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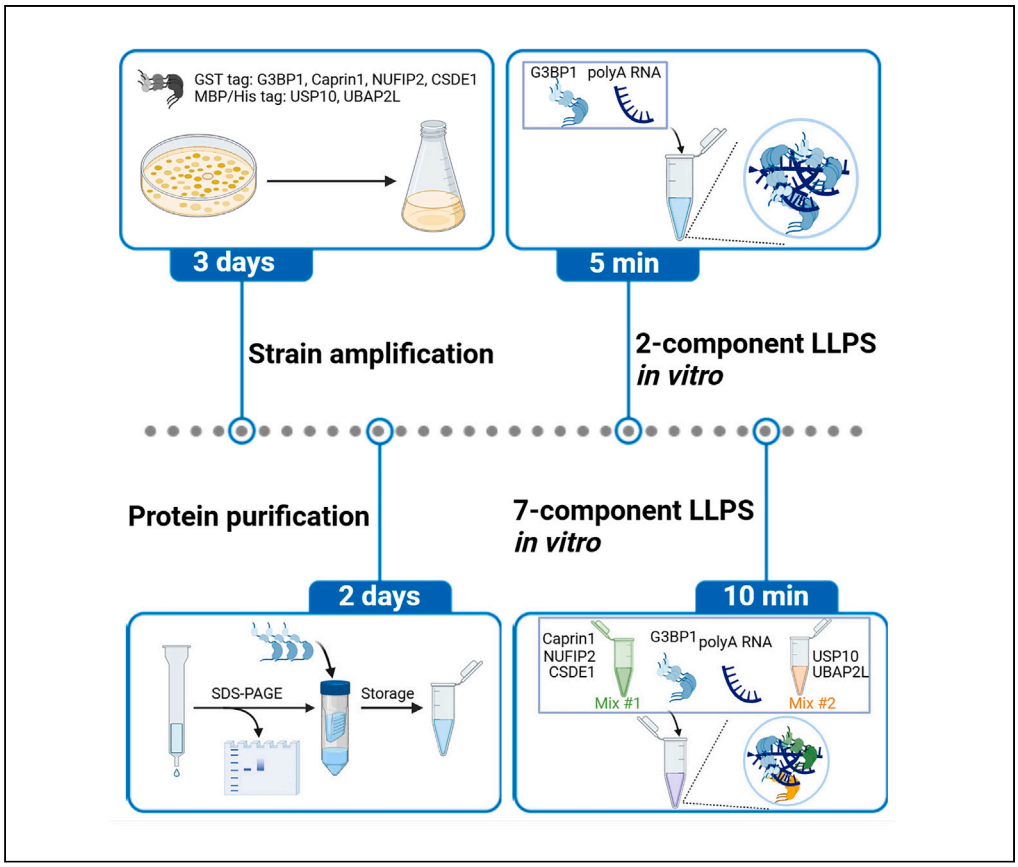
Protocol for multi-component reconstitution of stress granules *in vitro*



To date, stress granule studies mainly focus on cell models. The diversity of molecules in stress granules makes it challenging to uncover the function of each molecule in stress granule regulation. Here, we provide a protocol to reconstitute stress granules with multi-components *in vitro*. We describe steps for strain amplification, protein purification, and liquid-liquid phase separation (LLPS). The multi-component reconstitution system constructed in this protocol also provides a technique for other condensate reconstitution studies *in vitro*.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Purification of G3BP1, Caprin1, NUFIP2, USP10, CSDE1, and UBAP2L proteins

Instructions for multi-component reconstitution of stress granules *in vitro*

Guidance on applying the multi-component reconstitution system

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Protocol for multi-component reconstitution of stress granules *in vitro*

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SUMMARY

To date, stress granule studies mainly focus on cell models. The diversity of molecules in stress granules makes it challenging to uncover the function of each molecule in stress granule regulation. Here, we provide a protocol to reconstitute stress granules with multi-components *in vitro*. We describe steps for strain amplification, protein purification, and liquid-liquid phase separation (LLPS). The multi-component reconstitution system constructed in this protocol also provides a technique for other condensate reconstitution studies *in vitro*.

For complete details on the use and execution of this protocol, please refer to Yao et al.[1]

BEFORE YOU BEGIN

As the most critical node in the protein-RNA network for stress granules (SGs) assembly, G3BP1 protein can undergo liquid-liquid phase separation (LLPS) in the presence of RNA *in vitro*.[2–4] This two-component system has served as a simplified model to study the principles of G3BP1 and RNA involved in phase separation and is broadly used for SG study. However, the simplified two-component system fails to mimic stress granules due to the lack of other core network components for SGs assembly in cells, such as Caprin1 and USP10.[1] The protocol below describes the detailed steps for reconstituting liquid-liquid phase separation *in vitro* with 7-component, which are key components of the SG interaction network, including G3BP1, Caprin1, USP10, UBAP2L, CSDE1, NUFIP2, and polyA RNA. Our protocol can efficiently reflect the multi-valence mediating LLPS *in vitro*, such as dynamic and enrichment of G3BP1 protein in the liquid droplets, and thus presents a highly effective strategy to study the protein-protein interaction network in stress granules assembly *in vitro*. In addition, we will provide more technical details on purifying G3BP1 and other proteins in this protocol.

