Receptor tyrosine kinases regulate signal transduction through a liquid-liquid phase separated state

Graphical abstract

Highlights

- Phosphorylated RTKs undergo phase separation with downstream effectors
- Phosphorylation-dependent multivalent interaction drives FGFR2-SHP2 phase separation
- The FGFR2-SHP2 complex colocalizes PLCγ1 to its plasma membrane substrate
- Enzymatic activities are regulated within the FGFR2-SHP2-PLCγ1 membraneless droplets

Authors

Chi-Chuan Lin, Kin Man Suen, Polly-Anne Jeffrey, ..., Stephen D. Evans, Carmen Molina-París, John E. Ladbury

Correspondence

c.c.lin@leeds.ac.uk (C.-C.L.), j.e.ladbury@leeds.ac.uk (J.E.L.)

In brief

Lin et al. demonstrate that phosphorylated RTKs undergo liquid-liquid phase separation upon the recruitment of downstream proteins. Focusing on the RTK FGFR2, this process is shown to modulate enzymatic activities within the subcellular membraneless compartment.

Lin et al., 2022, Molecular Cell 82, 1089–1106

March 17, 2022 © 2022 The Author(s). Published by Elsevier Inc.

https://doi.org/10.1016/j.molcel.2022.02.005
Receptor tyrosine kinases regulate signal transduction through a liquid-liquid phase separated state

Chi-Chuan Lin, Kin Man Suen, Polly-Anne Jeffrey, Lukasz Wieteska, Jessica A. Lidster, Peng Bao, Alistair P. Curd, Amy Stainthorp, Caroline Seiler, Hans Koss, Eric Miska, Zamal Ahmed, Stephen D. Evans, Carmen Molina-París, and John E. Ladbury

School of Molecular and Cellular Biology, University of Leeds, Leeds LS2 9JT, UK
School of Mathematics, University of Leeds, Leeds LS2 9JT, UK
School of Physics and Astronomy, University of Leeds, Leeds LS2 9JT, UK
Wellcome Trust Cancer Research UK Gurdon Institute, University of Cambridge, Tennis Court Road, Cambridge CB2 1QN, UK
Institute of Structural and Molecular Biology, Division of Biosciences, University College London, Gower Street, London WC1E 6BT, UK
Francis Crick Institute, London NW1 1AT, UK
Department of Molecular and Cellular Oncology, University of Texas M D Anderson Cancer Center, Houston, TX 77030, USA
These authors contributed equally
Lead contact
Correspondence: c.c.lin@leeds.ac.uk (C.-C.L.), j.e.ladbury@leeds.ac.uk (J.E.L.)
https://doi.org/10.1016/j.molcel.2022.02.005

SUMMARY

The recruitment of signaling proteins into activated receptor tyrosine kinases (RTKs) to produce rapid, high-fidelity downstream response is exposed to the ambiguity of random diffusion to the target site. Liquid-liquid phase separation (LLPS) overcomes this by providing elevated, localized concentrations of the required proteins while impeding competitor ligands. Here, we show a subset of phosphorylation-dependent RTK-mediated LLPS states. We then investigate the formation of phase-separated droplets comprising a ternary complex including the RTK, (FGFR2); the phosphatase, SHP2; and the phospholipase, PLCγ1, which assembles in response to receptor phosphorylation. SHP2 and activated PLCγ1 interact through their tandem SH2 domains via a previously undescribed interface. The complex of FGFR2 and SHP2 combines kinase and phosphatase activities to control the phosphorylation state of the assembly while providing a scaffold for active PLCγ1 to facilitate access to its plasma membrane substrate. Thus, LLPS modulates RTK signaling, with potential consequences for therapeutic intervention.

INTRODUCTION

Receptor tyrosine kinases (RTKs) initiate signaling pathways, which regulate diverse cellular processes. On activation, multiple moderate affinity tyrosyl phosphate (pY) binding sites become available for the rapid recruitment of downstream effector proteins. However, the mechanism for expedient recruitment via random molecular diffusion through the cytoplasm is not fully understood. One way in which the probabilistic outcome associated with random diffusion could be alleviated is through the localized accumulation of high effective concentrations of signaling proteins in discrete pools in the cell (Cebecauer et al., 2010). The inclusion of interacting proteins into liquid-liquid phase-separated (LLPS) membraneless droplets maintains functionally relevant proteins at high concentrations in a liquid phase at the required point of action, enhancing equilibrium binding and enzyme activity (Banani et al., 2017; Bracha et al., 2018; Case et al., 2019a; Hyman et al., 2014; Wang et al., 2018). These LLPS states have been associated with a wide range of cellular functions including the regulation of signaling through, e.g., nephrin (Case et al., 2019a; Li et al., 2012), the T-cell receptor (Su et al., 2016), mTOR (Zhang et al., 2018), and RAS (Huang et al., 2019; Tulpule et al., 2021); however, whether LLPS extends to plasma membrane-bound RTK signal transduction has not been investigated. Here, we show that a subset of RTKs undergo LLPS with downstream effector proteins. We then demonstrate that one of these RTKs, fibroblast growth factor receptor 2 (FGFR2), forms a signaling-competent LLPS state with two downstream enzymes: a tandem Src homology 2 (SH2) domain-containing protein tyrosine phosphatase 2 (SHP2) and 1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase gamma 1 (PLCγ1). We show that these proteins assemble into a ternary complex, which exploits LLPS condensation to simultaneously modulate kinase, phosphatase, and phospholipase activities. Therefore, LLPS formation ensures that the requirement for prolonged, high-fidelity signaling is
achieved. Our work suggests that the formation of biological condensates might be a key organizing principle of RTK-mediated signaling, with broad implications for further mechanistic studies as well as therapeutic intervention.

RESULTS

Phosphorylated RTKs form droplets with signaling proteins

To understand whether droplet formation could play a role in the regulation of RTK-containing signaling complexes, in the first instance, the intracellular domains of a subset of RTKs were investigated: pEGFR\text{Kinase-Tail} (residues 712–1210), pHer2\text{Kinase-Tail} (720–1255), pHER4\text{Kinase-Tail} (718–1305), pFGFR1\text{Kinase-Tail} (478–822), pFGFR2\text{Cyt0} (400–821), pVEGFR1\text{Kinase-Tail} (827–1338), and pVEGFR2\text{Kinase-Tail} (834–1356) (a schematic of all constructs used in this work is given in Figure 1A and purity in Figure S1A). We investigated the droplet formation with phosphorylated RTK intracellular domains with two known downstream effector proteins: phosphatase SHP2 (the inactive C459S mutant, SHP2\text{C459S} [Agazie and Hayman, 2003]) and adaptor protein SHC (the PS2 isoform) (Figure S1A). Both effector proteins contain a pair of domains, which bind to pY-containing ligands. Visible droplets are apparent in an in vitro droplet formation assay using fluorescently labeled phosphorylated RTKs with SHP2\text{C459S} or SHC (Figure 1A) in 150-mM NaCl and using the optimum concentrations we obtained from the phase diagram analysis of different RTKs with SHP2\text{C459S} (Figure 1B) or SHC (Figure S1B). SHP2\text{C459S} and SHC were able to form droplets with all the RTKs we tested apart from the pVEGFR2\text{Kinase-Tail-SHP2C459S} pair, which could reflect differences in affinities between the RTK pY sequences and SHP2\text{C459S} or SHC.

We focused our investigation on droplet formation involving FGFR2 (pFGFR2\text{Cyt0}) and SHP2\text{C459S} (Chen et al., 2020). Active SHP2 resulted in lesser droplet formation than SHP2\text{C459S}, as did the use of unphosphorylated FGFR2\text{Cyt0} or the “kinase-dead” K517I mutant (FGFR2\text{Cyt0K517I}). Thus, the appearance of droplets depends on prolonged RTK phosphorylation (Figure S1C). To mitigate against the rapid phosphorylation-dephosphorylation cycles in in vitro experiments, we adopted the SHP2\text{C459S} phosphatase-dead trapping mutant in the following experiments, except where we have stated that WT protein is used.

The droplets of FGFR2-SHP2 exhibit liquid-like features in cells

Using optimal protein concentrations derived from phase diagrams (Figure 1B), we found that pFGFR2\text{Cyt0-SHP2C459S} droplets display the liquid-like features of mobilization and fusing upon encounter (Figure 2A), as well as in fluorescence recovery after photobleaching (FRAP) experiments where the fluorescence of pFGFR2\text{Cyt0-SHP2C459S} droplets recovered within minutes (Figure 2B). To determine whether the receptor forms droplets with SHP2\text{C459S} in fixed cells, we overexpressed full-length RFP-tagged FGFR2 and GFP-tagged SHP2\text{C459S} and stimulated with FGF9, a specific FGFR2 ligand, in HEK293T cells in which endogenous FGFR2 is negligible (Figure S2A). To remove the alternative mode of SHP2 recruitment to FGFR2 via FRS2, we adopted a FGFR2 mutant deleted for 428\text{VT}-429 (FGFR2\text{ΔVT}; see STAR Methods). Compared with the basal state, the stimulation of FGFR2 results in the coalescence of micrometer-sized clusters at the plasma membrane of complexes containing SHP2\text{C459S} (Figure S2A).

To understand how the droplets affect signaling, another known substrate protein of FGFR2, PLC\text{γ1} (Huang et al., 2016), was also investigated. Although it has been reported that the RTK-dependent phosphorylation of PLC\text{γ1} on Y783 abrogates its interaction with active RTKs (Bunney et al., 2012; Grexset et al., 2010), the phospholipase was still seen to condense into droplets on plasma membrane upon the stimulation of cells (Figure S2B), whereas no such clusters were observed in the absence of the receptor where both SHP2\text{C459S} and PLC\text{γ1} appear to be randomly diffusing in cells (Figure S2C). We also demonstrated that SHP2\text{C459S-RFP} exhibited condensate fluidity on the membrane when FGFR2\text{ΔVT} is expressed and activated by FGF9, as assessed by the fusion experiments in HEK293T cells (Figure 2C). These results indicate that the recruitment of SHP2\text{C459S} to active pFGFR2 promotes the formation of droplets that exhibit a dynamic liquid-like behavior.

Reconstituted FGFR2-SHP2\text{C459S} LLPS droplets promote the recruitment of active PLC\text{γ1}

PLC\text{γ1} is recruited into active FGFR2 through binding to pY769 on the receptor (Bunney et al., 2012). The concomitant phosphorylation of PLC\text{γ1} on Y783 causes an intramolecular interaction between pY783 and the CSH2 domain of the phospholipase; this intramolecular interaction induces a structural rearrangement and leads to the dissociation of phosphorylated PLC\text{γ1} from the recruiting RTK (Bunney et al., 2012; DeBell et al., 2007; Poulin et al., 2005). However, active PLC\text{γ1} needs to be retained proximal to the plasma membrane, where it can access and hydrolyze its substrate phosphatidylinositol 4,5-bisphosphate (PIP2). To explore a potential retention mechanism, we tested the ability of FGFR2-SHP2\text{C459S} LLPS droplets to recruit active PLC\text{γ1} in vitro.

Using the concentrations of pFGFR2\text{Cyt0}, SHP2\text{C459S}, and pPLC\text{γ1} established from phase diagrams (Figure S2D), we saw no droplet formation with the individual fluorescently labeled proteins (Figure 2Dii); however, submicrometer-sized droplets formed upon the addition of pPLC\text{γ1} to pFGFR2\text{Cyt0-SHP2C459S} (Figure 2Dii). Size exclusion chromatography revealed that these three proteins can form a high-molecular-weight complex (Figure S2E). The pairwise combination of pFGFR2\text{Cyt0-SHP2C459S} formed droplets (Figure 2Diii), but the lack of droplet formation between pFGFR2 and pPLC\text{γ1} is consistent with the reported abrogation of interaction upon Y783 PLC\text{γ1} phosphorylation (Figure 2Div) (Bunney et al., 2012; Grexset et al., 2010). Interestingly, SHP2\text{C459S-pPLC\text{γ1}} also exhibited droplet formation (Figure 2Dv). To provide a robust confirmation of LLPS formation, we employed two complementary methods that have emerged as current standards in the field (Alberti et al., 2019). First, increasing the concentration of NaCl from 150 mM to 250 mM inhibited the electrostatic interactions and, therefore, led to a decrease in the size of droplets (Figures 2Dii and 2Dvi). At 500-M NaCl concentration, the droplets completely dispersed (Figure 2Dvii). Second, the addition of 10% 1,6-hexanediol disrupted
Figure 1. Phosphorylated RTK-mediated condensation of protein complexes
(A) (Above) Images of recombinant phosphorylated receptors from the EGFR, FGFR, and VEGFR families (Atto-488 labeled) droplet formation upon adding SHP2C459S (top panel) or SHC (middle panel); phosphorylated RTK proteins alone do not form droplets (third panel). Concentrations of each RTK-SHP2C459S or SHC pair were shown (x axis: RTK concentration; y axis: SHP2C459S or SHC concentration) and scale bars, 10 μm. (Below) Schematic diagram with residue numbers shows the defined boundaries of RTK intracellular regions of SHP2, SHC, and PLCγ1 proteins and polypeptides used in this study.
(B) Phase diagrams of phosphorylated EGFR, FGFR, and VEGFR family proteins (Atto-488 labeled) with concentrations shown in x axis and SHP2C459S (y axis) in 20 mM HEPES (pH7.5), 150 mM NaCl, and 1 mM TCEP. The sizes of the circles represent the average sizes of droplets (μm²), and the color scale bars represent the number of droplets in a 0.0256-mm² area.
Figure 2. The dynamic LLPS properties of phosphorylated pFGFR2Cytot-SHP2C459S-pPLCγ1 condensates
(A) The dynamic LLPS properties of pFGFR2Cytot (10 μM)-SHP2C459S (60 μM) condensates was assessed by the fusion experiment. Images were taken every 5 min. Scale bars, 100 μm.
(B) Quantification of FRAP data (means ± SD, n = 2 experiments) for pFGFR2Cytot (10 μM)-SHP2C459S (60 μM) condensates.
(C) The dynamic LLPS property of full-length SHP2C459S-RFP condensates coexpressed with FGFR2DVT and stimulated with 10 ng/ml of FGF9 ligand in HEK293T cells. Images were taken every 30 s. Scale bars, 250 nm.
(D) Droplet formation observed between pFGFR2Cytot-Atto-488 (10 μM), SHP2C459S-Atto-550 (60 μM), and pPLCγ1 Atto-647 (12 μM). (i) Individual proteins showed no evidence of droplet formation. Droplet formation was observed after 1 min between different combinations of proteins: (ii) all three proteins; (iii) pFGFR2Cytot with

(legend continued on next page)
condensates (Figure 2Dvii). As an additional test of LLPS, lipoi-
mide and lipoic acid, which dissolve stress granules (Wheeler et al., 2019), appeared to do the same to pFGFR2Cyto- 
SHP2C459S-pPLCγ1 droplets (Figures 2Dix, 2Dx, and 2Dxii). Finally, the fusion experiment using the pFGFR2Cyto- 
SHP2C459S-pPLCγ1 condensates was consistent with a fluid 
state (Figure 2E).

Reconstituted pFGFR2Cyto-SHP2C459S-pPLCγ1 
assembles undergo LLPS on lipid membrane bilayers 

To mimic FGFR2 signaling complex assembly on a membrane and 
confirm that a complex can form as a part of the LLPS, we pre-
pared fluorescently labeled pFGFR2Cyto with an N-terminal 6x 
His tag that allowed attachment to the supported lipid bilayers con-
taining 2% Ni-NTA (Banjade and Rosen, 2014; Case et al., 2019a; 
Huang et al., 2019; Su et al., 2017; Zeng et al., 2018). pFGFR2Cyto 
alone was uniformly distributed, as observed by fluorescence mi-
croscopy (Figure 3Aii), and freely diffusing on the bilayers, as re-
vealed by FRAP analysis (Figure S3A). To initiate the condensate 
formation, we added labeled, untagged SHP2C459S and incubated 
for 1 min. After buffer exchange to remove the excess protein, sub-
micron-sized condensates were observed (Figure 3Aii). We added 
increasing concentrations of labeled, untagged pPLCγ1 (Figures 
3Aiii and 3Aiv). This led to the appearance of robust pFGFR2Cyto- 
SHP2C459S-pPLCγ1 clusters. Confocal imaging confirmed that 
pFGFR2Cyto, SHP2C459S, and pPLCγ1 colocalized within the 
clusters. The concentrations of the three proteins used were based on 
our phase diagrams derived in the context of 3D droplet formation; 
however, it should be noted that condensates do form at lower 
concentrations on the 2D membrane. FRAP analysis showed that 
all three proteins in the condensed clusters exhibited a slow 
dynamic exchange with their counterparts into and out of the con-
densates (Figure 3B). Unlike the typical liquid-like behavior seen in 
pFGFR2Cyto-SHP2C459S condensates that show more substantial 
fluorescence recovery, the addition of pPLCγ1 may drive the 
condensate properties toward more gel-like ones. This in vitro 
experiment indicated that both SHP2C459S and pPLCγ1 can be re-
cruited into membrane-bound pFGFR2 and form phase-sepa-
rated clusters.

Endogenous SHP2 and PLCγ1 form discrete membrane-
bound puncta in cells 

Next, we tested whether the formation of membrane-bound, 
phase-separated FGFR2-SHP2-PLCγ1 complex can occur with 
endogenous protein expression in cells. We used the human 
colorectal adenocarcinoma cell line Caco-2, which expresses all 
three proteins of interest. First, we confirmed that both endoge-
nous SHP2 and PLCγ1 colocalized to punctate structures at the 
plasma membrane upon FG9 stimulation by immunofluores-
cent (IF) staining (Figure 3C). Due to the lack of specific anti-
bodies against FGFR2, we were unable to directly demonstrate 
the formation of endogenous FGFR2-SHP2-PLCγ1 puncta by 
IF. However, knocking down FGFR2 in Caco-2 cells (Caco-2 
FGFR2-ko) (Figure S3B) resulted in decreased colocalization of 
endogenous SHP2 and PLCγ1 membrane-bound puncta (Figure 
3C), indicating the involvement of FGFR2 in the formation of 
such puncta. The prevailing SHP2 and PLCγ1 puncta in the 
Caco-2 FGFR2-ko cells could be due to the incomplete knockdown 
or the expression of other membrane-bound RTKs, such as 
FGFR3, which is also highly endogenously expressed in Caco-2 
cells (Erdem et al., 2017).

