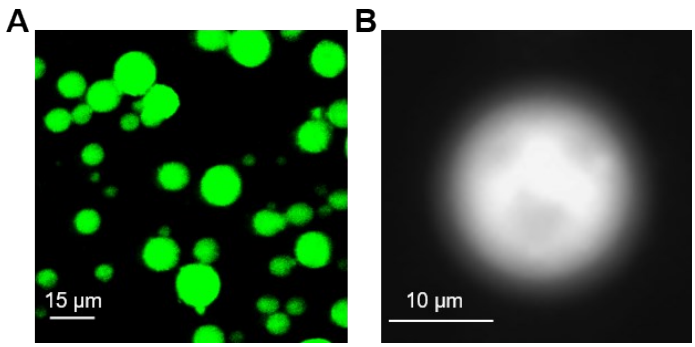

Figure S1. (A) Fluorescent image of liquid droplets formed by LAF1 (20 mM Tris, 150 mM NaCl, 1 mM TCEP, pH = 7.4) taken by confocal microscopy. (B) Zoom-in image of liquid droplet formed by LAF1 (20 mM Tris, 150 mM NaCl, 1 mM TCEP, pH = 7.4) recorded by light microscopy.

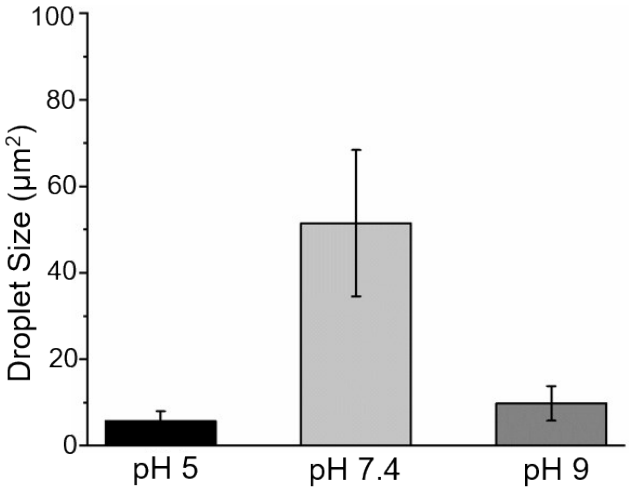

Figure S2. Statistics of average sizes of LAF1 droplets at pH 5, pH 7.4 and pH 9. The statistics was determined based on the images acquired (Figure 3A in the main text). Quantification was carried out using software ImageJ.

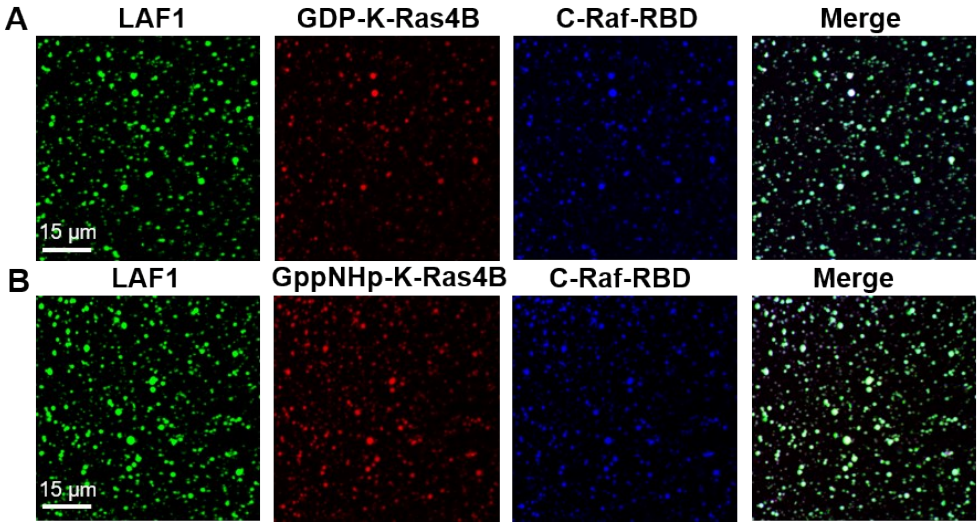

Figure S3. (A) C-Raf-RBD localized in the droplets by binding to the GDP-K-Ras4B via electrostatic interactions. (B) C-Raf-RBD localized in the droplets by binding to the GppNHp-K-Ras4B via electrostatic interactions. Droplets were formed after diluting concentrated LAF1 to low concentration LAF1 (5 μM) in Tris buffer (20 mM Tris, 100 mM NaCl, 1 mM TCEP, pH = 7.4). Both GDP-K-Ras4B and GppNHp-K-Ras4B was labeled with ATTO 550. LAF1 was tagged with EGFP. C-Raf-RBD was labeled with BDP 650/665 X NHS ester.

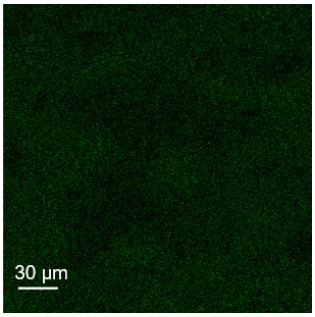

Figure S4. Fluorescent image of LAF1 (5 μM) in high salt concentration buffer (20 mM Tris, 1 M NaCl, 1 mM TCEP, pH = 7.4), showing that LAF1 (5 μM) in high salt concentration buffer does not form liquid droplets.

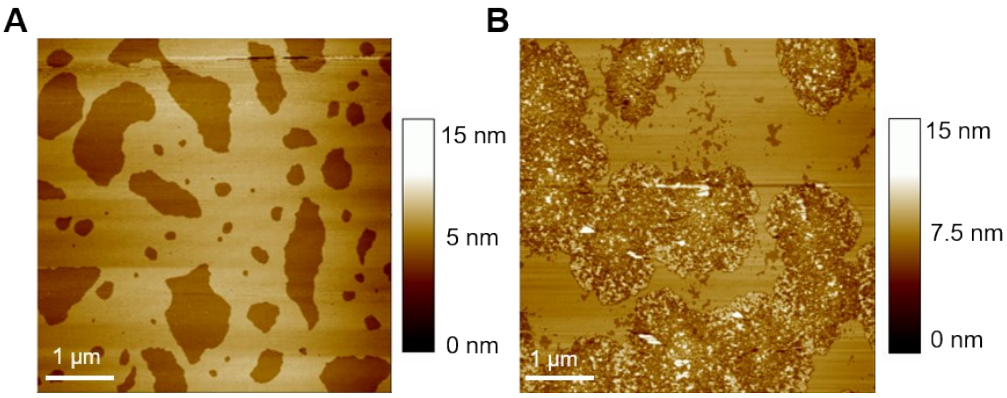

Figure S5. (A) AFM image of the heterogeneous lipid membrane shown before injection of K-Ras4B. (B) AFM image of the heterogeneous lipid membrane after injection of 200 μL LAF1 (1 μM) in Tris buffer (20 mM Tris, 1 M NaCl, 1 mM TCEP, pH 7.4).

References

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[5] L. Li, S. Möbitz, R. Winter, *Langmuir,* 2020, **36**, 5944–5953.