Before starting the reconstitution experiment, we describe the steps for construction of plasmids. G3BP1, Caprin1, NUFIP2, and CSDE1 are inserted in the pGEX-6p-1 vector with GST tag, and UBAP2L and USP10 are expressed in pMAL-c5x vector with MBP and His tag. Here, recombinant G3BP1, Caprin1, UBAP2L, and NUFIP2 proteins are purified using bacterial codon optimized constructs, while CSDE1 and USP10 are using human cDNA. All proteins are expressed in *E.coli* with rare tRNA transgenes. Next, we describe the steps to reconstitute stress granules *in vitro* with purified proteins and commercialized polyA RNA. We recommend using UBAP2L fused with MBP because it is prone to aggregate *in vitro* when the tag is removed. Finally, we assessed the 7-component reconstitution system by measuring the droplet area to represent the ability of LLPS of G3BP1 or its orthologs via labeling Caprin1 with EGFP.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Rosetta (DE3) chemically competent cell	BioMed	Cat# BC204-01
DH5α chemically competent cell	TsingKe	Cat# TSV-A07
Chemicals, peptides, and recombinant proteins		
L-glutathione	Sigma-Aldrich	Cat# G4251-50G
Imidazole	Sigma-Aldrich	Cat# I2399-100G
Ribonuclease A	Takara	Cat# 740505
Chloramphenicol	Inalco	Cat# 1758-9321
Ampicillin, sodium salt	Sangon Biotech	Cat# A610028-0025
IPTG	JSENB	Cat# JS0154
SimplyBlue SafeStain	Thermo Fisher Scientific	Cat# LC6060
Dithiothreitol (DTT)	Sigma-Aldrich	Cat# 43815
PMSF	Alpha Diagnostic	Cat# PMSF16-S-250
Polyadenylic acid	Sigma-Aldrich	Cat# 10108626001
TRIzol Reagent	Thermo Fisher Scientific	Cat# 15596018
Critical commercial assays		
Phanta Max Super-Fidelity DNA Polymerase	Vazyme	Cat# P505
HiScript III 1st Strand cDNA Synthesis Kit (+gDNA wiper)	Vazyme	Cat# R312
FastPure Gel DNA Extraction Mini Kit	Vazyme	Cat# DC301
ClonExpress II One-Step Cloning Kit	Vazyme	Cat# C112
EndoFree Mini Plasmid Kit II	TIANGEN	Cat# 4992422
Recombinant DNA		
pGEX-6P-1-G3BP1	Yao et al.[1]	WeKwikGene # 0000914
pGEX-6P-1-Caprin1	Yao et al.[1]	WeKwikGene # 0000944
pGEX-6P-1-CSDE1	Yao et al.[1]	WeKwikGene # 0000947
pGEX-6P-1-NUFIP2	Yao et al.[1]	WeKwikGene # 0000946
pMAL-c5x-UBAP2L	Yao et al.[1]	WeKwikGene # 0000949
pMAL-c5x-USP10	Yao et al.[1]	WeKwikGene # 0000950
pGEX-6P-1-Caprin1-EGFP	Yao et al.[1]	WeKwikGene # 0000945
Software and algorithms		
ImageJ		
GraphPad Prism9.5		
Photoshop CS6	Adobe	
Illustrator	Adobe	
SnapGene		
Expasy - ProtParam		
Other		
High-affinity Ni-charged resin FF	GenScript	Cat# L00666
Glutathione resin	GenScript	Cat# L00206
Affinity chromatography column empty column tube (60 mL)	Beyotime	Cat# FCL60
Econo-Pac chromatography columns, Pkg of 50	Bio-Rad	Cat# 7321010
Syringe filters, 33 mm, 0.22 μm, PES	Biosharp	Cat# BS-PES-22
Disposable experimental syringe (sterile, individually packaged, with needle, 10 mL)	Beyotime	Cat# FS810-60pcs
Amicon Ultra filter, 3 kDa MWCO	Millipore	Cat# UFC5003
Amicon Ultra filter, 10 kDa MWCO	Millipore	Cat# UFC8010
Cover glass	Citotest	Cat# 80340-0130
Slide	Citotest	Cat# 80312-2101
FuturePAGE 4%–20% 15 wells	ACE	Cat# ET15420Gel

MATERIALS AND EQUIPMENT

Note: All buffers detailed below should be filtered through a Biosharp PES (0.22 μm) prior to use.

Buffer A: lysis buffer for GST-tag proteins		
Reagent	Final concentration	Amount
Tris-HCl (pH 7.6, 1 M)	50 mM	50 mL
NaCl (5 M)	400 mM	80 mL
DTT (1 M)	1 mM	–
PMSF (100 mM)	1 mM	–
ddH_2O	N/A	870 mL
Total	**N/A**	**1000 mL**
DTT and PMSF are added directly before use and once added, the buffer is stable for 1 day at room temperature.		

Buffer B: lysis buffer for His-tag proteins		
Reagent	Final concentration	Amount
Tris-HCl (pH 7.6, 1 M)	50 mM	50 mL
NaCl (5 M)	400 mM	80 mL
DTT (1 M)	1 mM	–
PMSF (100 mM)	1 mM	–
Imidazole	30 mM	2 g
ddH_2O	N/A	870 mL
Total	**N/A**	**1000 mL**
DTT and PMSF are added directly before use and once added, the buffer is stable for 1 day at room temperature.		

Buffer C: elution buffer for GST-tag proteins		
Reagent	Final concentration	Amount
Tris-HCl (pH 7.6, 1 M)	50 mM	0.5 mL
NaCl (5 M)	400 mM	0.8 mL
DTT (1 M)	1 mM	–
L-glutathione	10 mM	30 mg
ddH_2O	N/A	8.7 mL
Total	**N/A**	**10 mL**
DTT and PMSF are added directly before use and once added, the buffer is stable for 1 day at room temperature. The L-glutathione is added before use and once added, the buffer is stored on the ice.		

Buffer D: elution buffer for His-tag proteins		
Reagent	Final concentration	Amount
Tris-HCl (pH 7.6, 1 M)	50 mM	0.5 mL
NaCl (5 M)	400 mM	0.8 mL
DTT (1 M)	1 mM	–
Imidazole	300 mM	200 mg
ddH_2O	N/A	8.7 mL
Total	**N/A**	**10 mL**
DTT and PMSF are added directly before use and once added, the buffer is stable for 1 day at room temperature.		