The puncta of FGFR2-SHP2-PLCγ1 exhibited liquid-like 
features in cells 

We next assessed whether FGFR2-SHP2-PLCγ1 puncta display 
any liquid-like features in cells using live cell fluorescence 
microscopy. The results of this assessment suggest that the for-
mation of FGFR2-SHP2-PLCγ1 condensates in vitro is highly 
dependent on SHP2 binding to FGFR2. To test this, while 
ruled out the endogenous SHP2 forming puncta and affecting 
the imaging results, fluorescent protein-tagged FGFR2AVT, 
SHP2C459S, and PLCγ1 were expressed in HEK293T SHP2 KO 
cells (Figure S3B) and the average expression level of SHP2C459S 
was adjusted to approximately the endogenous SHP2 expres-
sion level (Figure S3C). Using live cell imaging with a plasma 
membrane marker, we observed ligand-dependent, mem-
brane-localized puncta formation between independently 
fluorescent protein-tagged FGFR2AVT, SHP2C459S, and PLCγ1 
(Figures 3Di and 3Dii). The expression of all three fluorescent 
protein tags alone does not promote the puncta formation (Fig-
ure 3Diii). The mobilization of the puncta containing the three 
proteins was visible on the plasma membrane (Video S1 of red 
box from Figure 3Dii). The formation of FGFR2AVT-EGFP or 
PLCγ1-EGFP droplets on the plasma membrane upon FG9 stimu-
lation, as well as their dynamic liquid-like fusion/fission 
behavior, was further supported by highly inclined and laminated 
optical sheet (HILO) imaging (Figures S3D and S3E; Videos S2– 
S4). This indicates that the FGFR2AVT-SHP2C459S-PLCγ1 puncta 
activate a dynamic liquid-like behavior. In the absence of 
SHP2C459S and PLCγ1, FGFR2AVT was still able to form puncta 
on the membrane; these are likely to include other cellular pro-
teins, such as SHC, as we present in Figure 1A (Figure 3Dii). 
By contrast, without FGFR2AVT expression and activation, 
the SHP2C459S nor PLCγ1 can independently form puncta on the 
membrane (Figures 3Dv and 3Dvi); both proteins are 
randomly diffused in the cytosol. Importantly, without SHP2C459S 
expression, the FGFR2AVT is not able to recruit and retain PLCγ1 
on the membrane, highlighting the role of SHP2 in controlling 
the membrane localization of PLCγ1 (Figure 3Dvii).

SH2 domain interactions mediate the formation of 
complexes 

We next examined the molecular features of the complex(es) 
formed by the three components of the droplets. First, we
Figure 3. The formation of LLPS pFGFR2-SHP2C459S-pPLCγ1 condensates on supported lipid bilayers and plasma membranes
(A) pFGFR2Cyto-SHP2C459S-pPLCγ1 condensates on supported lipid bilayers. (i) Confocal images of homogeneously distributed pFGFR2Cyto Atto-488 (20 μM, 6xHis tagged) on membrane bilayers, (ii) pFGFR2Cyto Atto-488 gradually clustered upon the addition of SHP2C459S Atto-594 (60 μM, untagged), and (iii) pPLCγ1 Atto-647 (6 μM, untagged), followed by (iv) additional 36 μM of untagged pPLCγ1 Atto-647. Scale bars, 10 μm.
(B) FRAP analysis showing the dynamic nature of pFGFR2Cyto-SHP2C459S-pPLCγ1 condensates on supported lipid bilayers as all pFGFR2Cyto, SHP2C459S, and pPLCγ1 exchanged with their counterparts in the dilute phase. Data are presented as mean ± SD, n = 2 experiments.
(C) Immunofluorescence staining images showing colocalized SHP2-Alexa 488 and PLCγ1-Alexa 647 droplet formation on plasma membrane in FGF9-stimulated (10 ng/ml, 15 min) Caco-2 cells and Caco-2 FGFR2i cells. Inset image: magnification of regions shown to exemplify endogenous SHP2-PLCγ1 clusters on membranes. Graph (right of image): statistical analysis of droplet formation in parental Caco-2 cells and Caco-2 FGFR2i cells. Only the SHP2-Alexa 488 and...

(legend continued on next page)
characterized the pairwise protein interactions involved in mediating the ternary complex formation. A direct interaction between FGFR2 and SHP2 has not been reported. To explore the nature of this, we used two constructs, GST-SHP2C459S and GST-SHP2C58, in a pull-down assay to precipitate FGFR2 proteins from the HEK293T cells stably expressing FGFR2ΔVT or an enzymatically disabled version of FGFR2 (FGFR2ΔVT-KD; double Y/F mutants on the activation loop Y656/Y657 render FGFR2 kinase dead, KD) (Figure 4A). Cells were either serum-starved or stimulated with the FGF9 ligand to activate receptors. Both SHP2C459S and SHP2C58 were able to bind FGFR2ΔVT, but not the unphosphorylatable KD mutant. Growth factor stimulation had a modest impact on the interactions observed (Figure 4A). This can be attributed to previously observed basal receptor phosphorylation (Ahmed et al., 2013; Lin et al., 2012) providing constitutive recruitment site(s) for the SH2 domain-containing SHP2 constructs (Figure 4A, input).

To identify the pY binding site on FGFR2 and the recognition site on SHP2, we recombinantly produced and phosphorylated three different regions of the FGFR2: the intact cytoplasmic domain (pFGFR2Cyto; residues 400–821, contains the juxtamembrane region, the kinase domain, and the C-terminal tail), the kinase domain (pFGFR2kinase; 464–763), and the C-terminal tail (pFGFR2CT; 764–821). Using a GST pull-down assay with a variant of the SHP2 domains, we demonstrated the direct interaction of pFGFR2Cyto and pFGFR2C58 with full-length wild-type SHP2 (SHP2) or SHP2C459S (Figure 4B). The decrease in complex formation seen using GST-SHP2 compared with GST-SHP2C459S is likely due to the depletion of pY binding sites by phosphatase activity toward FGFR2. No interaction could be seen using the isolated PTP domains (GST-SHP2PTP and GST-SHP2PTPC459S) or the SHP2 C-terminal tail (GST-SHP2C58) (Figure 4B). The tandem SHP2 SH2 domains (GST-SHP2C58) also interacted with the pFGFR2Cyto and pFGFR2C58 (Figure 4B). The larger SHP2C459S construct was more efficient in precipitating FGFR2 proteins than GST-SHP2C58. The pFGFR2Cyto interacts with SHP2, SHP2C459S, and SHP2C58 with an apparent low affinity. The interactions of both SHP2C459S and SHP2C58 with pFGFR2Cyto were confirmed by microscale thermophoresis (MST; Figure S4A; Table S1). Bio-layer interferometry (BLI) provided further evidence of the interaction between immobilized GST-SHP2C58 and pFGFR2Cyto (5 μM; Figure S4B).

Having demonstrated that the C-terminal 58 residues of the receptor were sufficient to bind to SHP2C58, a polypeptide containing these residues was used to investigate the interaction of pFGFR2 with SHP2C58 using HSQC nuclear magnetic resonance (NMR) spectroscopy. In this case, pFGFR2C58 was titrated into 15N-SHP2C58, and major changes in chemical shifts indicate the direct interaction (Figure S4C). Isothermal titration calorimetry (ITC) confirmed the binding and gave a stoichiometry of 1:1 for the complex (Kd = 8 μM; Figure S4D), indicating that only one SH2 domain of the tandem SH2 domains is able to bind to one pY in the C-terminal tail. To identify the pY residue(s) in GST-tagged FGFR2C58 responsible for the recruitment of SHP2C58, each tyrosine residue was individually replaced by a phenylalanine residue. We prepared both tyrosine phosphorylated FGFR2C58 (GST-pC58 and its single Y/F mutants) and unphosphorylated FGFR2C58 (GST-C58 and its single Y/F mutants) for pulling down SHP2C58. For the pFGFR2C58 mutants, only the Y769F mutant (GST-pC58Y769F) was unable to pull down SHP2C58, even with a background of other pY residues (Figure 4C), indicating that Y769 is the major phosphorylation site on the FGFR2 C-terminal tail and serves as the binding site for SHP2C58. This is the same pY residue that is required to recruit PLCγ1 to the receptor prior to the phosphorylation of the phospholipase. This previously unrecognized direct interaction involving pY769 was confirmed using a GST-SHP2C459S pull-down assay in the HEK293T cells transfected with FGFR2ΔVT or the FGFR2ΔVT-Y769F mutant (Figure 4D). As seen in Figure 4A, the FGFR2-SHP2 interaction can occur in the absence of growth factor, consistent with Y769 being highly phosphorylated even under basal conditions (Chen et al., 2009). This basal phosphorylation has been observed on equivalent tyrosine residues on other FGFRs (Huang et al., 2016; Kostas et al., 2018; Krick et al., 2018). Finally, for mutations that disrupt the binding of pY residues in the SH2 domains (R32A in the NSH2 and R138A in CSH2), we identified that the CSH2 domain is required for the interaction with the receptor (Figures S4E and S4F); the NSH2 domain interacts with pFGFR2 with a low affinity. The recruitment of the CSH2 domain to pY769, therefore, leaves the NSH2 available for other pY-mediated interactions.

**Direct binding of SHP2 and PLCγ1 is mediated by their tandem SH2 domains in a phosphorylation-independent manner**

To characterize the interaction of SHP2 and PLCγ1, we first focused on the pY sites on PLCγ1 (Y774/Y777, Y775/Y782, Y784/Y787, Y788/Y791). Molecular Cell 82, 1089–1106, March 17, 2022 1095

---

**Figure 4**

**A** Live cell images showing FGFR2ΔVT-SHP2C459S-PLCγ1 LLPS droplet formation on plasma membrane upon FGFR2ΔVT expression and activation in HEK293T SHP2 KO cells. FGFR2ΔVT, SHP2C459S, and PLCγ1 were tagged with Neptune 2.5, mOrange, and mEGFP, respectively. Alexa 350-conjugated wheat germ agglutinin was used to stain the plasma membrane. (i) Serum-starved (-FGF9) cells show a low level of FGFR2ΔVT expression and activation in HEK293T SHP2 KO cells. FGFR2ΔVT, SHP2C459S, and PLCγ1 were tagged with Neptune 2.5, mOrange, and mEGFP, respectively. Alexa 350-conjugated wheat germ agglutinin was used to stain the plasma membrane. (ii) Serum-starved (-FGF9) cells show a low level of FGFR2ΔVT expression and activation in HEK293T SHP2 KO cells. FGFR2ΔVT, SHP2C459S, and PLCγ1 were tagged with Neptune 2.5, mOrange, and mEGFP, respectively. Alexa 350-conjugated wheat germ agglutinin was used to stain the plasma membrane. (iii) Expression of fluorescent tags alone does not initiate the droplet formation. (iv) In the absence of SHP2C459S, mOrange and PLCγ1-mEGFP, activated FGFR2ΔVT still forms droplets on the membrane with other endogenous cellular proteins. (v) SHP2C459S-mOrange does not form droplets in the absence of FGFR2ΔVT. (vi) PLCγ1-mEGFP does not form droplets in the absence of FGFR2ΔVT. (vii) FGFR2ΔVT cannot recruit active PLCγ1-mEGFP to the membrane in the absence of SHP2 expression, resulting in the random diffusion of PLCγ1-mEGFP. (b) Statistical analysis of FGFR2ΔVT-SHP2C459S-PLCγ1 LLPS droplet formation in the absence (light peach) or presence (dark peach) of FG9 stimulation. Sample numbers = 40 per condition from 3 independent experiments. Data are presented as mean ± SD.
Figure 4. Characterization of the interactions between FGFR2\textsubscript{VT}-SHP2\textsubscript{C459S} droplets
(A) (Left) Pull-down experiments using GST-SHP2\textsubscript{C459S} or GST-SHP2\textsubscript{2SH2} (see schematic in Figure 1A) show that the binding of SHP2 requires phosphorylation of FGFR2\textsubscript{VT}. FGFR2\textsubscript{VT} or FGFR2\textsubscript{VT-KD} (double mutant Y656/657F) stably expressing HEK293T cells were unstimulated or FGF9-stimulated (10 ng/ml, 15 min). Arrows highlight GST fusion as part of the SHP2 constructs. The lower level of interaction without FGF9 stimulation due to protein recruitment by the basally activated FGFR2 as shown in the pFGFR2 blot (Input). (Right) Densitometry analysis of GST pull down, n = 3. Data are presented as mean ± SD. Replicate data are shown in Data S1A.

(legend continued on next page)
and Y771, Y775, and Y783 (schematic, Figure 1A). This polypeptide permits the added assessment of the impact of the three C-terminal tyrosine residues, pY783, we used an extended tandem SH2 construct (residues 545–791) that includes three C-terminal tyrosine residues, namely Y771, Y775, and Y783 (schematic, Figure 1A). This polypeptide permits the added assessment of the impact of the phosphorylation state of Y783 on complex formation. Moderate affinity binding was shown to occur between the tandem SH2 domains of SHP2 and PLCγ1 (SHP2SH2–PLCγ1SH2; $K_{d}\sim 1.16 \pm 0.09 \mu M$) and also when PLCγ1 is phosphorylated (SHP2SH2–pPLCγ1SH2; $K_{d}\sim 0.48 \pm 0.04 \mu M$; Figure S5C; Table S1). This interaction is independent of the pY residues that are normally required to bind to SH2 domains because the SHP2 with pY recognition site mutants SHP2SH2R3213A81 retains the ability to interact with pPLCγ1SH2 ($K_{d}\sim 0.48 \pm 0.03 \mu M$; Figure S5D; Table S1).

Comparison of the NMR spectra of the isolated $^{15N}$-PLCγ1SH2 with those of the $^{15N}$-PLCγ1SH2–SHP2SH2 complex showed mostly minor, widely distributed changes (Figures S5E–S5G), consistent with small structural/dynamic changes. However, a limited number of residues showed pronounced chemical shift perturbations (CSPs) indicative of a specific binding event (Figure S5A). The mapping of the CSPs onto the structure of PLCγ1SH2 revealed that they localized to a potential binding region involving both SH2 domains (Figure 5B). The binding of SHP2 to this site would occlude the N-terminal pY binding pocket abrogating the binding of PLCγ1 to pY769 on FGFR2, thereby removing competition for this site with SHP2. The CSPs of residues connecting the CSH2 domain with the Y783 peptide region on PLCγ1 are known to be sensitive to any direct or allosteric interference because of the fast exchange equilibrium between bound and unbound states (Koss et al., 2018). The absence of any corresponding CSPs indicates that the previously observed intramolecular interactions of the CSH2 domain on PLCγ1 with the pY783 binding pocket remain largely unperturbed by SHP2 binding and, hence, can preserve the active state of the phospholipase (Figure S5H; Table S1). These data lead to the assumption that when both proteins are present, only SHP2 can engage the receptor.

Next, NMR was used to map the pPLCγ1SH2 binding interface on $^{15N}$-SHP2SH2; similar to the PLCγ1SH2 example, the $^{15N}$-SHP2SH2–pPLCγ1SH2 complex showed mostly minor, widely distributed changes (Figures 5C and 5D). The specific binding event is indicated by a limited number of residues that showed CSPs (F41, F71, E83, H84, S165, V170, and L212; Figures S5I and S5J). The binding of pPLCγ1SH2 appears to have no effect on either SHP2SH2 pY binding pockets that are capable of binding to pFGFR2 (i.e., CSH2 binding to pY769 and NSH2 binding to other potential pY sites, e.g., on the kinase domain).

**FGFR2, SHP2, and PLCγ1 form a stable ternary complex in the LLPS state**

The direct pairwise interactions between pFGFR2/SHP2 and SHP2/pPLCγ1 maintained through mutually exclusive interfaces suggests the formation of a ternary complex, which we postulate is the minimal complex required for the droplet formation involving the three interacting components. To investigate this, we conjugated FGFR2cyto or pFGFR2cyto on agarose beads and performed pull-down experiments using recombinant SHP2SH2, PLCγ1SH2, and pPLCγ1SH2. Unphosphorylated FGFR2cyto failed to recruit any protein as the pairwise interactions are pY-Sh2 domain-dependent (Figure 5E, lanes 4, 5, and 6). Consistent with the reported data (Bunney et al., 2012; Gresset et al., 2010), we observed an initial binding between Pgfgr2cyto and PLCγ1SH2 (Figure 5E, lane 8) that declined as the phospholipase was phosphorylated by the receptor in the presence of ATP/MgCl2 (Figure 5E, lane 11). We also observed SHP2SH2 binding to Pfgfr2cyto (Figure 5E, lane 7). The addition of ATP/MgCl2 to the pFGFR2cyto and SHP2SH2 mixture does not affect the binding (Figure 5E, lane 10). Importantly, when SHP2SH2 and PLCγ1SH2 were mixed with pFGFR2, both were found to bind the receptor concurrently (Figure 5E, lane 9), possibly due to the competition between SHP2SH2 and PLCγ1SH2 bindings to pY769 on pFGFR2cyto and/or the recruitment of a complex including both SHP2SH2 and PLCγ1SH2 into the receptor. In the presence of ATP/MgCl2, SHP2SH2 and the phosphorylated pPLCγ1SH2 can be concomitantly precipitated by pFGFR2cyto (Figure 5E, lane 12). Because pPLCγ1SH2 does not bind to pFGFR2, our data reveal that the phospholipase must be recruited into the receptor via SHP2SH2, thus inferring the existence of a ternary complex including pFGFR2, SHP2, and pPLCγ1.