Buffer E: LLPS reaction buffer *in vitro*	
Reagent	Final concentration
Tris-HCl (pH 7.6, 1 M)	50 mM
NaCl (5 M)	150 mM

STEP-BY-STEP METHOD DETAILS

Purification of G3BP1, CSDE1, NUFIP2, Caprin1, and Caprin1-EGFP proteins fused with GST tag

Timing: 7 days

The sequences of G3BP1, CSDE1, NUFIP2, Caprin1, and Caprin1-EGFP that are codon optimized for *E.coli* are inserted into pGEX-6p-1 vector that is linearized via XhoI and BamHI. GST tag is fused at the N-terminus. Fusion GST proteins are captured using glutathione resin, and the GST tag is removed by TEV protease between GST and each protein; the cleaved proteins are further purified using size exclusion chromatography (SEC), shown in Figures 1A–1C.

1. Construct the plasmids using the PCR reaction shown below.
   a. Prepare PCR reaction as the PCR master mix.

PCR reaction master mix	
Reagent	Amount
DNA template	1 μL
DNA Polymerase	1 μL
Primer 1	2 μL
Primer 2	2 μL
2× Phanta Max Buffer	25 μL
dNTP Mix (10 mM each)	1 μL
ddH_2O	18 μL

   b. Prepare PCR cycling reaction.

PCR cycling conditions			
Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	15 s	
Denaturation	95°C	15 s	32 cycles
Annealing	56°C	30 s	
Extension	72°C	1 min	
Final extension	72°C	3 min	
Hold	4°C	On hold	

   c. Linearize the pGEX-6p-1 vector with XhoI and BamHI.

Linearize vector conditions	
Reagent	Amount
Vector	2 μg
XhoI	1 μL
BamHI	1 μL
10× CutSmart Buffer	5 μL
ddH_2O	Up to 50 μL

   d. Assemble two linearized DNA fragments into a plasmid and transform it into the DH5α chemically competent cells to screen the single positive clone.
2. Select a single positive clone and amplify the DH5α chemically competent cells to extract plasmids. Transform the plasmid into Rosetta (DE3) chemically competent cells to express individual proteins. Select positive clone by plating on LB agar plate with 100 μg/mL Ampicillin.

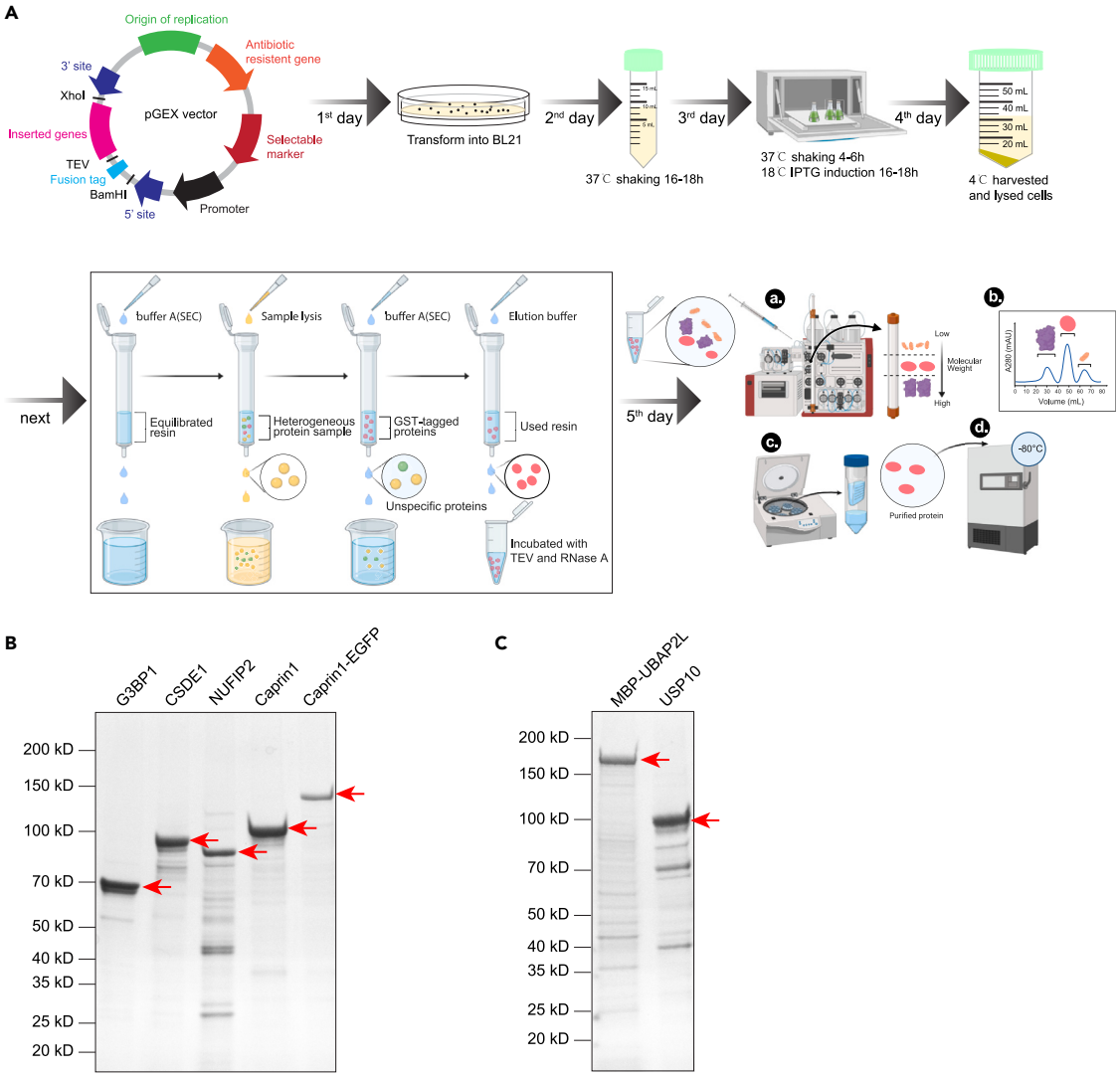

Figure 1. Purification of recombinant proteins from bacteria

(A) Steps for the protein purification in *E.coli*. The GST or MBP is cleaved with TEV protease.

(B) Coomassie-stained 4–20% acrylamide gel for proteins purified by GST tag. The expected size of G3BP1, CSDE1, NUFIP2, Caprin1, and Caprin1-EGFP is about 62, 90, 80, 100, and 126 kD, respectively.

(C) Coomassie-stained 4–20% acrylamide gel for proteins purified by MBP/His tag. The expected size of MBP-UBAP2L and USP10 is about 160 and 100 kD, respectively.

3. Inoculate a single clone into 10 mL of LB with 100 μg/mL Ampicillin and 25 μg/mL chloramphenicol, and place at 37°C with shaking at 200 rpm over 16 h.

   Note: The Rosetta (DE3) Chemically Competent Cell used contains the rare tRNA transgene, which confers chloramphenicol resistance.

4. The next day, transfer the 10 mL culture into 1 L of LB with 100 μg/mL Ampicillin and 25 μg/mL chloramphenicol. Place the culture at 37°C with shaking at 200 rpm for 4–6 h till OD_{600} is over 0.8.
5. Cool down the culture by placing it at 18°C with shaking at 200 rpm.
6. Add IPTG with a final concentration of 1 mM into the culture to induce protein expression at 18°C with shaking at 200 rpm for 18 h.
7. Next day: harvest and lyse the cells.

a. Harvest the cells by centrifugation at 8,000 × *g* for 15 min at 4°C.
b. Resuspend the cell pellet with 25 mL cold buffer A supplemented with DTT and PMSF and place it on the ice.
c. Lyse cells at 4°C via ultrasonication for 15 min with 2s of power on and 5s of power off, at 30% power energy output.

Note: The whole process should be performed on ice.

d. Harvest the lysate with centrifugation at 18,000 × g for 20 min at 4°C and collect the supernatant.
e. Filter the supernatant to remove the residual pellet with an empty gravity column.

8. Purify proteins with glutathione resin.
   a. Add 5 mL glutathione resin into the gravity column and wash the resin with 5 mL buffer A 3 times.

   Note: Consider performing this step in a cold room or refrigerator if the protein is temperature-sensitive. For proteins used in this protocol, the supernatant is kept cold on ice, and the gravity column purification is performed at 25°C. Washing and elution buffers are also kept cold on ice during the whole process.

   b. Transfer 30 mL supernatant (7e) into the column with resin gradually till all the samples are loaded and applied.
   c. Wash the resin with 5 mL buffer A 5 times.

   Note: Monitor the washing step by detecting protein in flow-through fraction by Coomassie Brilliant Blue G250. Usually, there is no protein detected after 5 times washes.

   d. Elute and collect proteins with buffer C into the 1.5 mL EP tube and place them on the ice till no proteins can be detected in the elution fraction by Coomassie Brilliant Blue G250. Most proteins can be eluted in about 6 mL elution buffer.
   e. Regenerate resin with 0.5 M NaOH and store the resin for reuse in 70% ethanol at 4°C.
9. SDS-PAGE detection and remove GST-tag
   a. Mix 5–10 μL of eluted fraction with SDS-PAGE sample buffer and load into SDS-PAGE gel. Confirm the expression pattern and purity of target proteins by Coomassie gel staining.
   b. Pool the fractions together and incubate the target proteins with 0.01 mg/mL of TEV protease and 10 μg/mL RNase A overnight at 4°C with gentle rotation to cleave GST. Save an aliquot before TEV addition for control to compare tag cleavage.

   Note: RNase A is added to remove RNA contamination for G3BP1 protein and other RNA-binding proteins because the presence of RNA significantly affects the phase separation of G3BP1 protein *in vitro*.

10. Next day: Isolate and purify proteins with Size Exclusion Chromatography using buffer A.
    a. Confirm the tag cleavage by SDS-PAGE.
    b. Filter proteins after cleaving the tag with a 0.22 μm PES syringe filter to remove aggregates.
    c. Load the proteins onto a Superdex 200 column equilibrated in buffer A using an AKTA Pure Protein Purification System with UV-Vis monitor.
    d. Collect the elution in 2 mL aliquots.
    e. Check the fractions with SDS-PAGE.
11. Collect and store the purified proteins.
    a. Concentrate G3BP1, CSDE1, and Caprin1 proteins to ~50 μL with Amicon Ultra Centrifugal Filters with 3 kD molecular weight cutoff. Centrifuge at 8,000 × g at 4°C for 20 min.

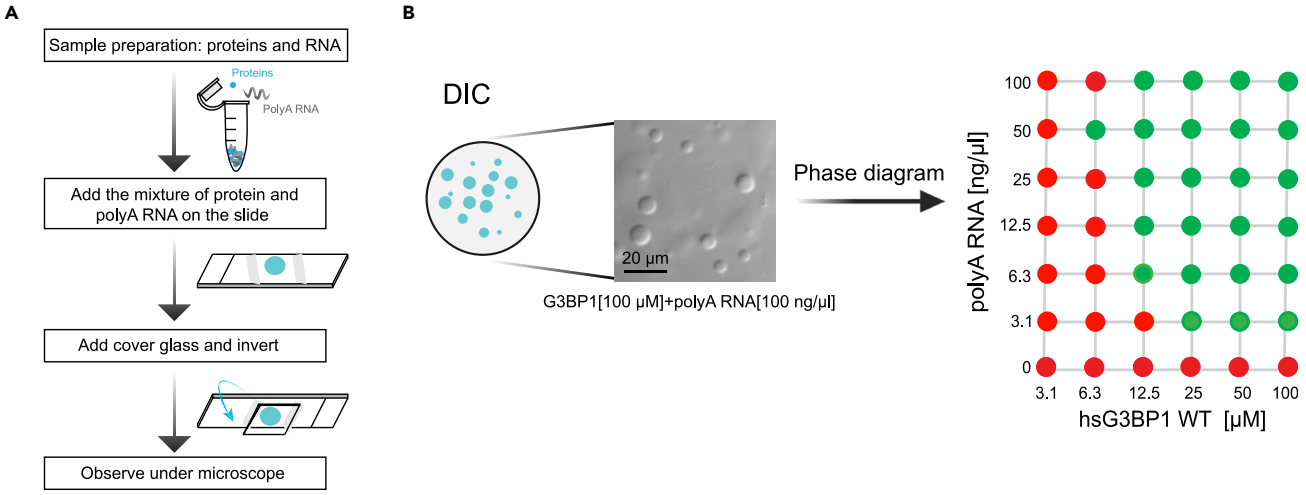

Figure 2. Liquid-liquid phase separation of G3BP1 and polyA RNA

(A) Steps for liquid-liquid phase separation *in vitro*.