The mechanism of formation of the ternary complex was elucidated by reconstituting the interactions on a BLI sensor with immobilized GST-pFGFR2cyto (Figure 5F, upper panel). To observe binding, the concentrations of the added proteins were typically
Figure 5. Interactions between SHP2 and PLCγ1 droplets and the formation of ternary complexes

(A) Plot of the chemical shift changes (ppm) of the backbone amide peaks of ¹H, ¹⁵N-labeled PLCγ1SH2 (200 μM) upon the addition of 3 mol L⁻¹ equivalent of SHP2SH2. The residue numbers are indicated on the x axis.

(B) CSP of residues mapped on to the crystal structure of the PLCγ1SH2 (PDB code: 4FBN). The gradient indicates the strength of the perturbation. The pY binding pockets for NSH2 and CSH2 are shown in cyan (R562, R586, S588, E589, T590, and T596) and green (R675, R694, R696 and A703), respectively. Left hand image shows putative binding region (highlighted by increasing CSP). Right hand image shows the structure rotated into plane by 180° to show the comparatively negligible CSP on the ‘non-binding’ surface.

(C) Plot of the chemical shift changes (ppm) of the backbone amide peaks of ¹H, ¹⁵N-labeled SHP2SH2 (100 μM) upon the addition of 6 mol L⁻¹ equivalent of PLCγ1SH2. The residue numbers are indicated on the x axis.

(legend continued on next page)
SHP2 tandem SH2 domains drive FGFR2-SHP2-PLCγ1 LLPS

Having identified the interactions that sustain the ternary complex, we sought to reconstitute the features of LLPS using full-length proteins, submicrometer-sized droplets were formed upon the addition of pPLCγ1 tandem SH2 domains that represent the minimal binding regions of the intact proteins. As with the intact proteins, SHP2 tandem SH2 domains were capable of driving LLPS with pFGFR2 and pPLCγ1 (see STAR Methods).

Figure 6Ai): the optimum concentrations are revealed by a phase diagram (Figure 6Ai), and the LLPS property was confirmed by FRAP (Figure 6Bi). As the pY769 of FGFR2 provides the binding site for the CSH2 domain of SHP2, the addition of a pY769-mimicking peptide, which inhibits SHP2 binding, resulted in a decrease in droplet number and size in pFGFR2Cyto–SHP2 LLPS condensates (Figures 6Aii, 6Aiii, and S6C). This is mirrored in condensates formed using full-length SHP2 (Figure S6D), thus confirming that the pY-SHP2 domain interaction(s) helps to sustain the LLPS. Finally, we used the pY-binding incompetent SH2 domain mutations in pPLCγ1 tandem SH2 domains to show that the blocking of the interaction of the CSH2 domain with pY769 on the C-terminal tail of pFGFR2Cyto was able to abrogate LLPS (pPLCγ1 R32A, pSH2 R138A, and SHP2 R32A/138A) (Figure 6B). Importantly, SHP2 tandem SH2 domains also abrogated phase separation, suggesting that an NSH2 domain-mediated, nonspecific, weak, pY-dependent interaction(s) is (are) necessary for phase separation. Thus, our combined binding data reveal multiple potential interactions between SHP2 and pFGFR2; i.e., SHC2 domain interacts with the phosphorylated FGFR2 C-terminal tail (Figure S4D), and also weak interactions between NSH2 from the SHP2 (SH2 R138A) and FGFR2 were observed (Figures S4E and S4F). The kinase domain of FGFR2 has six available tyrosine residues for phosphorylation. MST assays confirmed that SHP2 tandem SH2 domains can interact with any of these six pY residues (Figure S6E; Table S1). As a result, the interactions of SHP2 SH2 domains with pFGFR2Cyto can sustain the multivalent features of phase-separated molecules.

The results presented here provide a mechanistic model for how FGFR2-SHP2-PLCγ1 ternary complexes drive LLPS.
Figure 6. Characterization of the ternary complex formation

(A) (i) In vitro phase separation assay using Atto-labeled pFGFR2Cyto (10 μM) and truncated SHP2SH2 (30 μM). The addition of a pY769 peptide (ii) or a general pY peptide (ii) to compete SH2 domain binding reduces droplet formation. Scale bars, 10 μm.

(B) R to A mutation of residues 32 or/and 138 in the pY binding sites show that both wild-type SH2 domains of SHP2 are required (30 μM of each mutant) for LLPS with pFGFR2Cyto (10 μM). (i) Wild-type SHP2SH2. (ii) SHP2SH2 R32A. (iii) SHP2SH2 R138A. (iv) SHP2SH2 R32/138A. Scale bars, 10 μm.

(C) In vitro phase separation assay using Atto-labeled pFGFR2Cyto (10 μM), SHP2SH2 (30 μM), and pPLCγ1SH2 (12 μM). (i) Individual proteins showed no evidence of droplet formation. Droplet formation was observed after 1 min: (ii) with all three proteins; (iii) with pFGFR2Cyto and SHP2SH2, not with pFGFR2Cyto with...
two molecules to interact (Figure 6Civ). The addition of salt or 10% 1,6 hexanediol supported LLPS characteristics by the shrinkage of droplet size (Figures 6Cvi and 6vii). The observation of droplet fusion underscored the existence of pFGFR2Cyto-SHP2C459S-pPLCγ12SH2 in an LLPS state (Figure 6F).

The LLPS affects enzyme activities in the ternary complex

To demonstrate a potential functional outcome of LLPS formation in the context of FGFR2 signaling, we employed in vitro enzymatic assays. Increased tyrosine kinase activity was demonstrated through mixing an excess of the inactive K517I-mutated pFGFR2Cyto as the substrate (pFGFR2Cyto K517I; Figure 6D, lane 1), with an LLPS containing the unlabeled pFGFR2Cyto-SHP2C459S-pPLCγ1 complex in the presence of ATP/MgCl₂. The formation of LLPS droplets was confirmed using a light microscope (Figure 6G). The active pFGFR2Cyto in the solution efficiently phosphorylated the pFGFR2Cyto K517I substrate (Figure 6D, third panel, lane 3). Phosphorylation activity appears to be more efficient in the presence of all three components of the ternary complex in the droplet environment (Figure 6D, third panel, lane 5). The dissolution of the droplet by addition of 10% 1,6-hexanediol led to a reduction in kinase activity (Figure 6D, lane 9). Note that hexanediol does not affect the kinase activity of FGFR2 in this system (Figures 6D, lane 7 and 6H).

Replacing SHP2C459S with wild-type SHP2, we were also able to show that within the context of the pFGFR2Cyto-SHP2-pPLCγ1 LLPS, the phosphatase activity of wild-type SHP2 towards a GST-tagged phosphopeptide substrate was lowered (Figure 6E, lanes 1, 2, and 4). Again, the dissolution of the droplet by hexanediol increases the efficiency of phosphatase activity (Figure 6E lane 5) without itself affecting interactions of the phosphatase within the LLPS (Figures 6E, lane 3 and 6F). Moreover, condensed droplet formation may help to inhibit the effects of non-specific phosphatases (alkaline phosphatase, cisternal [CIP]) on the activated receptor and pPLCγ1 (Figure 6F).

Finally, using an artificial substrate 4-methylumbelliferyl myo-inositol-1-phosphate, N-methyl-morpholine salt in an in vitro assay (White et al., 2014), the lipolytic activity of pPLCγ1 was shown to be greatly enhanced when it is in the phase-separated pFGFR2Cyto-SHP2C459S-pPLCγ1 complex (Figures 6G and 6J). Thus, within the LLPS state, the functional output of the ternary complex is enhanced through increased kinase and phosphatase activities and the downregulation of the phosphatase activity.

SHP2-mediated assembly of the ternary complex on the membrane provides a scaffold for PLCγ1 downstream signaling

To confirm that our in vitro functional studies extend to the in cellulo context, we stably knocked out the SHP2 expression in MCF7 cells (MCF7 SHP2 KO) (Figure 7A) or the SHP2 expression in A431 cells (A431 SHP2i) (Figure 7A) and Caco-2 cells (Caco-2 SHP2i) (Figure 7A). All three cell lines endogenously express FGFR2. In the absence of SHP2, FGF9-stimulated cells displayed impaired dephosphorylation of PLCγ1 (Figures 7A and 7A). Although the knockdown of SHP2 should result in prolonged activation of the receptor, we observed that when the phospholipase is unable to bind to SHP2, phosphorylation of the PLCγ1 downstream effectors PKCζIII and AKT is significantly reduced in all cell lines (Figures 7A and 7A). The downregulation of PKCζII, a downstream protein phosphorylated on serine 660 (pS660) in response to diacyl glycerol production through the turnover ofPIP2 by PLCγ1, suggests that PLCγ1 function is compromised; i.e., despite being in an activated state, PLCγ1 is unable to access its membrane-localized substrate in the absence of the scaffolding function of the FGFR2-SHP2 complex. The decoupling of the phosphatase activity of SHP2 and PLCγ1-mediated signaling is further exemplified with the addition of the SHP2 inhibitor NSC87877 (50 μM) to A431 cells (Figure 7B). Here, the level of phosphorylated PKCζII remained unaffected by the inhibition of SHP2, underscoring the idea that it is the presence, rather than the activity of SHP2, that is required for PLCγ1 downstream signaling.

Further corroboration of this scaffolding role for SHP2 was shown by the depletion of SHP2 in MCF7 SHP2 KO cells and

---

**pPLCγ12SH2 and (iv) with SHP2C459S with pPLCγ12SH2. (v) Droplet size was diminished with increasing concentration of NaCl after incubation for 1 min and (vi) (250 mM compared with 150 mM in (i)) in the presence of 10% 1, 6-hexanediol for 1 min (viii). Scale bars, 10 μm.

(D) (Top panel) An excess of inactive FGFR2Cyto-K517I (FGFR2Cyto K517I [200 μM] pFGFR2Cyto [10 μM] = 2001) was used as the substrate to monitor kinase activity. 500 μM of ATP/MgCl₂ was added and incubated at room temperature for 15 min. In the context of phase-separated droplets (by adding SHP2C459S [60 μM] and pPLCγ1 [12 μM]; lane 5), the kinase activity of pFGFR2Cyto was enhanced (compare lanes 3 and 5). The addition of 10% 1,6-hexanediol (lane 9) results in the reduction of kinase activity by dissolving the phase-separated droplets (compare lane 5 and lane 9). (Bottom panel) Densitometry analysis of kinase activity, n = 5.

Data are presented as mean ± SD. Representative data are shown in Data S2C.

(E) (Top panel) A synthesized GST-phospho-substrate (600 μM, lane 1) was used to measure SHP2 (60 μM) activity in the context of phase-separated droplets. Upon the addition of FGFR2Cyto and pPLCγ1 (10 μM and 12 μM, respectively) and incubation for 15 min, the SHP2 activity is reduced in the droplets compared with the isolated phosphatase (compare lane 2 and lane 4). The addition of 10% 1,6-hexanediol (lane 5) results in the upregulation of activity by dissolving the droplets (compare lane 4 and lane 5). (Bottom panel) Densitometry analysis of phosphatase activity, n = 2. Data are presented as mean ± SD. Representative data are shown in Data S2C.

(F) Confocal images of the effect of external phosphatase CIP (10 μM) on pFGFR2Cyto-SHP2C459S-pPLCγ1 LLPS formation. Top panel: droplet formation without CIP. Middle panel: the addition of CIP to droplet formation has limited effect on the dephosphorylation of proteins, hence the droplets are still present. Lower panel: the addition of CIP to pFGFR2Cyto before droplet formation (before the addition of SHP2C459S and pPLCγ1) efficiently dephosphorylates pFGFR2Cyto; therefore, no droplet can form at the unphosphorylated state.

Western blots (below left) confirmed that pFGFR2Cyto-SHP2C459S-pPLCγ1 (10 μM, 60 μM, and 12 μM, respectively) droplet formation prevents the dephosphorylation of pPLCγ1 and pFGFR2Cyto by 10 μM of CIP (exposure 0.5 h). (Below right) Densitometry analysis of the phosphorylation state of pPLCγ1 (salmon) and pFGFR2Cyto (dark green), n = 2. Data are presented as mean ± SD. Representative data are shown in Data S2D.

(G) The lipase activity of PLCγ1 (50 μM) was dramatically enhanced in the phase-separated environment (by adding pFGFR2Cyto [100 μM] and SHP2C459S [60 μM]; green curve) compared with PLCγ1 alone (magenta curve). Sample sizes n = 4. Data are presented as mean ± SD.
Figure 7. Phase transition of FGFR2-SHP2-PLCγ1 upregulates downstream signaling

(A) (Left) Depletion of SHP2 upregulates PLCγ1 through phosphorylation of Y783 but downregulates its downstream effectors (shown by reduced phosphorylation of PKCβII-S660 and AKT-S473) in FGF9-stimulated (10 ng/ml) MCF7 cells and A431 cells. (Right) Densitometry analysis of SHP2 expression and the activation levels of various signaling proteins (dark green: parental cells; light green: SHP2 depletion cells). MCF7 cells: n = 3; A431 cells: n = 2. Data are presented as mean ± SD. Replicate data are shown in Data S3A.

(legend continued on next page)
A431 SHP2i cells, which led to depressed Ca\textsuperscript{2+} concentrations as a result of the downregulation of PLC\textsubscript{1} mediated signaling (Figure 7B). In agreement with this, we also observed a reduction in the functional output of cell motility in MCF7 SHP2 KO cells and A431 SHP2i cells (Figure 7C), consonant with the loss of PLC\textsubscript{1} function.

To confirm whether the SHP2 scaffold or enzymatic function is responsible for impaired PLC\textsubscript{1} signaling, we transiently transfected three different SHP2 constructs (SHP2, SHP2\textsubscript{C459S}, and SHP2\textsubscript{2SH2}) into A431 SHP2i cells. The expression of wild-type SHP2 in A431 SHP2i cells results in the rescue of PKC\textsubscript{II} activity as we expected; however, the expression of phosphatase-dead mutant (SHP2\textsubscript{C459S}) or just the tandem SH2 domains (SHP2\textsubscript{2SH2}) is also sufficient to restore the activity of PKC\textsubscript{II} (Figure 7D).

**DISCUSSION**

RTK function depends on the ability to recruit downstream, cytoplasmic signaling components to the plasma membrane. In the cytoplasm, proteins diffuse randomly to their receptor targets largely unaided energetically. Thus, it is expected that the propagation of a mutually exclusive signal needs to be supported by mechanisms that ensure timely delivery of the correct proteins to the RTK with greater reliability than the probability-dependent diffusion process.

Here, we report the formation of LLPS condensates sustained by phosphorylated RTKs upon the recruitment of the signaling protein SHP2 or SHC. We honed in on a functional plasma membrane-localized LLPS, the basic element of which is a ternary protein complex composed of active pFGFR2, SHP2, and pPLC\textsubscript{1} (Figures 7Fi, 7Fii, and 7Fiii). The assembly of the three proteins to form the condensate requires the phosphorylation-dependent interactions of FGFR2 and the tandem SH2 domains of SHP2, which provide multivalency and drive the higher-order FGFR2-SHP2 complex assembly. The FGFR2-SHP2 condensate further recruits activated PLC\textsubscript{1} through the tandem SH2 domain heterodimerization; this process ensures the membrane localization of the activated PLC\textsubscript{1} facilitating access to the phospholipid substrate.

A unique aspect of the RTK-LLPS complex observed in this work is that, unlike previously reported membraneless organelles or particles (Amaya et al., 2018; Brangwynne et al., 2009; Delarue et al., 2018; Sheu-Gruttadauria and MacRae, 2018; Shin et al., 2018), it is sustained by a plasma membrane-bound receptor. This membrane association limits the extent of the expansion of the droplet into the cytoplasm (Su et al., 2016; Case et al., 2019b), resembling a droplet of condensed protein.
The idea that LLPS can function as a switch for enzyme activity (Li et al., 2012) in RTK-mediated pathways is observed here in controlling RTK FGFR2 signaling. The restriction of SHP2 phosphatase and amplification of both FGFR2 and PLCγ1 activities drive PKCζ signaling with a potential cancer outcome (El-Gamal et al., 2014; Sledge and Gökmen-Polar, 2006; Teicher, 2019). Alongside the elevated apparent local concentrations associated with the condensed state, it is likely that within the highly charged, multivalent milieu of a globule, catalytically favorable conformations are more easily accessible. Indeed, LLPS-component mutant forms of SHP2 have been seen to show elevated activity in vitro based on conformational perturbation (Zhu et al., 2020).

**Limitations of the study**
The assembly of membraneless RTK-LLPS complexes may be a general phosphorylation- and concentration-dependent mechanism for activating RTKs and recruiting signal proteins. Additional work is needed to ascertain the biological roles of LLPS in RTK signaling, as well as to investigate its potential links to the manifold disease states associated with the perturbations of RTK function. It will also be of interest how the LLPS formation affects the efficacy of therapeutic agents that target RTKs or their effectors.

**STAR★METHODS**
Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **RESOURCE AVAILABILITY**
  - Lead contact
  - Materials availability
  - Data and code availability
- **EXPERIMENTAL MODEL AND SUBJECT DETAILS**
  - Mammalian cell culture
  - Escherichia coli strains
- **METHOD DETAILS**
  - Cloning, expression and purification of recombinant proteins
  - Cleavage of affinity tags
  - Plasmids transfection and viral infection
  - CRISPR knockdown/knockout
  - In vitro phosphorylation of purified proteins
  - Protein fluorescent labelling
  - Confocal microscopy for in vitro droplet formation
  - Supported lipid bilayer assay
  - Fluorescence recovery after photobleaching (FRAP) assay
  - Immunofluorescence
  - Plasma membrane staining and live cell imaging
  - Highly inclined and laminated optical sheet (HILO) microscopy
  - Pulldown and western blots
  - Bimolecular fluorescence complementation (BiFC)
  - Bio-layer interferometry (BLI)
  - Microscale thermophoresis (MST)
  - Isothermal titration calorimetry (ITC)
  - Nuclear magnetic resonance (NMR)
  - In vitro FGFR2 kinase assay
  - In vitro SHP2 phosphatase assay
  - In vitro phosphatase assay
  - Dephosphorylation by CIP
  - In vitro PLCγ1 activity assay
  - Wound-healing assay
  - Calcium concentration assay
  - Mathematical model
  - Reaction Figure:

**SUPPLEMENTAL INFORMATION**
Supplemental information can be found online at https://doi.org/10.1016/j.molcel.2022.02.005.

**ACKNOWLEDGMENTS**
We thank A. Zhuravleva, A. Kalverda (BioNMR Facility, University of Leeds), and M. Peckham (Bio-imaging Facility, University of Leeds). This work was funded by Cancer Research UK (grant C57233/A22356), MRC, UK (grant MR/K015613/1), and BBSRC, UK (grant BB/S015787/1).

**AUTHOR CONTRIBUTIONS**
C.-C.L., K.M.S., L.W., J.A.L., P.B., A.P.C., A.S., C.S., H.K., E.M., S.D.E., Z.A., and J.E.L. conceived and/or performed experiments and analyzed data. P.-A.J. and C.M.-P. performed the mathematical analysis. C.-C.L., K.M.S., and J.E.L. wrote the manuscript.

**DECLARATION OF INTERESTS**
The authors declare no competing interests.

Received: October 23, 2019  
Revised: November 2, 2021  
Accepted: February 1, 2022  
Published: February 28, 2022

**REFERENCES**
Agazie, Y.M., and Hayman, M.J. (2003). Development of an efficient “substrate-trapping” mutant of Src homology phosphotyrosine phosphatase 2 and identification of the epidermal growth factor receptor, Gab1, and three other proteins as target substrates. J. Biol. Chem. 278, 13952–13958.
Ahmed, Z., Lin, C.C., Suen, K.M., Melo, F.A., Levitt, J.A., Suhling, K., and Ladbury, J.E. (2013). Grb2 controls phosphorylation of FGFR2 by inhibiting receptor kinase and SHP2 phosphatase activity. J. Cell Biol. 200, 493–504.
Alberti, S., Gladfelter, A., and Mittag, T. (2019). Considerations and challenges in studying liquid-liquid phase separation and biomolecular condensates. Cell 176, 419–434.
Amaya, J., Ryan, V.H., and Fawzi, N.L. (2018). The SH3 domain of Fyn kinase interacts with and induces liquid–liquid phase separation of the low-complexity domain of hnRNP2. J. Biol. Chem. 293, 19522–19531.
Banani, S.F., Lee, H.O., Hyman, A.A., and Rosen, M.K. (2017). Biomolecular condensates: organizers of cellular biochemistry. Nat. Rev. Mol. Cell Biol. 18, 285–298.
Banjade, S., and Rosen, M.K. (2014). Phase transitions of multivalent proteins can promote clustering of membrane receptors. eLife 3, e04123.
Bracha, D., Walls, M.T., Wei, M.T., Zhu, L., Kurian, M., Avalos, J.L., Toettcher, J.E., and Brangwynne, C.P. (2018). Mapping local and global liquid phase behavior in living cells using photo-oligomerizable seeds. Cell 175, 1467–1480, e13.

Brangwynne, C.P., Eckmann, C.R., Courson, D.S., Rybarska, A., Hoege, C., Gharakhani, J., Jülicher, F., and Hyman, A.A. (2009). Germline P granules are liquid droplets that localize by controlled dissolution/condensation. Science 324, 1729–1732.

Bunney, T.D., Esposito, D., Mas-Droux, C., Lamber, E., Baxendale, R.W., Martins, M., Cole, A., Svergun, D., Driscoll, P.C., and Katan, M. (2012). Structural and functional integration of the PLCγ interaction domains critical for regulatory mechanisms and signaling deregulation. Structure 20, 2062–2075.

Burgar, H.R., Burns, H.D., Elsden, J.L., Lalioti, M.D., and Heath, J.K. (2002). Association of the signaling adaptor FRS2 with fibroblast growth factor receptor 1 (FGFR1) is mediated by alternative splicing of the juxtamembrane domain. J. Biol. Chem. 277, 4018–4023.

Case, L.B., Ditte, J.A., and Rosen, M.K. (2019a). Stoichiometry controls activity of phase-separated clusters of actin signaling proteins. Science 363, 1093–1097.

Cebeaucar, M., Spitaler, M., Sergé, A., and Magee, A.I. (2010). Signalling complexes and clusters: functional advantages and methodological hurdles. J. Cell Sci. 123, 309–320.

Chen, H., Libring, S., Rudderaraju, K.V., Miao, J., Solorio, L., Zhang, Z.Y., and Wendt, M.K. (2020). SHP2 is a multifunctional therapeutic target in drug resistant metastatic breast cancer. Oncogene 39, 7166–7180.

Chen, H., Xu, C.F., Ma, J., Eliseenkova, A.V., Li, W., Pollock, P.M., Petteloud, N., Miller, W.T., Neubert, T.A., and Mohammad, M. (2008). A crystallographic snapshot of tyrosine trans-phosphorylation in action. Proc. Natl. Acad. Sci. USA. 105, 19660–19665.

DeBell, K., Graham, L., Reischl, I., Serrano, C., Bonvini, E., and Rellahan, B. (2005). Structural basis for inhibition of the insulin receptor by the adaptor protein Grb14. Mol. Cell. Biol. 20, 325–333.

Edelstein, A., Armodil, N., Hoover, K., Vale, R., and Stuurman, N. (2010). Computer control of microscopes using μManager. Curr. Protoc. Mol. Biol. 92, 14–20.

El-Gamil, D., Williams, K., LaFollette, T.D., Cannon, M., Blachy, J.S., Zhong, Y., Voych, J.A., Williams, E., Awan, F.T., Jones, J., et al. (2014). PKC-β as a therapeutic target in CLL: PKC inhibitor AEB071 demonstrates preclinical activity in CLL. Blood 124, 1481–1491.

Erdem, Z.N., Schwarz, S., Drev, D., Heinze, C., Reti, A., Heffter, P., Hudec, X., Holzmann, K., Grafl-Kraupp, B., Berger, W., et al. (2017). Irinotecan upregulates fibroblast growth factor receptor 3 expression in colorectal cancer cells, which mitigates irinotecan-induced apoptosis. Transl. Oncol. 10, 332–339.

Evenias, J., Tugarinov, V., Skrynnikov, N.R., Goto, N.K., Muhandiram, R., and Kay, L.E. (2001). Ligand-induced structural changes to maltodextrin-binding protein as studied by solution NMR spectroscopy. J. Mol. Biol. 309, 961–974.

Fresse, S., Schubert, W.D., Findeis, A.C., Marquardt, T., Roske, Y.S., Stradal, T.E.B., and Heinz, D.W. (2006). The phosphorytrosine peptide binding specificity of Nck1 and Nck2 Src homology 2 domains. J. Biol. Chem. 281, 18236–18245.

Gresset, A., Hicks, S.N., Harden, T.K., and Sondek, J. (2010). Mechanism of phosphorylation-induced activation of phospholipase C-γ isozymes. J. Biol. Chem. 285, 35836–35847.

Hadar, Y.R., Kouhara, H., Lax, I., and Schlessinger, J. (1998). Binding of SHP2 tyrosine phosphatase to FRS2 is essential for fibroblast growth factor-induced PC12 cell differentiation. Mol. Cell. Biol. 18, 3966–3973.

Hu, J., Liu, J., Ghirlando, R., Saltel, A.R., and Hubbard, S.R. (2003). Structural basis for recruitment of the adaptor protein APS to the activated insulin receptor. Mol. Cell. 12, 1379–1389.

Huang, W.Y.C., Alvarez, S., Kondo, Y., Lee, Y.K., Chung, J.K., Lam, H.Y.M., Biswas, K.H., Kuriyan, J., and Groves, J.T. (2019). A molecular assembly phase transition and kinetic proofreading modulate Ras activation by SOS. Science 363, 1098–1103.

Huang, Z., Marsigilia, W.M., Basu Roy, U., Rahimi, N., Ilghari, D., Wang, H., Chen, H., Gai, W., Blais, S., Neubert, T.A., et al. (2016). Two FGF receptor kinase molecules act in concert to recruit and transphosphorylate phospholipase C-γ1. Mol. Cell. 61, 98–110.

Huculescu, R., Garcia-Pino, A., Buts, L., Lenaerts, T., and van Nuland, N. (2015). Stoichiometry insights into the intertwined dimer of lyn SH2. Protein Sci. 24, 1964–1978.

Hyman, A.A., Weber, C.A., and Jülicher, F. (2014). Liquid-liquid phase separation in biology. Annu. Rev. Cell Dev. Biol. 30, 39–58.

Kodama, Y., and Hu, C.D. (2012). Bimolecular fluorescence complementation (BiFC): a 5-year update and future perspectives. Biotechniques 53, 285–298.

Koss, H., Bunney, T.D., Esposito, D., Martins, M., Katan, M., and Driscoll, P.C. (2018). Dynamic allostery in PLCγ1 and its modulation by a cancer mutation revealed by MD simulation and NMR. Biophys. J. 115, 31–45.

Kostas, M., Haugsten, E.M., Zhen, Y., Sörensen, V., Szymbowska, P., Fiorito, E., Lorenz, S., Jones, N., de Souza, G.A., Wedeloch, A., and Wesche, J. (2018). Protein tyrosine phosphatase receptor type G (PTPRG) controls fibroblast growth factor receptor (FGFR) 1 activity and influences sensitivity to FGFR kinase inhibitors. Mol. Cell. Proteomics 17, 850–870.

Kruck, S., Helton, E.S., Hutcheson, S.B., Blumhoff, S., Garth, J.M., Denson, R.S., Zaharias, R.S., Wickham, H., and Barnes, J.W. (2018). FGF23 induction of O-linked N-acetylgalosaminyl regulates IL-6 secretion in human bronchial epithelial cells. Front. Endocrinol. 9, 708.

Lee, C.R., Park, Y.H., Min, H., Kim, Y.R., and Seok, Y.J. (2019). Determination of protein phosphorylation by polyacylamide gelelectrophoresis. J. Microbiol. 57, 93–100.

Lescop, E., Schanda, P., and Brutscher, B. (2007). A set of BEST triple-resonance experiments for time-optimized protein resonance assignment. J. Magn. Reson. 187, 163–169.

Li, P., Banjade, S., Cheng, H.C., Kim, S., Chen, B., Guo, L., Llaguno, M., Hollingsworth, J.V., King, D.S., Banani, S.F., et al. (2012). Phase transitions in the assembly of multivalent signalling proteins. Nature 483, 336–340.

Lin, C.C., Melo, F.A., Ghosh, R., Suen, K.M., Stagg, L.J., Kirkpatrick, J., Arold, S.T., Ahmed, Z., and Ladbury, J.E. (2012). Inhibition of basal FGF receptor phosphorylation by dimeric Grb2. Cell 149, 1514–1524.

Maciejewski, M.W., Schuyler, A.D., Gryn, M.R., Moraru, I., Romero, P.R., Ulrich, E.L., Eghbalnia, H.R., Livny, M., Delaglio, F., and Hoch, J.C. (2017). NMRFox: a resource for biomolecular NMR computation. Biophys. J. 112, 1529–1534.

Ong, S.H., Guy, G.R., Hadari, Y.R., Laks, S., Gotoh, N., Schlessinger, J., and Lax, I. (2000). FRS2 proteins recruit intracellular signaling pathways by binding to diverse targets on fibroblast growth factor and nerve growth factor receptors. Mol. Cell. Biol. 20, 979–989.

Ong, S.H., Lim, Y.P., Low, B.C., and Guy, G.R. (1997). SHP2 associates directly with tyrosine phosphorylated p90 (SNT) protein in FGF-stimulated cells. Biochem. Biophys. Res. Commun. 238, 261–266.

Purvashin, K., Riek, R., Wider, G., and Wüthrich, K. (1997). Attenuated T2 relaxation by mutual cancellation of dipole-dipole coupling and chemical shift
anisotropy indicates an avenue to NMR structures of very large biological macromolecules in solution. Proc. Natl. Acad. Sci. USA. 94, 12366–12371.

Poulin, B., Sekiya, F., and Rhee, S.G. (2005). Intramolecular interaction between phosphorylated tyrosine-783 and the C-terminal Src homology 2 domain activates phospholipase C-γ1. Proc. Natl. Acad. Sci. USA. 102, 4276–4281.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., et al. (2012). Fiji: an open-source platform for biological-image analysis. Nat. Methods 9, 676–682.

Schulte-Herbrüggen, T., and Sørensen, O.W. (2000). Clean TROSY: compensation for relaxation-induced artifacts. J. Magn. Reson. 144, 123–128.

Sheu-Gruttadauria, J., and MacRae, I.J. (2018). Phase transitions in the assembly and function of human miRISC. Cell 173, 946–957, e16.

Shin, Y., Chang, Y.-C., Lee, D.S.W., Berry, J., Sanders, D.W., Ronceray, P., Wingreen, N.S., Haataja, M., and Brangwynne, C.P. (2018). Liquid nuclear condensates mechanically sense and restructure the genome. Cell 175, 1481–1491, e13.

Skinner, S.P., Fogh, R.H., Boucher, W., Ragan, T.J., Mureddu, L.G., and Vuister, G.W. (2016). CcpNmr AnalysisAssign: a flexible platform for integrated NMR analysis. J. Biomol. NMR 66, 111–124.

Sledge, G.W., Jr., and Gökmen-Polar, Y. (2006). Protein kinase C-beta as a therapeutic target in breast cancer. Semin. Oncol. 33 (Supplement 9), S15–S18.

Stein, E.G., Ghirlando, R., and Hubbard, S.R. (2003). Structural basis for dimerization of the Grb10 Src homology 2 domain. Implications for ligand specificity. J. Biol. Chem. 278, 13257–13264.

Su, X., Dittey, J.A., Hui, E., Xing, W., Banjade, S., Okrut, J., King, D.S., Taunton, J., Rosen, M.K., and Vale, R.D. (2016). Phase separation of signaling molecules promotes T cell receptor signal transduction. Science 352, 595–599.

Su, X., Dittey, J.A., Rosen, M.K., and Vale, R.D. (2017). Reconstitution of TCR signaling using supported lipid bilayers. Methods Mol. Biol. 1584, 65–76.

Suen, K.M., Lin, C.C., George, R., Melo, F.A., Biggs, E.R., Ahmed, Z., Drake, M.N., Arur, S., Arolfo, S.T., and Ladbury, J.E. (2013). Interaction with Shc prevents aberrant Erk activation in the absence of extracellular stimuli. Nat. Struct. Mol. Biol. 20, 620–627.

Teicher, B.A. (2006). Protein kinase C as a therapeutic target. Clin. Cancer Res. 12, 5336–5345.

Tokunaga, M., Imamoto, N., and Sakata-Sogawa, K. (2008). Highly inclined thin illumination enables clear single-molecule imaging in cells. Nat. Methods 5, 159–161.

Tulpule, A., Guan, J., Neel, D.S., Allegakoen, H.R., Lin, Y.P., Brown, D., Chou, Y.T., Heslin, A., Chatterjee, N., Perati, S., et al. (2021). Kinase-mediated RAS signaling via membraneless cytoplasmic protein granules. Cell 184, 2649–2664, e18.

Vranken, W.F., Boucher, W., Stevens, T.J., Fogh, R.H., Pajon, A., Liinas, M., Ulrich, E.L., Markley, J.L., Ionides, J., and Laue, E.D. (2005). The CCPN data model for NMR spectroscopy: development of a software pipeline. Proteins 59, 435–439.

Wang, Z., Ma, J., Miyoshi, C., Li, Y., Sato, M., Ogawa, Y., Lou, T., Ma, C., Gao, X., Lee, C., et al. (2018). Quantitative phosphoproteomic analysis of the molecular substrates of sleep need. Nature 558, 435–439.

Wheeler, R.J., Lee, H., Poser, I., Pal, A., Doelemans, T., Kishigami, S., Kour, S., Anderson, E.N., Marrone, L., Murthy, A.C., and Jahn, M. (2019). Small molecules for modulating protein driven liquid-liquid phase separation in treating neurodegenerative disease. Preprint at bioRxiv. https://doi.org/10.1101/721001.

White, M.J., Boyd, J.M., Horwill, A.R., and Nauseef, W.M. (2014). Phosphatidylinositol-specific phospholipase C contributes to survival of Staphylococcus aureus USA300 in human blood and neutrophils. Infect. Immun. 82, 1559–1571.

Zeng, M., Chen, X., Guan, D., Xu, J., Wu, H., Tong, P., and Zhang, M. (2018). Reconstituted postsynaptic density as a molecular platform for understanding synapse formation and plasticity. Cell 174, 1172–1187, e16.

Zhang, G., Wang, Z., Du, Z., and Zhang, H. (2018). mTOR regulates phase separation of PGL granules to modulate their autophagic degradation. Cell 174, 1492–1506, e22.