(B) Phase diagram of LLPS of G3BP1 in the presence of polyA RNA *in vitro*. Scale bar 20 μm. The green dots indicate the presence of LLPS, and the red dots indicate the absence of LLPS.

b. Monitor protein concentration by the method as following:

$$\text{Protein Concentration}_{[\mu M]} = [A280/(\text{Extinction coefficients})] \times 10^6$$

Note: The value of the Extinction coefficient for each protein is calculated by https://web.expasy.org/protparam/.

c. Store proteins in aliquots in 0.2 mL PCR tubes.

d. Flash-freeze in liquid nitrogen and store at −80°C.

Purification of USP10 and UBAP2L proteins fused with MBP/His tag

Timing: 7 days

The sequences of USP10 and UBAP2L are inserted into pMAL-c5x vector that is linearized via EcoRV and HindIII. MBP tag is fused at the N-terminus to increase the solubility of proteins, and 6 × His tag is fused at the C-terminus for purification. The proteins are captured using Ni-resin. The MBP tag is removed by TEV protease expressed between MBP and each protein. The target proteins are further purified using size exclusion chromatography (SEC). The protocol for purification of MBP/His tag proteins is the same as the above steps 1-11, shown in Figures 1A–1C, except that different buffers are used. For fusion MBP/His tag proteins purification, buffers B and D are used during the lysis and elution process, respectively.

Liquid-liquid phase separation assay *in vitro*

Timing: ~2 h

Induce LLPS of G3BP1 in the presence of polyA RNA. The process is referred to in Figures 2A and 2B. All reagents are prepared on ice, and all LLPS processes are performed at 25°C.

12. Thaw the protein aliquots on ice.

13. Dilute G3BP1 to a high concentration stock, such as 200 μM, using Tris-HCl pH 7.6 to reach a final concentration of 300 mM NaCl.
14. Make a serial dilution of the above G3BP1 stock with a working buffer (Tris-HCl pH 7.6, 300 mM NaCl) to reach 100, 50, 25, 12.5, and 6.25 μM, respectively.
15. Dissolve polyA RNA (Sigma Cat# 10108626001) with lengths ranging from 2.1 to 10 kb in H_2O. Aliquot the dissolved polyA and store at −80°C. Dilute polyA RNA using basic working buffer Tris-HCl pH 7.6 to reach a concentration of 200 ng/μL
16. Observe the *in vitro* LLPS reaction under sandwiched slides (shown in Figure 2A) composed of a slide, double-sided tape, and cover glass.
17. Add 1 μL polyA RNA with a different concentration gradient into 1 μL G3BP1 with different concentration gradients and mix in a 0.2 mL PCR tube to induce LLPS of G3BP1 in the final buffer with 150 mM salt concentration.

Note: A thorough mix is critical for generating a homogenous sample.

18. Add 2 μL LLPS mixture on the slide and gently cover with the cover glass. Flip the slide, letting droplets set on the cover glass under gravity.
19. Capture pictures with the Olympus IX73 60X oil lens under wide field within 3–5 min, with representative image shown in Figure 2B.

Note: We perform the DIC imaging on an Olympus IX73 wide-field microscope with a 60X oil lens. For multi-color imaging, we conduct the experiments on an Olympus FV3000 laser scanning confocal microscope equipped with a 60X oil-immersion objective. However, for both assays, an equivalent wide-field microscope or point-scanning microscope may also be sufficient.

20. Draw the diagram of LLPS of G3BP1+polyA RNA two-component system, shown in Figure 2B.

Note: Complete the image capture step within 3–5 min to reduce G3BP1-droplets wetting on the cover glass. It is critical to set a comparable time window for different experimental groups, as the G3BP1-RNA droplets are highly dynamic and undergo wetting on cover glass very fast. The number and size of droplets vary for the same sample observed at longer time windows.

LLPS of the three-component system is composed of G3BP1, polyA RNA, and another protein

Timing: ~2 h

21. Dilute G3BP1, Caprin1, CSDE1, UBAP2L, NUFIP2, and USP10 proteins to the stock concentration as 200, 400, 400, 54, 100 and 200 μM using basic working buffer Tris-HCl pH 7.6, respectively. The stock salt concentration of NaCl is 300 mM.
22. Dilute Caprin1, CSDE1, UBAP2L, NUFIP2 and USP10 using working buffer (Tris-HCl pH 7.6, 300 mM NaCl) to a 2× final concentration.
23. Dilute polyA RNA as in step 15 and assemble the chamber slides for LLPS assay as in step 16.
24. Add 1 μL protein with a different concentration into 1 μL G3BP1 with a concentration of 200 μM and mix in a 0.2 mL PCR tube.
25. Add 2 μL polyA RNA with a concentration of 200 ng/μL into the above protein system and mix in the 0.2 mL PCR tube to induce LLPS in the final buffer with 150 mM salt concentration.
26. Observe and capture pictures referring to steps 18-20.

7-Component reconstitution LLPS *in vitro*

Timing: ~3 h

Each protein in this multi-component reconstitution system promotes LLPS of G3BP1 *in vitro* within the appropriate concentration range, including USP10, which inhibits stress granule assembly in cells with a high concentration. Since there is a direct interaction between each protein and G3BP1, combined with the expression level of each protein in the cells and the efficiency of each protein in mediating SG assembly, we chose the stoichiometric ratio of G3BP1: Caprin1: CSDE1: NUFIP2: UBAP2L: USP10 = 5:1:1:0.5:0.5:0.5 to reconstitute SG *in vitro*.

Note: The roles of each component in regulating stress granule assembly.

Name	Estimated conc. [μM]/HeLa cells[5]	Estimated conc. [μM]/HEK 293T cells[6]	SG assembly	LLPS of G3BP1
G3BP1	0.624	3.062	Key node	Key node
Caprin1	0.352	2.332	promoting	promoting
CSDE1	0.317	1.622	promoting	promoting
UBAP2L	0.655	1.415	promoting	promoting
NUFIP2	0.179	0.486	promoting	promoting
USP10	0.095	0.473	localize to SG at basal level, inhibiting SG with overexpression	Weakly promoting

27. Dilute proteins as step 21, making the concentration of G3BP1, Caprin1, CSDE1, UBAP2L, NUFIP2, and USP10 is 100, 160, 160, 40, 40, and 40 μM, respectively.
28. Mix proteins as follows, shown in Figure 3A.
    a. Mix equal volumes of Caprin1 and CSDE1 to a final concentration of 80 μM both in 300 mM NaCl, 50 mM Tris-HCl pH 7.6 buffer in a 0.2 mL PCR tube.
    b. Add 2 μL of 40 μM of NUFIP2 into the mixture of Caprin1+CSDE1 to obtain an intermediate solution with Caprin1+CSDE1+NUFIP2 = 40 μM + 40 μM+20 μM in the 300 mM NaCl and 50 mM Tris-HCl pH 7.6 buffer and label as mix #1.
    c. In parallel, mix USP10 and UBAP2L to a final 20 μM concentration for both in 300 mM NaCl, 50 mM Tris-HCl pH 7.6 buffer in a new 0.2 mL PCR tube and label as mix #2.
    d. Mix equal volumes of mix #1 and #2 in a new 0.2 mL PCR tube to obtain Caprin1+CSDE1+NUFIP2+USP10+UBAP2L = 20 μM + 20 μM+10 μM + 10 μM+10 μM as mix #3.
    e. Further mix an equal volume of 100 μM of G3BP1 with mix #3 to obtain the 6-component SG protein mixture in 300 mM NaCl and 50 mM Tris-HCl pH 7.6 buffer.
29. Add 200 ng/μL polyA RNA in 50 mM Tris-HCl pH 7.6 buffer or control Tris-HCl buffer alone to the 6-component protein mixture to induce liquid-liquid phase separation.
30. Observe and capture pictures referring to steps 18-20.

Note: Capture pictures at ~5 min to ensure comparable phase separation behavior across groups.

Test the differential effect of G3BP1 orthologs with the 7-component system

Timing: ~2 h

The LLPS of the 7-component system composed of G3BP1, Caprin1, CSDE1, UBAP2L, NUFIP2, USP10, and polyA RNA is used as a model to confirm the regulation of molecular interactional network in SG assembly using G3BP1 orthologs. Referred to this paper,[1] we have found that the interactional network induced by G3BP1 plays a key role in mediating SG assembly and also SFV virus replication by comparing the differences among G3BP1 orthologs from different species, including *humans*, *mice*, *zebrafish*, *Drosophila*, *C. elegans*, *yeast*, and *Arabidopsis*. Caprin1, CSDE1, UBAP2L, NUFIP2, and USP10 proteins are used in the 7-component reconstitution system with a high abundance enrichment in the G3BP1-network.[1,3,4] We chose G3BP1 orthologs from

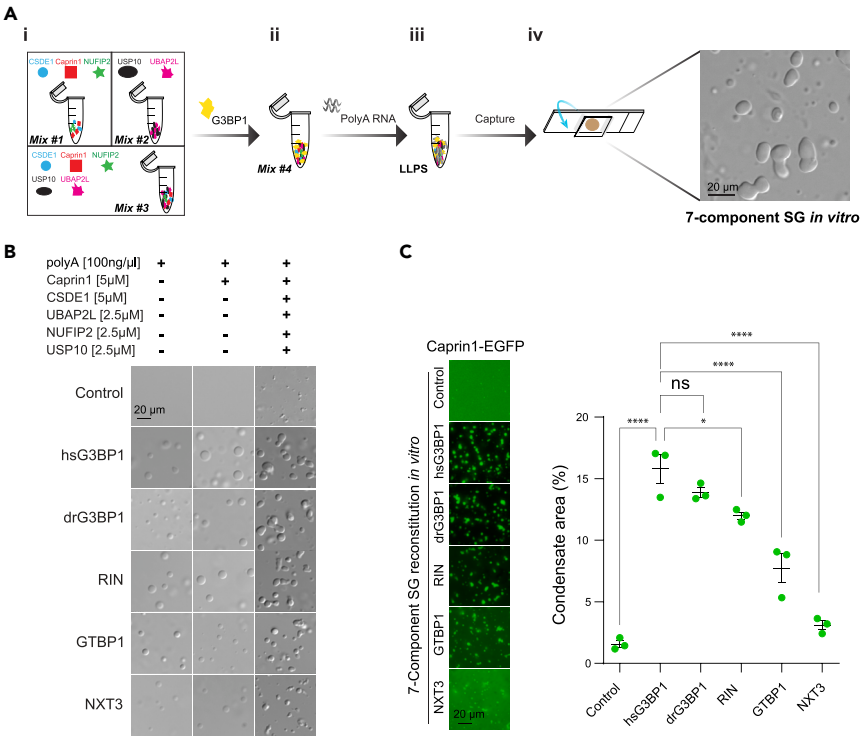

Figure 3. Reconstitution of SG with 7 components *in vitro*

(A) Steps for reconstitution of SG with 7 components *in vitro*. Scale bar 20 μm.

(B) LLPS of G3BP1 orthologs in the 7-component reconstitution system. Scale bar 20 μm. Figures are reprinted and adapted with permission from Yao et al., 2024.[1]

(C) Quantification of phase separation in the multicomponent system *in vitro* by labeling Caprin1. Scale bar 20 μm. The total droplet area was quantified according to Caprin1-EGFP to represent each droplet in one image (2048 × 2048 ppi), and the data were analyzed by ImageJ software. The mean value of the 3 repeats of the experiment was plotted. Error bars indicate SEM, $n = 3$. ****$p < 0.0001$, *$p < 0.05$, ns = no significant difference by one-way ANOVA; Tukey's multiple comparison test. Figures are reprinted and adapted with permission from Yao et al., 2024.[1]

humans, *zebrafish*, *Drosophila*, *C. elegans*, and *yeast* to verify the functions of the network *in vitro*. Results are shown in Figures 3B and 3C.[1]

Note: Here, Caprin1 protein is labeled by fusing EGFP at the C-terminus to calculate the area of the droplets to characterize the LLPS ability of G3BP1 orthologs in the reconstitution system *in vitro*.