Zhu, G., Xie, J., Kong, W., Xie, J., Li, Y., Du, L., Zheng, Q., Sun, L., Guan, M., Li, H., et al. (2020). Phase separation of disease-associated SHP2 mutants underlies MAPK hyperactivation. Cell 183, 490–502, e18.
### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Anti-FGFR Rabbit Polyclonal | Santa Cruz Biotechnology | Cat#: sc-122; RRID:AB_631509 |
| Anti-Phospho-FGF Receptor (Tyr653/654) Rabbit Polyclonal | Cell Signaling Technology | Cat#: 3471; RRID:AB_331072 |
| Anti-SHP2 Mouse Monoclonal | Santa Cruz Biotechnology | Cat#: 271053; RRID:AB_10612217 |
| Anti-SHP2 Rabbit Polyclonal | Sigma-Aldrich | Cat#: SAB1300500; RRID:AB_10610174 |
| Anti-SHP2 Goat Polyclonal, knockdown validated | Invitrogen | Cat#: PA5-17956; RRID:AB_10984971 |
| Anti-Phospho-p44/42 MAPK (Erk1/2) Rabbit Polyclonal | Cell Signaling Technology | Cat#: 9101; RRID:AB_331646 |
| Anti-p44/42 MAPK (Erk1/2) Rabbit Monoclonal | Cell Signaling Technology | Cat#: 4695; RRID:AB_390779 |
| Anti-PLCγ1 Rabbit Polyclonal | Santa Cruz Biotechnology | Cat#: SC-81; RRID:AB_632202 |
| Anti-PLCγ1 Rabbit Polyclonal - KO Validated | Abcam | Cat#: ab107455; RRID:AB_11156766 |
| Anti-Phospho-PLCγ1 (Tyr783) Rabbit Polyclonal | Cell Signaling Technology | Cat#: 2821; RRID:AB_330855 |
| Anti-PLCγ1 Rabbit Polyclonal, KO validated | Cell Signaling Technology | Cat#: 2822; RRID:AB_2163702 |
| Anti-Phospho-PKC (pan) (III Ser660) Rabbit Polyclonal | Cell Signaling Technology | Cat#: 9371; RRID:AB_2168219 |
| Anti-PKC (pan) Mouse Monoclonal | Santa Cruz Biotechnology | Cat#: SC-13149; RRID:AB_628144 |
| Anti-Phospho-Akt (Ser473) Rabbit Monoclonal | Cell Signaling Technology | Cat#: 3787; RRID:AB_331170 |
| Anti-Akt Rabbit Polyclonal | Cell Signaling Technology | Cat#: 9272; RRID:AB_329827 |
| Anti-α-Tubulin Rabbit Monoclonal | Cell Signaling Technology | Cat#: 2125; RRID:AB_2619646 |
| Anti-GST Rabbit Polyclonal | Cell Signaling Technology | Cat#: 2622; RRID:AB_331670 |
| Anti-p-Tyr Mouse Monoclonal | Santa Cruz Biotechnology | Cat#: 7020; RRID:AB_628123 |
| Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor Plus 488 | Invitrogen | Cat#: A2731; RRID:AB_2653280 |
| Donkey anti-Goat IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor Plus 594 | Invitrogen | Cat#: A2758; RRID:AB_2762828 |
| **Bacterial and virus strains** | | |
| BL21 (DE3) | New England Biolabs | Cat#: C2527H |
| **Chemicals, peptides, and recombinant proteins** | | |
| Recombinant Human FGF-9 Protein, CF | R&D Systems | Cat#: 273-F9/CF |
| Protease Inhibitor Cocktail Set III, EDTA-Free | Merck | Cat#: 539134 |
| Metafectene reagent | Biontex | Cat#: T020-1.0 |
| TransIT-2020 reagent | Mirus | Cat#: MIR 5404 |
| X-tremeGENE HP DNA transfection reagent | SIGMA | Cat#: 6366236001 |
| TransfeX transfection reagent | ATCC | Cat#: ATCC ACS-4005 |
| NSC 87877 | Santa Cruz Biotechnology | Cat#: sc-204139 |
| DMSO | SIGMA | Cat#: D2650 |
| 1, 6-Hexanediol | SIGMA | Cat#: 240117 |
| Lipoic acid | SIGMA | Cat#: 62320 |
| Lipoamide | Santa Cruz Biotechnology | Cat#: sc-239160 |
| 4-methylumbelliferyl myo-inositol-1-phosphate, N-methyl-morpholine salt | Carbosynth Limited | Cat#: M-5717 |

(Continued on next page)
**Continued**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Alkaline Phosphatase, Calf Intestinal (CIP) | New England Biolabs | Cat#: M0290 |
| DOPC | Avanti | Cat#: 850375 |
| Ni2+-NTA DOGS | Avanti | Cat#: 790404 |
| PEG5000 PE | Avanti | Cat#: 880230 |
| Atto-488 NHS ester | SIGMA | Cat#: 41698 |
| Atto-594 NHS ester | SIGMA | Cat#: 79636 |
| Atto-647 NHS ester | SIGMA | Cat#: 07376 |
| NTA-Atto 550 | SIGMA | Cat#: 94159 |
| Ammonium Chloride (15N, 99%) | Goss Scientific | Cat#: NLM-467 |
| D-Glucose (U-13C6, 99%; 1,2,3,4,5,6,6-D7, 97-98%) | Goss Scientific | Cat#: CDLM-3813 |
| FGFR2 pY769 peptide: TTNEE{pY}LDLSQP | Genscript | Customized |
| PLCγ1 pY771 peptide: TAEPD{pY}GALYEG | Genscript | Customized |
| PLCγ1 pY775 peptide: DYGAL{pY}EGRNPQ | Genscript | Customized |
| PLCγ1 pY783 peptide: RNPGF{pY}VEANPM | Genscript | Customized |

**Experimental models: Cell lines**

| HEK293T | ATCC | CRL-1573; RRID:CVCL_0045 |
| HEK293T SHP2 KO | This manuscript | N/A |
| A431 SHP2i | Ahmed et al., 2013 | N/A |
| A431 | ATCC | CRL-1555; RRID:CVCL_0037 |
| Caco-2 | ATCC | HTB-37; RRID:CVCL_0025 |
| Caco-2 SHP2i | This manuscript | N/A |
| MCF7 | ATCC | HTB-22; RRID:CVCL_0031 |
| MCF7 SHP2 KO | This manuscript | N/A |

**Recombinant DNA**

| EGFR kinase-Tail | This manuscript, residue 712-1210, cloned in pET28b | N/A |
| Her2 kinase-Tail | This manuscript, residue 720-1255, cloned in pET28b | N/A |
| Her4 kinase-Tail | This manuscript, residue 718-1308, cloned in pET28b | N/A |
| FGFR1 kinase-Tail | This manuscript, residue 478-822, cloned in pET28b | N/A |
| FGFR2 cyto | This manuscript, residue 400-821, cloned in pET28b | N/A |
| VEGFR1 kinase-Tail | This manuscript, residue 827-1338, cloned in pET28b | N/A |
| VEGFR2 kinase-Tail | This manuscript, residue 834-1356, cloned in pMAL-c5X | N/A |
| SHP2 | This manuscript, residue 1-593, cloned in pET28b | N/A |
| SHP2 C459S | This manuscript, residue 1-593, with C459S mutant, cloned in pET28b | N/A |
| SHC (p52) | Suen et al., 2013 | N/A |
| PLCγ1 | This manuscript, residue 1-1291, cloned in pET28b | N/A |
| FGFR2 cyto K517I | This manuscript, residue 00-821, with K517I mutant, cloned in pET28b | N/A |
| FGFR2 kinase | This manuscript, residue 464-763, cloned in pET28b | N/A |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| FGFR2<sub>C58</sub> | This manuscript, residue 764-821, cloned in pET28b | N/A |
| SHP2<sup>2SH2</sup> | This manuscript, residue 1-221, cloned in pET28b | N/A |
| SHP2<sup>2SH2 R32A</sup> | This manuscript, residue 1-221, with R32A mutant, cloned in pET28b | N/A |
| SHP2<sup>2SH2 R138A</sup> | This manuscript, residue 1-221, with R138A mutant, cloned in pET28b | N/A |
| SHP2<sup>2SH2 R32/138A</sup> | This manuscript, residue 1-221, with R32/138A mutant, cloned in pET28b | N/A |
| PLC<sup>Y12SH2</sup> | This manuscript, residue 545-791, cloned in pET28b | N/A |
| GST-SHP2 | This manuscript, residue 1-593, cloned in pGEX-4T1 | N/A |
| GST-SHP2<sub>C459S</sub> | This manuscript, residue 1-593, with C459S mutant, cloned in pGEX-4T1 | N/A |
| GST-SHP2<sup>2SH2</sup> | This manuscript, residue 1-221, cloned in pGEX-4T1 | N/A |
| GST-SHP2<sup>2SH2 R32A</sup> | This manuscript, residue 1-221, with R32A mutant, cloned in pGEX-4T1 | N/A |
| GST-SHP2<sup>2SH2 R138A</sup> | This manuscript, residue 1-221, with R138A mutant, cloned in pGEX-4T1 | N/A |
| GST-SHP2<sup>2SH2 R32/138A</sup> | This manuscript, residue 1-221, with R32/138A mutant, cloned in pGEX-4T1 | N/A |
| GST-SHP2<sub>PTP</sub> | This manuscript, residue 247-525, cloned in pGEX-4T1 | N/A |
| GST-SHP2<sub>PTP C459S</sub> | This manuscript, residue 247-525, with C459S mutant, cloned in pGEX-4T1 | N/A |
| GST-SHP2<sub>C69</sub> | This manuscript, residue 526-593, cloned in pGEX-4T1 | N/A |
| GST-PLC<sup>Y12SH2</sup> | This manuscript, residue 545-791, cloned in pGEX-4T1 | N/A |
| GST-FGFR2<sub>C58</sub> | This manuscript, residue 763-821, cloned in pGEX-4T1 | N/A |
| GST-FGFR2<sub>C58 Y769F</sub> | This manuscript, residue 763-821, with Y769F mutant, cloned in pGEX-4T1 | N/A |
| GST-FGFR2<sub>C58 Y779F</sub> | This manuscript, residue 763-821, with Y779F mutant, cloned in pGEX-4T1 | N/A |
| GST-FGFR2<sub>C58 Y783F</sub> | This manuscript, residue 763-821, with Y783F mutant, cloned in pGEX-4T1 | N/A |
| GST-FGFR2<sub>C58 Y805F</sub> | This manuscript, residue 763-821, with Y805F mutant, cloned in pGEX-4T1 | N/A |
| GST-FGFR2<sub>C58 Y812F</sub> | This manuscript, residue 763-821, with Y812F mutant, cloned in pGEX-4T1 | N/A |
| GST-FGFR2<sub>Cyto</sub> | This manuscript, residue 400-821, cloned in pGEX-4T1 | N/A |
| GST-substrate | This manuscript, gene fragment encodes 4xDADYLILPOQG, cloned in pGEX-4T1 | N/A |
| CN173-FGFR2<sub>Cyto</sub> | This manuscript, residue 400-821, N-terminally fused with residue 1-173 of CFP, cloned in pET28b | N/A |

(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, John E. Ladbury (j.e.ladbury@leeds.ac.uk)

Materials availability
Plasmids generated in this study will be available upon request.

Data and code availability
- Original western data and microscopy data for figures in this paper have been deposited at Mendeley Data and are publicly available as of the date of publication. The DOI is in the key resources table.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mammalian cell culture
HEK293T, A431 and MCF7 cells were maintained in DMEM (Dulbecco’s modified Eagle’s high glucose medium) supplemented with 10% (v/v) FBS (foetal bovine serum) (Caco-2 cells were maintained in EMEM (Eagle’s minimum essential medium with 20% FBS) and 1% antibiotic/antimycotic (Lonza) in a humidified incubator with 10% CO₂. shRNA control cells (A431 Ci) and SHP2 knockdown cells (A431 SHP2i) were maintained as described previously (Ahmed et al., 2013).

Escherichia coli strains
E. coli BL21(DE3) cells were used in this study for the production of recombinant proteins. Cells were cultured in 2x YT medium.
#### METHOD DETAILS

**Cloning, expression and purification of recombinant proteins**

SHP2 is known to be recruited to FGFRs through the scaffold protein FRS2 upon receptor activation (Hadari et al., 1998; Ong et al., 1997; Ong et al., 2000). To mitigate this in our cell-based assays the FGFR2
VT variant, which lacks the critical
VT
3
motif was adopted (Burgar et al., 2002). The full length SHP2, EGFR, Her2, and Her4 plasmid templates were obtained from Addgene (SHP2: #8381, EGFR: #81926, Her2: #16257, Her4: #29527). The full-length PLCγ1 mEGFP expression vector was a kind gift from Dr. Matilda Katan (University College London, UK). The full-length VEGFR1 and VEGFR2 mEGFP expression vector was a kind gift from Dr. Sreenivasan Ponnambalam (University of Leeds, UK). Gene fragments that encode different regions of SHP2 or RTK proteins as we described in the text and key resources table were amplified using standard PCR and cloned into prokaryotic or eukaryotic expression vectors as designed.

All His-tagged, GST-tagged, or MBP-tagged recombinant proteins were purified from BL21(DE3) cells. A single colony was used to transform 100 ml of 2xYT which was grown overnight at 37°C. 1L of 2xYT were inoculated with 10 ml of this overnight culture and were allowed to grow at 37°C until the OD
160=0.8 at which point the culture was cooled down to 20°C and expression was induced with 1 mM IPTG. Cultures were allowed to grow for a further 12 hours before harvesting by centrifugation. Cells were re-suspended in 20 mM Tris, 150 mM NaCl, 10% glycerol, pH 8.0 in the presence of protease inhibitors and lyzed by sonication. Insoluble material was removed by centrifugation (40,000 g at 4°C for 60 min).

For the purification of His-tagged proteins, the soluble fraction was applied to a Talon column. Following a wash with 10 times column volume of washing buffer (20 mM Tris, pH 8.0, 150 mM NaCl, and 1mM β-mercaptoethanol) protein was eluted from the column with elution buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 150 mM imidazole, and 1mM β-mercaptoethanol). Eluted proteins were concentrated to 5 ml and applied to a Superdex75 gel filtration column in buffer containing 20 mM HEPES, 150 mM NaCl and 1 mM TCEP pH 7.5. Analysis of pure proteins on SDS-PAGE showed greater than 98% purity. For the preparation of untagged SHP2C459S and PLCγ1 proteins, the purified proteins were incubated with 1 ml of talon beads for the cleavage procedure as described below.

For the purification of GST-tagged proteins, the soluble fraction was applied to a GST column. Following a wash with 10 times column volume of washing buffer (20 mM Tris, pH 8.0, 150 mM NaCl, and 1mM β-mercaptoethanol) protein was eluted from the column with elution buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 20 mM glutathione, and 1mM β-mercaptoethanol). Eluted proteins were concentrated to 5 ml and applied to a Superdex75 gel filtration column in buffer containing 20 mM HEPES, 150 mM NaCl and 1 mM TCEP pH 7.5. Analysis of pure proteins on SDS-PAGE showed greater than 98% purity.

For the purification of MBP-VEGFR2
VT protein, the soluble fraction was applied to 1 ml of Amylose agarosed. Following a wash with 20 times column volume of washing buffer (20 mM Tris, pH 8.0, 150 mM NaCl, and 1mM β-mercaptoethanol), the purity of MBP-VEGFR2
VT on beads was analysed on SDS-PAGE and showed greater than 98% purity.

Transformed E. coli for the expression of
1H, 15N, 13C-labelled protein for backbone resonance assignment was initially grown in normal LB broth overnight at 37°C. The next day cells were harvested and transferred to 100 ml M9 media containing deuterated D-glucose (U-13C6, 99%, 1, 2, 3, 4, 5, 6-D7, 97-98%), 15N-labeled ammonium chloride (15NH4Cl), and grew overnight at 30°C. The next day the pre-culture was transferred to 1000 ml of labelled M9 media (starting OD
160=0.1) and incubated at 37°C, 220rpm. When the OD
160 was reached ~0.8, IPTG was added to the final concentration of 1 mM. The protein was expressed for 16 hr at 20°C before harvesting.

**Cleavage of affinity tags**

Purified proteins were rebound to their affinity agarose beads for the cleavage of tags. Briefly, protein on agarose beads was prepared as a 50% slurry. Thrombin (1 unit for 1mg of protein) was added to the slurry and rotate gently at 4°C overnight. On the following day, thrombin was removed by passing the solution from the slurry through 1 ml of benzamidine agarose beads and the untagged target protein was collected from the benzamidine beads flowthrough. The untagged proteins were further purified using a Superdex 75 gel filtration column as described above.

**Plasmids transfection and viral infection**

Transfection of plasmids into HEK293T cells was performed using Metafectene transfection reagent. Transfection of plasmids into MCF7 cells was performed using TransIT transfection reagent. Transfection of plasmids into A431 cells was performed using X-tremeGENE HP DNA transfection reagent. Transfection of plasmids into Caco-2 cells was performed using TransfeX transfection reagent. Plasmids transfection and viral infection were carried out according to manufacture’s protocols and all plasmid DNA was prepared using QIAprep Spin Miniprep Kit (QIAGEN).

**CRISPR knockdown/knockout**

FGFR2 or SHP2 expression in different cell lines was knocked down or knockout using CRISPR. Briefly, pLentiCRISPR v2 plasmid containing FGFR2 gRNA target sequence (GTACCGTAAACCAGTTGCTCAG) of SHP2 gRNA target sequence (GAGACTCTCA CACTTTCCGTT) were purchased from GenScript. pLentiCRISPR v2 was co-transfected with the packaging plasmids pMD2.G and psPAX2 (2:1:1 ratio) into HEK293T cells. Collect virus-containing medium 48 hours after transfection and pass viral media through a 0.45μM low protein-binding filter. The viral supernatant can be used to infect cells or frozen at -80°C. For the infection, cells
were treated with 10 μg/ml polybrene and 1 ml of virus solution was used to infect cells in one well of a 6-well plate with 1 ml of culture medium. Infected cells were incubated for 72 hours, selection was performed by changing the medium containing puromycin (1 μg/ml for HEK293T, 2 μg/ml for A431, MCF7, and Caco-2 cells) every 2-3 days. Protein expression levels were confirmed using western blotting.

### In vitro phosphorylation of purified proteins

Purified RTK proteins were autophosphorylated by incubating 10μM of protein with 5mM of ATP/MgCl2 at room temperature for 2 hours. Reactions were quenched by adding 50mM EDTA. Phosphorylated RTK proteins were further purified using a Superdex 75 gel filtration column (in 20 mM HEPES, pH 7.5, 150 mM NaCl, and 1 mM TCEP) as described above.

Phosphorylated PLCγ1 proteins were prepared by incubating with recombinant FGFR2cyto conjugated on agarose beads with 5 mM ATP and 5 mM MgCl2 for 2 hours. The phosphorylation reactions were quenched by adding EDTA (prepared in 20 mM HEPES, pH 7.5) to a final concentration of 50mM, and FGFR2cyto Protein (on beads) was removed after the phosphorylation and quenching reactions by centrifugation. The supernatant solution that contains phosphorylated PLCγ1 proteins was further purified using a Superdex 75 gel filtration column (in 20 mM HEPES, pH 7.5, 150 mM NaCl, and 1 mM TCEP) as described above.

The phosphorylation states of proteins were analysed by gel-shift assays (Lee et al., 2019) on SDS-PAGE and immunoblotting using an anti-phosphotyrosine antibody and showed high degrees of homologues.

### Protein fluorescent labelling

Highly purified FGFR2 and PLCγ1 proteins (in 20 mM HEPES, pH 7.5, 150 mM NaCl, and 1 mM TCEP) were prepared in 100 mM NaHCO3 buffer (pH 8.3) at 0.5 mg/ml and labelled with Atto-488 NHS ester or Atto-647 NHS ester (Sigma) respectively and incubated at room temperature for 1 hr (fluorophore to protein molar ratio was 1:1). Highly purified SHP2 proteins (in 20 mM HEPES, pH 7.5, 150 mM NaCl, and 1 mM TCEP) were directly labelled with NTA Atto-550 at room temperature for 10 minutes (fluorophore to protein molar ratio was 1:1). For untagged SHP2 proteins (used in support lipid bilayers experiment) highly purified SHP2 proteins (in 20 mM HEPES, pH 7.5, 150 mM NaCl, and 1 mM TCEP) were prepared in 100 mM NaHCO3 buffer (pH 8.3) at 0.5 mg/ml and labelled with Atto-594 NHS ester and incubated at room temperature for 20 minutes to reduce non-specific labelling that affects the SH2 domain binding ability. Excess dye was removed using G-15 desalting chromatography. Proteins were concentrated and labelling efficiency was measured by Nanodrop 2000 (ThermoFisher).

### Confocal microscopy for in vitro droplet formation

Purified proteins were mixed or diluted with buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, and 1 mM TCEP) to desired combinations and concentrations, and incubated at room temperature for 1 min before imaging. To monitor the effect of NaCl, 1, 6-hexanediol, lipoic acid, or lipoamide in the formation of droplets, mixtures were prepared by adding compounds at last and incubated for 1 minutes. Finally, 4μl of each sample was pipetted onto a 3-well chambered cover glass slides (Thermo Scientific, ER-303B-CE24). Images were acquired on either Leica SP8 or Zeiss LSM880 microscopes. Super-resolution imaging was performed on Zeiss LSM880 using the AiryScan settings.

### Supported lipid bilayer assay

Methods for preparing supported lipid bilayers have been described (Banjade and Rosen, 2014; Case et al., 2019a; Huang et al., 2019; Su et al., 2017; Zeng et al., 2018). In general, phospholipids containing 98% POPC (AvantiLipids), 2% DGS-NTA-Ni (AvantiLipids) and 0.1% PEG 5000 PE (AvantiLipids) were mixed and dried under a stream of nitrogen and resuspended in PBS. Finally, the vesicle solution was sonicated for 90 seconds in an ice-water bath to make small unilamellar vesicles (SUVs) and the solution was subjected to a centrifugation at 33,500 g for 45 min at 4°C. Supernatant containing SUVs was collected. The membrane reconstitution system was prepared on a homemade chambered cover glass (cleaned with Hellmanex III (Hellma Analytics) overnight, thoroughly rinsed with H2O, followed by 15 minutes incubation in 1:1 IPA/H2O, followed by excessive rinsing of H2O. Supported lipid bilayers were formed on the cleaned glass slides by incubating the SUVs mixed with PBS for at least 30 min. The chambers were then rinsed with PBS buffer, followed by incubation of 1 mg/mL BSA in PBS for 10 min to block defects in supported membranes. Supported lipid membranes were further washed with the protein buffer for twenty times.

pFGFR2Cyto (20μM; His-tagged) was added to the membrane and incubated for 5 minutes, solution was buffer exchanged into protein buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, and 1 mM TCEP) to remove excess protein. After imaging, untagged SHP2 and PLCγ1 proteins were sequential added for confocal imaging. Between all incubation steps, the chamber were washed with protein buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, and 1 mM TCEP) thoroughly to remove unbound proteins. All preparations were done at room temperature.

### Fluorescence recovery after photobleaching (FRAP) assay

FRAP assay was performed on a Zeiss LSM 880 inverted confocal microscope at room temperature. Fluorescent signals were bleached using the appropriate corresponding laser beam. Fluorescence intensity was recorded in two regions: a region that was bleached and a region of an equal size that was not bleached. The unbleached region was used as a control for the stability of fluorescence signal throughout the FRAP experiment. Fluorescence signal from the bleached region was normalised to the unbleached
region, which was then expressed as a fraction of the normalised signal prior to bleaching. The fluorescence intensity difference between pre-bleaching and at time 0 (the time point immediately after photobleaching pulse) was normalized to 1. The experimental control was based on quantification of fluorescence intensities of similar droplet/membrane regions without photobleaching.

**Immunofluorescence**

HEK293T cells expressing fluorescent tagged FGFR2, SHP2, or PLCγ1 and Caco-2 cells that endogenously express all three proteins were seeded on coverslips in a 24-well plate. After serum-starvation for 16 hours, cells were stimulated with 10 ng/ml FGF9 for 15 minutes or remained untreated. Cells were fixed for 15 minutes with 4% paraformaldehyde, washed, and incubated in blocking buffer for 1 hour (1X PBS with 1% BSA and 0.5% Triton X-100). Blocking buffer was aspirated and cells were incubated with primary antibody overnight in the dark at 4°C. The following day, cells were washed, incubated with fluorophore-conjugated secondary antibodies for 1 hour at room temperature in the dark, washed, and then mounted. Slides were analyzed using a Zeiss LSM 880 inverted confocal microscope and images were analyzed using ImageJ software.

**Plasma membrane staining and live cell imaging**

HEK293T SHP2 KO cells expressing FGFR2ΔV1-Neptune 2.5, SHP2ΔC459S-mOrange, and PLCγ1-mEGFP or expressing fluorescent proteins as control experiments were seeded in Ibidi μ-Dish (ibidi, 81156) coated with Poly-D-Lysine (sigma, P4832). Cells were serum-starved for 16 hours. For plasma membrane staining, wheat germ agglutinin (Alexa 350 conjugated, Invitrogen, W11263) was prepared in PBS as a 1 mg/ml stock. Before imaging experiment, cells were washed 3 times with PBS and labelling was performed using 0.01 mg/ml wheat germ agglutinin at 37°C for 10 min. When the labelling is complete, labelling solution was removed and cells were washed 3 times with PBS and starvation medium was added. Cells were direct subjected to imaging experiment or stimulated with FGF9 (10 ng/ml) for 15 minutes before performing confocal imaging.

**Highly inclined and laminated optical sheet (HILO) microscopy**

HILO microscopy (Tokunaga et al., 2008) was performed on a home-built system based on an open stage for mounting the sample and fluorescence microscopy optics (RM21, Mad City Labs, with XYZ-nanopositioner). The illumination beam (488 nm laser, LBX-488-150-CSB-PP, Oxxius) was expanded 14x (aspheric lens A220TM-A, pinhole P20D, collimator AC254-150-A-ML, Thorlabs), and translated across and focussed on the back focal plane of the objective (60X, NA 1.5: UPLAPO60XOHR, Olympus) using a motorised mirror and lens stage (TIRF Module, Mad City Labs). A spectral filter (Di01-405/488/561/635-25x36, Semrock) separated excitation and emission light. Images were formed with a tube lens (TTL180-A, f = 180 mm, Thorlabs) and captured on a cooled sCMOS camera (Prime BSI, Photometrics Teledyne). The system was controlled with Micro-Manager 2.0 (Edelstein et al., 2010).

Image processing was carried out in FIJI (Schindelin et al., 2012). The Z-stack (depth step: 0.25 μm) was depth-coded (colour map: “Ice”) with a macro based on K_TimeRGBcolorcode.ijm by Kota Miura. Maximum intensity projections are shown. Contrast stretching aids the visibility of the protein clusters and cellular area. For the XZ view, the image was scaled in Z so that the scale bar applies isotropically to both the XY and XZ images (XY pixels: 0.108 μm). Images were acquired after stimulation of FGFR2 with FGF9 ligand.

**Pulldown and western blots**

GST-tagged proteins immobilized on Glutathione Sepharose (GE Healthcare Life Science) as a 50% slurry. For pulldown experiments using mammalian cells, cells were grown in 10cm dishes, serum starved overnight and stimulated with 10 ng/ml FGF9 for 15 minutes. The beads were then spun down at 4,000 rpm for 3 minutes, supernatant was removed and the beads were washed with 1 ml lysis buffer (0.5mg per vial). 50 μl of the slurry beads were added to the lysates and incubated at 4°C overnight with gentle rotation. For pulldown experiments using purified proteins, 1 μg of purified proteins were prepared in a 50 μl volume (in 20 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM PMSF and Protease Inhibitor Cocktail Set III (Calbiochem)). The cell debris was removed by centrifugation at 13,000 rpm for 20 minutes. Cell lysates were washed at a concentration of 1mg/ml in the lysis buffer (0.5mg per vial), 50 μl of the slurry beads were added to the sloutions and incubated at 4°C overnight with gentle rotation. The beads were then spun down at 4,000 rpm for 3 minutes, supernatant was removed and the beads were washed with 1 ml lysis buffer. This washing procedure was repeated five times in order to remove non-specific binding. After the last wash, 50 μl of 2x Laemmli sample buffer were added, the sample was boiled and subjected to SDS-PAGE and western blot assay using specific antibodies. Immune complexes were detected with horseradish peroxidase conjugated secondary antibodies and visualized by enhanced chemiluminescence reagent according to the manufacturer’s instructions (Pierce).

**Bimolecular fluorescence complementation (BiFC)**

The sequences encoding residues 1-173 (CN173) and 174-238 (CC173) of CFP were fused upstream of sequences encoding FGFR2ΔCyto and PLCγ1ΔSH2 in pET28b vectors for expression in E.coli (Kodama and Hu, 2012). Both proteins were purified and phosphorylated as described above in the presence of FGFR2ΔCyto, ATP and MgCl2. The fluorescence complementation was measured using the Monolith NT.115 (NanoTemper Technologies, Gmbh) as described above. Protein samples were filled into capillaries and the change in fluorescence intensity upon the adding of SHP2ΔSH2 domain was monitored. Data were plotted using Origin 7.0.
Bio-layer interferometry (BLI)
BLI experiments were performed using a FortéBio Octet Red 384 using Anti-GST biosensors (18-5096). Assays were done in 384 well plates at 25°C. Association was measured by dipping sensors into solutions of analyte protein for a designed time period as indicated in each figure, and was followed by moving sensors to wash buffer for a designed time period as indicated in each figure to monitor the dissociation process. Raw data shows a rise in signal associated with binding followed by a diminished signal after application of wash buffer. In experiments in which the ternary complex was reconstituted on the sensor ATP/MgCl₂ was added as described in the Results.

Microscale thermophoresis (MST)
The binding affinities were measured using the Monolith NT.115 (NanoTemper Technologies, GmbH). Proteins were fluorescently labelled with Atto488 according to the manufacturer’s protocol. Labelling efficiency was determined to be 1:1 (protein:dye) by measuring the absorbance at 280 and 488 nm. A 16 step dilution series of the unlabelled binding partner was prepared and mixed with the labelled protein at 1:1 ratio and loaded into capillaries. Measurements were performed at 25°C in 20 mM HEPES, 150 mM NaCl and 1 mM TCEP pH 7.5 buffer containing 0.01% Tween 20. Data analyses were performed using Nanotemper Analysis software, v.1.2.101 and were plotted using Origin 7.0. All measurements were conducted as triplicates and the errors were presented as the standard error of the triplicates. Equilibrium dissociation constants (Kₐ’s) are reported as ‘apparent’ values because for the interaction of FGFR2pCyto with SHP2 and PLCγ1 because the MST binding profiles appear to include two independent binding sites. The second of which is much weaker and is likely to be due to non-specific effects. Nonetheless the possibility of competing equilibria is flagged by the use of the term apparent. Reported data fits are based on the initial tight binding event. Where the Kₐ is reported without the superscript ‘app’ the data has been fit to the standard 1:1 binding model.

Isothermal titration calorimetry (ITC)
ITC experiments were carried out using a MicroCal ITC200 (Malvern) at 25°C. 20 15µl injections of 100 µM FGFR2pC58 were made into 10 µM SHP2pCyto in the calorimeter cell. A control experiment involving the injection of 100 µM FGFR2pC58 into buffer was performed. The heat per injection was determined and subtracted from the binding data. Data was analysed using a single independent site model using Origin software.

Nuclear magnetic resonance (NMR)
All NMR spectroscopic experiments were carried out on Bruker Avance III (700, 800 and 950 MHz) NMR spectrometers equipped with cryogenically cooled triple resonance probes with a z-axis pulse field gradient coil were used. Resonance assignment spectra were recorded in 25 mM NaH₂PO₄/Na₂HPO₄, pH 6.5, 50 mM NaCl, 5 mM DTT, 1 mM EDTA. For the ¹H,¹⁵N,¹³C labelled construct, a standard set of 3D backbone resonance assignment experiments (HNCA, HNCOCA, HNCAACB, CACBCONH, HNCO and HNCACO) using standard Bruker library pulse sequences (with Watergate water suppression) or BEST versions (Lescop et al., 2007; Schulte-Herbrüggen and Sørensen, 2000) of amide transverse relaxation optimized spectroscopy (TROSY) (Pervushin et al., 1997) pulse sequences applying Non-Uniform Sampling (17-25%) were used to obtain a high-resolution spectra.

The NMR titration of SHP2pCyto into PLCγ1pCyto experiments were recorded at 25°C using 200 µM uniformly ¹⁵N-labelled PLCγ1pCyto in 20 mM HEPES (pH 7.5) containing 150 mM NaCl, 1mM TCEP and 10% (v/v) D₂O. 0, 150, 300, 450 600 and 900 µM unlabelled SHP2pCyto were added and an average BEST TROSY pulse sequence recorded.

The NMR titration of PLCγ1pCyto into SHP2pCyto experiments were recorded at 25°C using 100 µM uniformly ¹⁵N-labelled SHP2pCyto in 20 mM HEPES (pH 7.5) containing 150 mM NaCl, 1mM TCEP and 10% (v/v) D₂O. 600 µM unlabelled PLCγ1pCyto were added and an average BEST TROSY pulse sequence recorded.

All NMR data was processed with NMRPipe (Delaglio et al., 1995) and analyzed with CcpNmr Analysis software package (Skinner et al., 2016; Vranken et al., 2005) available in-house and on NMRBox platform (Maciejewski et al., 2017). Chemical shift perturbations (CSPs) for individual residues were calculated from the chemical shift for the backbone amide ¹H (Δδ₁H) and ¹⁵N (Δδ₁⁵N) using the following equation: CSP = √[(Δδ₁H₂ + (0.154 Δδ₁⁵N)) (Evenäs et al., 2001)].

In vitro FGFR2 kinase assay
A kinase-dead mutant FGFR2pCyto K517I was expressed and purified as a substrate for active FGFR2pCyto kinase assay (in 20 mM HEPES, pH 7.5, 150 mM NaCl, and 1 mM TCEP). To accurately measure the kinase activity in a LLPS environment as we described in the text, 200-time access of FGFR2pCyto K517I substrate was mixed with 10 µM of the active FGFR2pCyto in the solution state, in the LLPS state, or in other control conditions. 500 µM of ATP/MgCl₂ was added and incubated at room temperature for 15 minutes. The phosphorylation states of substrate under various conditions were analysed using immunoblotting.

In vitro SHP2 phosphatase assay
A 4xDADELYIPQQG peptide was cloned as a GST-fusion (GST-substrate) and used for in vitro SHP2 phosphatase assay. Briefly, GST-substrate on GST beads was phosphorylated by FGFR2pCyto in the presence of 5mM ATP/MgCl₂ for 2 hours. After washing with PBS to remove FGFR2pCyto and ATP/MgCl₂, GST-substrate was eluted from GST beads using 20 mM glutathione. Eluted phosphorylated GST-substrate was passed through a Superdex 75 column for further purification and buffer exchange (in 20 mM HEPES, pH 7.5).
pH 7.5, 150 mM NaCl, and 1 mM TCEP). 10-time access of phosphorylated GST-substrate was mixed with 60 µM of wild type SHP2 in the solution state, in the LLPS state, or in other control conditions at room temperature for 15 minutes. The phosphorylation states of substrate under various conditions were analysed using immunoblotting.

**In vitro phosphatase assay**

The effect of 10% 1, 6-hexanediol on SHP2 activity (60 µM) was measured using the non-radioactive phosphatase assay system (Promega V2471) according to the manufacture’s protocol.