31. Insert sequence of EGFP at the C-terminus of Caprin1 in pGEX-6p-1 vector by homologous recombination.
32. Purify and condense Caprin1-EGFP protein as above steps 1-11.
33. Further mix an equal volume of 100 μM of G3BP1 orthologs with mix #3 to obtain the 6-component SG protein mixture in 300 mM NaCl, 50 mM Tris-HCl pH 7.6 buffer. BSA is used as the control.

Note: According to step 28, described as "a to d", we get mix #3 in a 0.2 mL PCR tube. Here, Caprin1 is replaced by Caprin1-EGFP.

34. Add 200 ng/μL polyA RNA in 50 mM Tris-HCl pH 7.6 buffer or control Tris-HCl buffer alone to the 6-component protein mixture to induce liquid-liquid phase separation.
35. Observe and capture pictures referring to steps 18-20.

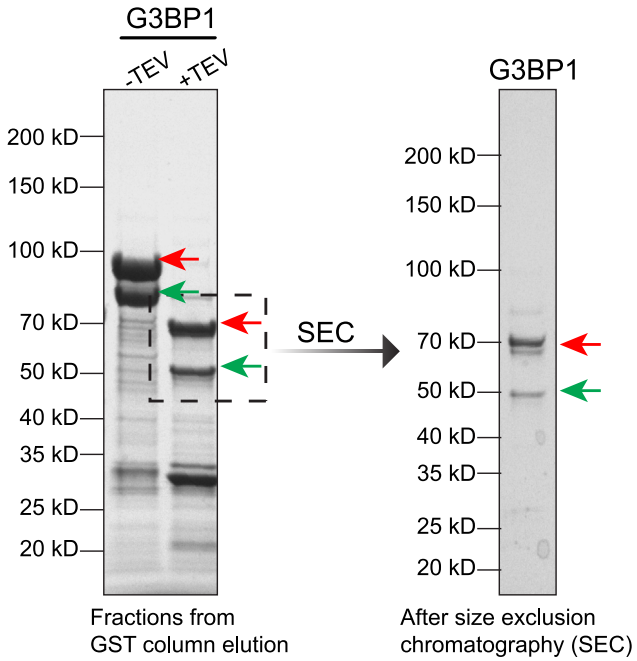

Figure 4. The stability of G3BP1 without bacterial codon optimization *in vitro*

Coomassie-stained 4–20% acrylamide gel for G3BP1 with or without TEV. The molecular weight of G3BP1 is about 62 kD. The red and green arrows indicate the full length and truncation of G3BP1, respectively.

Quantification of the droplet area *in vitro*

Timing: ~1 h

36. Analyze the data with ImageJ software.
    a. Open the image with ImageJ.
    b. Convert image format to "RGB stack".
    c. Mark the fluorescence area. Click "Auto" and "Set" on the "threshold" screen, and then click the "OK".
    d. Set measurement parameters on the "Analyze" screen. Click "Set Measurement" and select "Area", "Mean", "IntDen (Integrated Density)", "Area fraction", "Mean gray value", "Limited to threshold", and "Display label" as measurement parameters, then click the "OK".
    e. Measure the parameters. Click "Measure" on the "Analyze" screen.
    f. Save the results.
37. Display the data by GraphPad Prism9.5.

EXPECTED OUTCOMES

The dimerization induced by G3BP1's N-terminus and the RNA-binding affinity mediated by its C-terminus are necessary for LLPS of G3BP1 *in vitro* and *in vivo*. Especially *in vitro*, we have verified that G3BP1 alone failed to trigger phase separation even with a high concentration, as shown in the phase diagram in Figure 2B. So, the quality of G3BP1 is important during purification.

It potentially triggers truncations in its C-terminus, shown in Figure 4, resulting in its failure to bind with RNA and induce LLPS *in vitro*. The bacterial codon optimization improves the purity of the G3BP1 protein and avoids truncations, as shown in Figure 1B.

QUANTIFICATION AND STATISTICAL ANALYSIS

Microsoft Excel, GraphPad Prism9.5, and ImageJ-Win64 were used for statistical analysis. Statistical values are displayed as the means ± SD (standard deviation)/SEM (standard error of the mean). The details are included in the corresponding figure legends. Multiple group comparisons were performed using a one-way analysis of variance (ANOVA) followed by post-hoc tests. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$; ns. not significant.

LIMITATIONS

In this protocol, all proteins we used are expressed with *E.coli,* and the lack of post-translational modifications potentially affects their functions in inducing LLPS *in vitro*. In addition, the 7-component reconstitution system for SG is composed of several key G3BP1 network proteins, lacking other components, such as the 40S ribosome, regulators of RNA chaperone components, ATP, etc. Thus, further optimization of the *in vitro* system is needed to reflect other aspects of the SG assembly process in cells.

TROUBLESHOOTING

Problem 1

The yield of purified NUFIP2 and USP10 is low.

Potential solution

Increase the volume of LB to 2 L or more to start with more materials for purification. Shorten the tag cleavage time and add more TEV protease for cleavage. A small test can be made to find an optimal cleavage time. With a 1% TEV mass addition into the eluted proteins, we can observe a >90% cleavage ~6 h at room temperature. Do not incubate protein and TEV for more than 24 h, even at 4°C, because this potentially increases protein degradation and aggregation. Try not to concentrate the protein before the tag cleavage. We find that higher concentrations can enhance the aggregation and precipitation of the cleavage proteins.

Problem 2

The protein UBAP2L is prone to aggregates without the MBP tag.