**Dephosphorylation by CIP**

10 µM of phosphatase CIP (NEB, M0290) was incubated with individual protein (10 µM of pFGFR2*Cytot, 60 µM of SHP2*C459S, or 12 µM of pPLC*) for 30 minutes before droplet formation, or added after the pFGFR2*Cytot, SHP2*C459S and PLC*1 droplets were formed (incubation with CIP for 15 minutes). The effects of CIP in the dephosphorylation of individual protein and pFGFR2*Cytot- SHP2*C459S-PLC*1 droplets were examined using immunoblotting.

**In vitro PLC*1 activity assay**

The lipolytic activity of pPLC*1 was determined using the artificial substrate 4-methylumbelliferyl myo-inositol-1-phosphate, N-methyl-morpholine salt (Biosynth). Briefly, reaction mixtures consisted of 10 mM Tris, pH 6.8, 0.8 mM substrate, and 50% of protein mixtures (50 µM of pPLC*1 alone or other protein mixtures as we described in the text). The reaction mixtures were placed in a 96-well plate, and fluorescence was measured using 350 nm excitation/450 nm emission filters on a plate reader. Reactions were allowed to proceed for 45 min at room temperature, with measurements taken every 5 min.

**Wound-healing assay**

Cells were seeded in a 96 well plate. After overnight serum starvation the cells were scratched using IncuCyte® WoundMaker and stimulated with 10 ng/ml FGF9, then incubated for further 24 hr (MCF7 cells; 16 hr for A431 cells). Images were taken at 0 hr and after incubation by a microscope gantry inside a cell incubator (Incucyte, Essen Bioscience, Ann Arbor, MI, USA). The identical experiment was also performed using HEK293T cells stably transfected with FGFR2*DVT.

**Calcium concentration assay**

The calcium assay was perform using Fluo-4 NW Calcium Assay Kit (F36206, ThermoFisher Scientific) according to the manufactural manual. Briefly, cells were seeded in a 96 well plate. After FGF9 stimulation, medium was removed and cells were exposed to the dye loading solution and incubated for 30 minutes at 37 °C then 30 minutes at 25 °C. The Ca²⁺ level (fluorescence) was measured using instrument settings for excitation at 490 nm and emission at 510 nm.

**Mathematical model**

We have developed a deterministic mathematical model to describe the in vitro interactions between FGFR2*Cytot (F), PLC*1*SH2 (P) and SHP2*2SH2 (S). The model has been formulated to include the reactions depicted in Reaction Figure, which involve the following chemical species

- F (or n₁) = unphosphorylated FGFR2,
- pF (or n₂) = phosphorylated FGFR2,
- S (or n₃) = SHP2,
- pF · S (or n₄) = phosphorylated FGFR2-SHP2 complex,
- P (or n₅) = unphosphorylated PLCγ,
- pF · P (or n₆) = phosphorylated FGFR2-PLCγ complex,
- pF · pP (or n₇) = phosphorylated FGFR2-phosphorylated PLCγ complex,
- pP (or n₈) = SHP2-phosphorylated PLCγ,
- S · P (or n₉) = SHP2-unphosphorylated PLCγ complex,
- S · pP (or n₁₀) = SHP2-phosphorylated PLCγ complex,
- pF · S · P (or n₁₁) = phosphorylated FGFR2-SHP2-unphosphorylated PLCγ complex,
- pF · P · S (or n₁₂) = phosphorylated FGFR2-PLCγ-SHP2 complex,
- pF · S · pP (or n₁₃) = phosphorylated FGFR2-SHP2-phosphorylated PLCγ complex, and
- pF · pP · S (or n₁₄) = phosphorylated FGFR2-phosphorylated PLCγ-SHP2 complex,

where in every row above the first symbol is the abbreviated species name provided in Reaction Figure and the symbol in parentheses is used in the differential equations describing the variables of the mathematical model. We have assumed that there are no allosteric binding effects between SHP2, PLCγ, or the phosphorylated receptor. From the reactions in Reaction Figure and assuming mass-action kinetics, a set of ordinary differential equations (ODEs) for the concentrations (in units of µM) for each molecular species can be written as follows:
\[ \frac{dn_1}{dt} = -k_{\cdot 4}n_1, \]  
\[ \frac{dn_2}{dt} = k_{\cdot 1}n_1 - k_{\cdot 2}n_2(n_3 + n_9 + n_{10}) + k_{\cdot 3}(n_4 + n_{11} + n_{13}) - k_{\cdot 3}n_2(n_5 + n_9) + k_{\cdot 3}(n_6 + n_{12}) + k_{\cdot 5}n_7 + k_{\cdot 5}n_{14}, \]
\[ \frac{dn_3}{dt} = -k_{\cdot 3}n_2n_3 + k_{\cdot 3}n_4 - k_{\cdot 6}n_3n_5 + k_{\cdot 7}n_8 + k_{\cdot 12}n_5, \]
\[ \frac{dn_4}{dt} = k_{\cdot 2}n_2n_3 - k_{\cdot 2}n_4 - k_{\cdot 7}n_3n_5 + k_{\cdot 7}n_{13}, \]
\[ \frac{dn_5}{dt} = -k_{\cdot 3}n_2n_5 + k_{\cdot 3}n_6 - k_{\cdot 6}n_3n_5 + k_{\cdot 6}n_9, \]
\[ \frac{dn_6}{dt} = k_{\cdot 3}n_2n_5 - k_{\cdot 3}n_6 - k_{\cdot 4}n_6, \]
\[ \frac{dn_7}{dt} = k_{\cdot 4}n_6 - k_{\cdot 5}n_7, \]
\[ \frac{dn_8}{dt} = k_{\cdot 5}n_7 - k_{\cdot 7}n_6(n_3 + n_9) + k_{\cdot 7}(n_{10} + n_{13}), \]
\[ \frac{dn_9}{dt} = k_{\cdot 6}n_3n_5 - k_{\cdot 6}n_9 - k_{\cdot 2}n_2n_9 + k_{\cdot 3}n_11 - k_{\cdot 3}n_2n_9 + k_{\cdot 3}n_{12}, \]
\[ \frac{dn_{10}}{dt} = k_{\cdot 7}n_8n_3 - k_{\cdot 7}n_{10} - k_{\cdot 2}n_2n_{10} + k_{\cdot 2}n_{13} + k_{\cdot 5}n_{14}, \]
\[ \frac{dn_{11}}{dt} = k_{\cdot 2}n_2n_9 - k_{\cdot 2}n_{11}, \]
\[ \frac{dn_{12}}{dt} = k_{\cdot 2}n_2n_9 - k_{\cdot 3}n_{12} - k_{\cdot 4}n_{12}, \]
\[ \frac{dn_{13}}{dt} = k_{\cdot 7}n_8n_9 + k_{\cdot 3}n_2n_{10} - k_{\cdot 5}n_{13}, \] and
\[ \frac{dn_{14}}{dt} = -k_{\cdot 3}n_{14} + k_{\cdot 4}n_{12}. \]

We note that in the previous set of equations \( n_j \equiv n_j(t) \) for \( j = 1, \ldots, 14 \). Rate constants corresponding to phosphorylation events \( (k_{\cdot 1}, k_{\cdot 4}) \) or molecular dissociation events \( (k_{\cdot 2}, k_{\cdot 3}, k_{\cdot 5}, k_{\cdot 6}, k_{\cdot 7}) \) have dimensions of inverse time, and thus, units of \( s^{-1} \). Rate constants corresponding to molecular association events \( (k_{\cdot 2}, k_{\cdot 3}, k_{\cdot 6}, k_{\cdot 7}) \) have dimensions of inverse concentration and time, and thus, units of \( \mu M^{-1} s^{-1} \).
We have assumed that at time $t = 0$, the initial time for the system under consideration, only $F$, $S$ and $P$ are present. Thus, the initial concentrations for all other chemical species vanish. Given the timescales to be studied in the experimental model, the mathematical model does not include protein synthesis, degradation or trafficking of any of the molecular species and hence, the total number of molecules of $F$, $S$ and $P$ are constant in time. We can write down conservation expressions for the total concentration of $F$ ($n_F$), $S$ ($n_S$) and $P$ ($n_P$), since we assume the experimental volume of the system does not change with time. We, therefore, can write

$$n_F = n_1 + n_2 + n_3 + n_6 + n_7 + n_11 + n_{12} + n_{13} + n_{14},$$  \hspace{1cm} (Equation 15)

$$n_S = n_3 + n_4 + n_8 + n_{10} + n_{11} + n_{12} + n_{13} + n_{14},$$  \hspace{1cm} (Equation 16)

$$n_P = n_5 + n_6 + n_7 + n_8 + n_9 + n_{10} + n_{11} + n_{12} + n_{13} + n_{14}.$$  \hspace{1cm} (Equation 17)

We note that in the previous set of constraints $n_j \equiv n_j(t)$ for $j = 1, \ldots, 14$. The above equations hold since the total concentration of a molecule at any time $t$ is the sum of the concentrations of all species containing this molecule at that time point.

It is of interest to see which ternary complex species will prevail in the system for sufficiently late times, and thus, we have analysed the steady states of the mathematical model. Steady states can be found by setting the right hand sides of the differential equations to zero and simultaneously solving the resulting equations for the different molecular species in the system. In this case, due to the complexity of the equations, we cannot find explicit expressions for the species at steady state but we find the following implicit equations for a stable steady state solution (denoted by $n^*$)

$$n_1^* = n_2^* = n_3^* = n_4^* = n_5^* = n_6^* = n_7^* = n_8^* = n_{10}^* = n_{11}^* = n_{12}^* = n_{13}^* = n_{14}^* = 0,$$  \hspace{1cm} (Equation 18)

$$n_2^* \neq 0,$$  \hspace{1cm} (Equation 19)

$$n_3^* \neq 0,$$  \hspace{1cm} (Equation 20)

$$n_4^* \neq 0,$$  \hspace{1cm} (Equation 21)

$$n_5^* = \frac{k_{+2} n_2^* n_3^*}{k_{-2}},$$

$$n_6^* = \frac{k_{+7} n_2^* n_3^*}{k_{-7}},$$

$$n_7^* = \frac{k_{+7} n_2^* n_3^*}{k_{-7}},$$

$$n_8^* = \frac{k_{+7} n_2^* n_3^*}{k_{-7}}.$$  \hspace{1cm} (Equation 22)

Constraints (15), (16) and (17), together with equations (18), (19), (20), (21), (22) and (23) provide a set of implicit polynomial equations for $n_2^*$, $n_3^*$ and $n_4^*$. It is interesting to observe that the stable steady state defined by the previous equations only provides a non-vanishing value for the ternary complex $pF \cdot s \cdot pP$ (or $n_{13}^* \neq 0$), and that the other ternary complexes, $pF \cdot s \cdot pP$, $pF \cdot pP \cdot S$ and $pF \cdot pP \cdot S$, are such that $n_{11}^* = n_{12}^* = n_{14}^* = 0$. This is in agreement with the experimental results presented in this manuscript.

The parameters $k_{+2}, k_{-2}, k_{+6}, k_{-6}, k_{+7}$ were fixed by the experimental $k_d$ values where, $k_{d,2} = 25.1 \mu M$, $k_{d,3} = 0.223 \mu M$, $k_{d,6} = 1.16 \mu M$, $k_{d,7} = 0.48 \mu M$.

$k_{+1} = k_{-4} = 10^9 s^{-1}$, $k_{-2} = k_{-3} = k_{+5} = k_{-6} = k_{-7} = 10^{-1} s^{-1}$, and the molecular association rates $k_{+2}, k_{+3}, k_{+6}$ and $k_{+7}$ with units of $\mu M^{-1}s^{-1}$ are fixed by the experimental $k_d$ values where, $k_{d,2} = 25.1 \mu M$, $k_{d,3} = 0.223 \mu M$, $k_{d,6} = 1.16 \mu M$ and $k_{d,7} = 0.48 \mu M$. 

Molecular Cell 82, 1089–1106.e1–e12, March 17, 2022  e11
QUANTIFICATION AND STATISTICAL ANALYSIS

Data for quantification analyses are presented as mean ± standard deviation (SD). The protein levels in the western blot images were quantified by ImageJ (Fiji) and the number of replicates is shown in the figure legends. In vitro condensates were quantified using the Analyse Particle function in ImageJ (Fiji). Average of the number of condensates from 4 independent areas are shown. Colocalisation of cellular condensates were determined by using the "Coloc 2" function in ImageJ (Fiji). Degree of colocalization of endogenous PLCγ1 and SHP2 in Caco-2 cells is determined by Pearson’s R value.
Supplemental information

Receptor tyrosine kinases regulate signal transduction through a liquid-liquid phase separated state

Chi-Chuan Lin, Kin Man Suen, Polly-Anne Jeffrey, Lukasz Wieteska, Jessica A. Lidster, Peng Bao, Alistair P. Curd, Amy Stainthorp, Caroline Seiler, Hans Koss, Eric Miska, Zamal Ahmed, Stephen D. Evans, Carmen Molina-París, and John E. Ladbury
SUPPLEMENTAL INFORMATION

Receptor tyrosine kinases regulate signal transduction through a liquid–liquid phase separated state

Chi-Chuan Lin, Kin Man Suen, Polly-Anne Jeffrey, Lukasz Wieteska, Jessica A. Lidster, Peng Bao, Alistair P. Curd, Amy Stainthorp, Caroline Seiler, Hans Koss, Eric Miska, Zamal Ahmed, Stephen D. Evans, Carmen Molina-Paris, and John E. Ladbury
Supplementary Figure 1

A

B

C
Figure S1. Preparation of recombinant proteins and phosphorylation-dependent FGFR2-SHP2 droplet formation, Related to Figure 1.

(A) Coomassie blue staining of purified recombinant proteins used in this study. Proteins were expressed and purified from *E. coli* (see Material and Methods) and the purity was greater than 95% examined by SDS-PAGE electrophoresis.

(B) Phase diagrams of phosphorylated EGFR, FGFR, and VEGFR family proteins (Atto-488 labelled) with concentrations shown in X-axis and SHC (Y-axis) in 20 mM HEPES (pH 7.5), 150 mM NaCl, and 1 mM TCEP. The sizes of the circles represent the average sizes of droplets (μm²) and the colour scale bars represent the numbers of droplets in a 0.0256 mm² area.

(C) (Top panel) Replacement of SHP2<sub>C459S</sub> with wild type SHP2 under identical conditions results in the reduction of droplet size. Unphosphorylated FGFR2<sub>Cyro</sub> (depFGFR2<sub>Cyro</sub> or FGFR2<sub>Cyro K517I</sub> (the kinase-dead mutant)) cannot form droplets with SHP2<sub>C459S</sub>. These images highlight the requirement for prolonged phosphorylation on FGFR2<sub>Cyro</sub> for droplet formation.

(Below) The phosphorylation state of FGFR2<sub>Cyro</sub> was examined by a gel shift assay (left, coomassie blue staining) and an immunoblotting (right) using a specific phospho-antibody (pY656/657) against the activation loop of FGFR2.
Supplementary Figure 2

A

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
& SHP2\textsubscript{C495S}-GFP & FGFR2\textsubscript{cyto}-RFP & Merged \\
\hline
Basal & & & \\
FGF9 & & & \\
\hline
\end{tabular}
\end{table}

B

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
& PLCy1-GFP & FGFR2\textsubscript{ext}-RFP & Merged \\
\hline
Basal & & & \\
FGF9 & & & \\
\hline
\end{tabular}
\end{table}

C

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
& PLCy1-GFP & SHP2\textsubscript{C495S}-RFP & Merged \\
\hline
Basal & & & \\
FGF9 & & & \\
\hline
\end{tabular}
\end{table}

D

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
pPLCy1 (μM) & pFGFR2\textsubscript{cyto} (μM) & SHP2\textsubscript{C495S} (μM) \\
\hline
0 & 0 & 0 \\
10 & 10 & 10 \\
20 & 20 & 20 \\
30 & 30 & 30 \\
40 & 40 & 40 \\
50 & 50 & 50 \\
60 & 60 & 60 \\
70 & 70 & 70 \\
80 & 80 & 80 \\
90 & 90 & 90 \\
100 & 100 & 100 \\
110 & 110 & 110 \\
120 & 120 & 120 \\
\hline
\end{tabular}
\end{table}

E

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
Elution volume (ml) & Absorbance 280nm & \\
\hline
40 & 60 & 100 & 140 & 180 & 220 \\
\hline
\end{tabular}
\end{table}
Figure S2. FGFR2-SHP2-PLCγ1 complex formation in cells and \textit{in vitro}, Related to Figure 2.

\textbf{(A)} Cell images showing FGFR2ΔVT-RFP and SHP2C459S-GFP cluster formation upon FGFR2 expression and activation (stimulated by FGF9) in HEK293T cells. Insets: magnification of regions shown by arrow to exemplify FGFR2ΔVT-RFP and SHP2C459S-GFP clusters.

\textbf{(B)} Cell images showing FGFR2ΔVT-RFP and PLCγ1-GFP cluster formation upon FGFR2 expression and activation (stimulated by FGF9) in HEK293T cells. Insets: magnification of regions shown by arrow to exemplify FGFR2ΔVT-RFP and PLCγ1-GFP clusters.

\textbf{(C)} No evidence of SHP2 or PLCγ1 cluster formation in the absence of FGFR2ΔVT; both SHP2 and PLCγ1 appear to randomly diffuse in cytosol.

\textbf{(D)} Phase diagrams of phosphorylated pFGFR2\textsubscript{Cyto}-SHP2\textsubscript{C459S}-pPLCγ1 in 20 mM HEPES (pH7.5), 150 mM NaCl, and 1 mM TCEP. pFGFR2\textsubscript{Cyto} was labelled with Atto 488, SHP2\textsubscript{C459S} was labelled with Atto 550 and pPLCγ1 was labelled with Atto 647. 10 µM of pFGFR2\textsubscript{Cyto} was mixed with various concentrations of SHP2\textsubscript{C459S} (0, 10, 60, and 120 µM) first before the addition of pPLCγ1 (from 100 – 12 µM). The sizes of the circles represent the average sizes of droplets (µm\(^2\)) and the colour scale bars represent the numbers of droplets in a 0.0256 mm\(^2\) area. In the absence of SHP2\textsubscript{C459S}, pFGFR2\textsubscript{Cyto} does not form droplets with pPLCγ1.

\textbf{(E)} Size exclusion chromatography was used to isolate the pFGFR2\textsubscript{Cyto}-SHP2\textsubscript{C459S}-pPLCγ1 complex. The components of each elution fraction were examined by coomassie gel staining. A complex of greater than 1:1:1 of pFGFR2\textsubscript{Cyto}-SHP2\textsubscript{C459S}-pPLCγ1 ternary complex was observed with a calculated molecular weight of 406 kDa (peak 2). The binary pFGFR2\textsubscript{Cyto}-SHP2\textsubscript{C459S} complex was identified as peak 3 (237kDa).
Figure S3. The formation of FGFR2-SHP2-PLCγ1 complex on membranes, Related to Figure 3.

(A) FRAP of pFGFR2_{Cyto} (labelled with Atto 488) on supported lipid bilayers to demonstrate the liquid-like property. Left panel: photobleaching, middle panel: fluorescent recovery after bleaching, and right panel: real time quantification of FRAP data for pFGFR2_{Cyto}.

(B) The knockdown levels of endogenous FGFR2 and SHP2 was examined by immunoblotting.

(C) The average exogenous expression level of SHP2_{C459S} in HEK293T SHP2 KO cells was adjusted in order to mimic the endogenous SHP2 expression.

(D) HILO image showing that the FGFR2_{ΔVT-EGFP} droplets (coexpressed with untagged SHP2_{C459S} and PLCγ1; stimulated with 10ng/ml FGF9) are localised close to the coverslip (improved contrast in HILO microscopy. Data were presented as depth-coded images ((i): XY view and (ii): XZ view), colour bar: 0-14µm. In the XZ view (ii), it is clear that many of the droplets, at different brightnesses, are visible along the flat line of the coverslip, and hence localised on the plasma membrane.

(E) HILO image showing that: (i) fusion event of FGFR2_{ΔVT-EGFP} droplets (coexpressed with untagged SHP2_{C459S} and PLCγ1; stimulated with 10 ng/ml FGF9) on the membrane. The four time lapse images were monitored from the red box shown in the whole cell image. Scale bar for the whole cell image: 5 µm; scale bar for the time lapse image: 500 nm. (ii) fusion/fission event of PLCγ1-EGFP droplets (coexpressed with untagged FGFR2_{ΔVT} and SHP2_{C459S}; stimulated with 10 ng/ml FGF9) on the membrane. The four time lapse images were monitored from the red box shown in the whole cell image. Scale bar for the whole cell image: 5 µm; scale bar for the time lapse image: 500 nm. (iii) EGFP alone control. No droplet formation. Scale bar: 5 µm
Figure S4. The molecular interaction of FGFR2 with SHP2, Related to Figure 4.

(A) MST isotherm for the binding of pFGFR2Cyto to full length SHP2\textsubscript{C459S} (K\textsubscript{d} = 0.52±0.0 µM; red curve) or the truncated tandem SH2 domain SHP2\textsubscript{2SH2} (K\textsubscript{d} = 25.1±1.63 µM; green curve).

(B) BLI experiments were used to confirm the phosphorylation-dependent interaction and the specific domains required for binding. GST-SHP2\textsubscript{2SH2}, GST-SHP2\textsubscript{PTP C459S} and GST-SHP2\textsubscript{C69} were immobilized on GST sensors and pFGFR2\textsubscript{Cyto} (5 µM) and pFGFR2\textsubscript{Kinase} (5 µM) were used to test the binding. pFGFR2\textsubscript{Cyto} clearly interacts with GST-SHP2\textsubscript{2SH2}. The expected weak binding to pFGFR2\textsubscript{Kinase} is not visible due to the concentration of this reagent being below the K\textsubscript{d} for the interaction. Black - immobilized GST-SHP2\textsubscript{2SH2} with pFGFR2\textsubscript{Cyto}; red - immobilized GST-SHP2\textsubscript{2SH2} with pFGFR2\textsubscript{Kinase}; blue - immobilized GST-SHP2\textsubscript{PTP C459S} with pFGFR2\textsubscript{Cyto}; pink - immobilized GST-SHP2\textsubscript{PTP C459S} with pFGFR2\textsubscript{Kinase}; green - immobilized GST-SHP2\textsubscript{C69} with pFGFR2\textsubscript{Cyto}; dark blue - immobilized GST-SHP2\textsubscript{C69} with pFGFR2\textsubscript{Kinase}; grey - immobilized GST with pFGFR2\textsubscript{Cyto}. Ligand-analyte association (0 sec) and dissociation (buffer washing, 120 sec) are indicated by red arrows.

(C) NMR spectra of \textsuperscript{1}H, \textsuperscript{15}N-labelled isolated SHP2\textsubscript{2SH2} (100 µM, black) and with added pFGFR2\textsubscript{C58} (300 µM, red). The chemical shifts for individual residues (i.e. movement of peaks from black to red positions) confirm direct interaction across a broad interface.

(D) Binding of SHP2\textsubscript{2SH2} (10 µM) with the pFGFR2\textsubscript{C58} (100 µM) shown by ITC. The top panel shows raw data for the titration; the bottom panel shows integrated peaks plotted on axes with molar heat of binding versus the molar ratio of titrated protein fitted to a single-site binding model. Heats of dilution were measured in a separate control experiment and subtracted from binding data prior to fitting. Importantly the stoichiometry of the interaction 1:1 confirms that only CSH2 from the tandem SH2 domains recognizes pY769 on the receptor.

(E) (Top) GST-SHP2\textsubscript{2SH2}, GST-SHP2\textsubscript{2SH2 R32A}, GST-SHP2\textsubscript{2SH2 R138A} and GST-SHP2\textsubscript{2SH2 R32/138A} were used to pull down FGFR2\textsubscript{ΔVT} from HEK293T cells stimulated with FGF9 (10 ng/ml). Mutation of R138 abrogates binding of FGFR2 confirming the requirement of the wild type
CSH2 domain for binding to receptor. (Bottom) Densitometry analysis of GST pull down. n=2. Data were presented as mean ± SD. Replicate data shown in Data S1E.

(F) GST-SHP2Sh2 R32A, GST-SHP2Sh2 R138A and GST-SHP2Sh2 R32/R138A were used to pull down recombinant pFGFR2Cyo. Mutation of R138 abrogates binding of FGFR2 confirming the requirement of a wild type CSH2 domain for binding to receptor.
**Figure S5.** The molecular interaction of SHP2 with PLCγ1, Related to Figure 5.

(A) MST isotherm for the binding of synthesized PLCγ1-derived tyrosyl phosphopeptides containing pY771, pY775 or pY783 to labelled SHP2_{2SH2}. No significant interaction was found with any of the phosphopeptides.

(B) ITC isotherms of SHP2_{2SH2} binding to PLCγ1 pY771, pY775, and pY783 tyrosyl phosphopeptides. Twenty 3 µl injections of each phosphopeptide (100 µM) were titrated into SHP2_{2SH2} (10 µM) at 25°C. Top, baseline-corrected power-versus-time plot for the titration. Bottom, integrated heats and curve fitting using Origin™ software.

(C) MST measurement of SHP2_{2SH2} binding to labelled PLCγ1_{2SH2}, blue curve; or labelled pPLCγ1_{2SH2}, red curve. No significant difference was observed in their binding affinities to SHP2_{2SH2}.

(D) MST isotherm for the interaction of labelled SHP2_{2SH2} R32/138A mutant and pPLCγ1_{2SH2}, showing that the interaction is not based on the canonical binding of pY to an SH2 domain.

(E) NMR 1\textsuperscript{H} and 15\textsuperscript{N} chemical shift changes on addition of SHP2_{2SH2} (600 µM) to 15\textsuperscript{N}-labelled PLCγ1_{2SH2} (200 µM). Blue squares highlight some of the residues on the spectrum showing shift changes; which are magnified in (F).

(G) 1\textsuperscript{H}, 15\textsuperscript{N} peak assignments mapped onto the space-filling model crystal structure of PLCγ1_{2SH2} (PDB code: 4FBN). The orientations of the structure are as shown in Fig. 5B. The coverage of assigned residues of PLCγ1_{2SH2} is 46.5% (red, assigned residues).

(H) MST isotherm for the interaction of labelled SHP2_{2SH2} and a preformed complex between PLCγ1_{2SH2} and a tyrosyl phosphopeptide containing pY783 showing that the binding of pY783 does not hinder the tandem SH2 domain interface.

(I) NMR 1\textsuperscript{H} and 15\textsuperscript{N} chemical shift changes on addition of PLCγ1_{2SH2} (600 µM) to 15\textsuperscript{N}-labelled SHP2_{2SH2} (100 µM). Blue squares highlight some of the residues on the spectrum showing shift changes; which are expanded in (J).
Figure S6. Characterization of FGFR2-SHP2-PLCγ1 droplets properties, Related to Figure 6.

(A) Phase diagram of pFGFR2<sub>Cyto</sub>-Atto 488 and SHP2<sub>2SH2</sub>-Atto 550 droplet formation in a buffer containing 20 mM HEPES (pH7.5), 150 mM NaCl, and 1 mM TCEP. The sizes of the circles represent the average sizes of droplets (μm<sup>2</sup>) and the colour scale bars represent the numbers of droplets in a 0.0256 mm<sup>2</sup> area.

(B) FRAP recovery curve for pFGFR2<sub>Cyto</sub>-Atto 488 and SHP2<sub>2SH2</sub> (means ± SD, n = 2 experiments).

(C) Phase diagram of pFGFR2<sub>Cyto</sub>-Atto 488 and SHP2<sub>2SH2</sub>-Atto 550 droplet formation with 20 µM of pY769 peptide and SH2 inhibitor peptide in a buffer containing 20 mM HEPES (pH7.5), 150 mM NaCl, and 1 mM TCEP. The addition of both inhibitor abolishes the formation of droplets. The sizes of the circles represent the average sizes of droplets (μm<sup>2</sup>) and the colour scale bars represent the numbers of droplets in a 0.0256 mm<sup>2</sup> area.

(D) Inhibition of pFGFR2<sub>Cyto</sub>-Atto 488 (10 µM) and SHP2<sub>C459S</sub>-Atto 550 (30 µM) droplet formation by 20 µM or 100 µM of pY769 peptide or SH2 inhibitor peptide. Scale bar = 10 µm.

(E) MST measurements of SHP2<sub>2SH2</sub>R138A binding to pFGFR2<sub>Kinase</sub> with single Y to F mutants of all of the individual tyrosines on the kinase domain. This result shows multivalent, weak binding between SHP2 NSH2 domain and any of the available pY residues.

(F) The dynamic LLPS property of pFGFR2<sub>Cyto</sub> (10 µM)-SHP2<sub>2SH2</sub> (30 µM)-PLCγ1<sub>2SH2</sub> (12 µM) droplets was monitored by the fusion experiment. This experiment demonstrated the tandem SH2 domains of SHP2 and PLCγ1 are the minimum requirement to form LLPS droplets with pFGFR2<sub>Cyto</sub>. Scale bar = 10 µm.

(G) The white light image of pFGFR2<sub>Cyto</sub>-SHP2-PLCγ1 droplets used for enzymatic assay.

(H) Purified pFGFR2<sub>Cyto</sub> (10 µM) was incubated with ATP/MgCl<sub>2</sub> (5 mM) in the presence or absence of 10% 1,6-hexanediol in a buffer containing 20 mM HEPES (pH7.5), 150 mM NaCl,
and 1 mM TCEP. The effect of 1,6-hexanediol on pFGFR2<sub>Cyto</sub> activity was monitored by pFGFR2 immunoblotting at different time points as indicated. This result demonstrated that 10% 1,6-hexanediol does not affect kinase activity in the LLPS condition.

(I) Purified SHP2 (60 µM) was incubated with a phospho-substrate (Methods: *In vitro* phosphatase assay) in the presence or absence of 10% 1,6-hexanediol in a buffer containing 20 mM HEPES (pH7.5), 150 mM NaCl, and 1 mM TCEP. The effect of 1,6-hexanediol on SHP2 activity was monitored by the turnover rate of the substrate. This result demonstrated that 10% 1,6-hexanediol does not affect phosphatase activity in the LLPS condition. n=2. Data were presented as mean ± SD.

(J) Purified pFGFR2<sub>Cyto</sub> (10 µM), SHP2<sub>C459S</sub> (60 µM), and pPLCγ1 (50 µM) were incubated with artificial pPLCγ1 substrate 4-methylumbelliferyl myo-inositol-1-phosphate, N-methylmorpholine salt in a buffer containing 20mM HEPES (pH7.5), 150mM NaCl, and 1mM TCEP to test the specificity of pFGFR2<sub>Cyto</sub>, SHP2<sub>C459S</sub>, and pPLCγ1 with the substrate. SHP2<sub>C459S</sub> shows a low level of nonspecific reaction (blue and brown curves). The addition of 10% 1,6-hexanediol upregulates pPLCγ1 activity (purple curve) comparing with pPLCγ1 alone (green curve). Sample sizes = 8, Data were presented as mean ± SD.
Supplementary Figure 7

A

Caco-2  Caco-2 SHP2

FGF9

100 kDa + 15 min

75 kDa + 15 min

50 kDa + 60 min

DMSO

IB: anti-pPKCβII (pS660)

IB: anti-pAKT (pS473)

IB: anti-αTubulin

B

A431 DMSO  A431 NSC87877

IB: anti-pPKCβII (pS660)

IB: anti-pPLCγ1 (pY783)

IB: anti-pAKT (pS473)

IB: anti-αTubulin

C

MCF7 transfection

Mock  RFP  RFP-C58

IB: anti-pPKCβII (pS660)

IB: anti-pPLCγ1 (pY783)

IB: anti-pAKT (pS473)

IB: anti-αTubulin

IB: anti-RFP

D

Caco2 transfection

FGF9

75 kDa

50 kDa

10 kDa

IB: anti-pPKCβII (pS660)

IB: anti-pPLCγ1 (pY783)

IB: anti-pAKT (pS473)

IB: anti-αTubulin

IB: anti-Myc

E

SHP2 WT  FGF2 WT

SHP2 WT  FGF2 WT

$K_f = 5.82 \pm 0.077 \mu M$

$K_f = 1.29 \pm 0.09 \mu M$
Figure S7. FGFR2-SHP2-PLCγ1 signalling regulated by phase separation, Related to Figure 7.

(A) (Top) Western blot showing Caco-2 cells with knock down of SHP2 (Caco-2 SHP2i). As seen in MCF7 SHP2 KO and A431 SHP2i cells the phosphorylation of PLCγ1 Y783 is increased on FGF9 stimulation (10 ng/ml) of FGFR2. Phosphorylation of S660 on PKCβII and S473 on AKT act as markers for up-regulation of PLCγ1 signalling. This is suppressed in the absence of SHP2 in the Caco-2 SHP2i cells. (Bottom) Densitometry analysis of SHP2 expression and the activation levels of various signalling proteins (dark cyan: parental cells; light cyan: SHP2 depletion cells). n=2. Data were presented as mean ± SD. Replicate data shown in Data S3D.

(B) (Left) Western blot showing the presence of phosphorylated downstream effector proteins in A431 cells without (using only DMSO vehicle) or with 50 µM of NSC87877 SHP2 inhibitor. The negligible change of phosphorylation of PKCβII and AKT upon FGF9 stimulation (10 ng/ml) shows that SHP2 phosphatase activity does not affect PLCγ1 activity. (Right) Densitometry analysis of the activation levels of various signalling proteins (dark blue: DMSO treatment; light blue: NSC87877 SHP2 inhibitor treatment). n=3. Data were presented as mean ± SD. Replicate data shown in Data S3E.

(C) (Left) Transfection of RFP-tagged FGFR2c58 to FGF9-stimulated (10 ng/ml) MCF7 cells results in the down regulation of PKCβII activity. (Right) Densitometry analysis of PKCβII activity. n=3. Data were presented as mean ± SD. Replicate data shown in Data S3F.

(D) Transfection of Myc-tagged FGFR2 pY769 peptide to Caco-2 cells followed by stimulation of FGF9 (10 ng/ml) results in the down regulation of PKCβII activity, but has no effect on AKT activity.

(E) MST isotherm for the binding of SHP22SH2 NMR. The mutations on the interface decrease the interaction with pPLCγ12SH2 with an affinity of 5.83±0.077 µM (green curve) compared with wild
type SHP2_{2SH2} \ (K_d = 0.48\pm0.0 \ 4\mu M, \text{Fig. S5C}), \text{ but it does not affect the interaction with pFGFR2_{Cyto} \ (K_d = 15.7\pm0.291 \ \mu M; \text{red curve}).}
Data S1. Replicated western blots, Related to Figure 4 and Figure S4.

A) Figure 4A replicate 2

B) Figure 4B replicate 2

C) Figure 4C replicate 2

D) Figure 4D replicate 2

E) Figure S4E replicate 2

Data S1. Replicated western blots, Related to Figure 4 and Figure S4.
Data S2. Replicated western blots, Related to Figure 5 and Figure 6.

A

Figure 5E replicate 2

Figure 5E replicate 3

B

Figure 6D replicate 2

Figure 6D replicate 3

Figure 6D replicate 4

Figure 6D replicate 5

C

Figure 6E replicate 2

D

Figure 6F replicate 2
Data S3. Replicated western blots, Related to Figure 7 and Figure S7.