Potential solution

UBAP2L protein is a large protein, composed of 1087aa, and may cause it difficult to be expressed in *E.coli*. UBAP2L contains more disordered domains, and it can undergo LLPS in 50 mM Tris-HCl pH 7.6, and 150 mM NaCl buffer. The solubility of UBAP2L is increased when fused with the MBP tag. The MBP-UBAP2L fusion protein can still show the cooperativity effect with G3BP1 *in vitro*. Try to induce UBAP2L expression at 37°C with a shorter time to increase solubility. To reduce the aggregation of UBAP2L during purification and at any concentration step, supplement the buffer with a higher salt concentration, such as 0.6–1 M/L NaCl.

Problem 3

The quality of the full length of G3BP1 is important to induce LLPS and assemble SG *in vitro*.

Potential solution

G3BP1 is a protein with 466aa and can be expressed in large quantities in bacteria cells. One thing we noticed is that there is usually a lower band ~10 kD smaller than the main band when using the human cDNA as template for bacteria expression. We later find out that the codon is a main problem. Bacterial codon optimization of G3BP1 construct is used in our studies, and we can see reduced protein truncation. The purity of G3BP1 protein is important for LLPS assay because the C-terminal of G3BP1 contains RRM and RGG motifs for RNA binding. The truncation usually happens at the C-terminus during protein translation and leads to the shortening of RGG motifs. Also, expressing the proteins in Rosetta (DE3) and related competent cells containing rare tRNA transgenes can

further improve the purity of proteins. The RNA is another key factor in assembling SG and inducing LLPS of G3BP1 *in vitro*. Nucleic acid contamination may happen for G3BP1 preparation if large quantities of bacterial cells and a lower salt buffer are used in the protocol. We add RNase A during the TEV cleavage step, and it can dramatically reduce RNA contamination from the G3BP1 purification process, and the value of A260/A280 measured on nanodrop for G3BP1 should be around ~0.6. For this reason, we do not recommend performing the cleavage at a concentrated state with a longer incubation time. For all the SG-related RNA binding proteins, it is better to shorten the time between purification start and protein freezing/storage for consistency between batches.

Problem 4

Here, polyA RNA is used as a tool to induce LLPS of G3BP1, and the quality of polyA RNA is important to maintain its functions. If total RNA is used to trigger G3BP1 LLPS, make sure the integrity of total RNA is good.

Potential solution

All reagents and experiments are kept in a low-temperature condition. Put the aliquots for LLPS on ice. The polyA RNA is diluted before use, and avoid reuse of the aliquot, for it may be degraded. So, we generally keep a small volume of aliquot for each RNA stock and will not freeze the aliquot after use.

Problem 5

In vitro LLPS assembly of the observing chamber and timing of observation are important. We describe the detailed practice for reconstituting SG with G3BP1 and related RBPs *in vitro*. We use simple untreated glass coverslips and glass slides to assemble the sandwiched chamber slides for microscopy imaging. The system is easy to perform and less in cost. However, we do see variations can happen and attention is needed for the timing of observation.

Potential solution

Find a suitable catalog of the coverslips and glass slides and clean them before use if necessary. When assembling the chamber for observation, do not touch the center of the slide for sample loading. We usually apply 1–2 μL of samples on the slide first and all the coverslips on top. Try to put the coverslip evenly on the sample and make sure the samples are of equal thickness by slightly applying a pushing force on the coverslip. Then, invert the chamber slide to let the droplets settle toward the coverslip. It is not recommended for longer observations, for example, 1 h after assembly of the sample, as this is an open chamber, and the liquid would evaporate and the samples are concentrated. A sealed chamber or 384-well plate with a coverslip bottom is better suited for longer observations.

For G3BP1-RNA LLPS *in vitro*, we usually made the observation 3–5 min after LLPS is triggered after mixing G3BP1 protein and RNA in the test tube. It may take 30 s to 1 min to assemble the chamber slide and adjust the microscope to image the droplets. We tend to focus near the surface of coverslips as more droplets are settling down due to gravity. A comparable time window for imaging is essential as the number and size of G3BP1-RNA droplets formed *in vitro* vary a lot at different times. Due to the higher liquidity of G3BP1-RNA droplets, they start to wet the coverslip and spread, making the DIC imaging challenging. We find that multiple components of SG proteins tend to form droplets that are more stable for imaging. Nevertheless, try to narrow the imaging time window for comparison of different groups.

Problem 6

Hardening of *in vitro* droplets by fluorophore-labeled protein.

We have used Alexa Fluor labeled G3BP1 for LLPS *in vitro*. Laser illumination can drastically harden the droplets, consistent with previous indications.[7] G3BP1-RNA droplets can wet the coverslip very

fast, and the hardening phenomenon caused by laser illumination can be easily observed in this system.

Potential solution

Fluorescent protein fusion can be used instead of chemical labeling. Also, reduce the number of chemical fluorophores used for labeling and only spike ~1–5% of labeled proteins into unlabeled proteins for experiments. Add 5 mM DTT to reduce the oxidation during imaging. Importantly, be mindful that some features of the droplets observed with labeled proteins could be caused by this problem.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Peiguo Yang (yangpeiguo@westlake.edu.cn).

Technical contact

Any information regarding the details of the protocol should be directed to and will be fulfilled by the technical contact, Zhiying Yao (yaozhiying@westlake.edu.cn).

Materials availability

All unique or stable reagents generated in this study are available from the lead contact with a completed materials transfer agreement. All the plasmids used in this study have been deposited to WeKwikGene (https://wekwikgene.wllsb.edu.cn/), a non-profit plasmid repository run by Westlake Laboratory. Plasmids encoding each protein used in the study could be obtained from WeKwikGene.

Data and code availability

All data reported in this paper will be shared by the lead contact upon request. This paper does not report original code.

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AUTHOR CONTRIBUTIONS

Z.Y. performed protein purification and *in vitro* reconstitution experiments and participated in writing the manuscript. P.Y. conceived and supervised the project and wrote the manuscript with input from Z.Y. and Y.L.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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