# Supplementary information

## Electrostatic interactions drive phase separation in Pup protein.

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## Materials

Sodium chloride (cat #ASS270), Imidazole (cat #ASI2835), SDS (cat #ASS2711), and Acetic acid (cat #ASA1550) were purchased from Avra Synthesis (Hyderabad, India). Tryptone (cat #RM9111), Yeast extract (cat #RM027), Coomassie brilliant blue R-250 (cat #MB153), Ammonium persulphate (cat #MB003), TEMED (cat #MB026), Acrylamide/Bisacrylamide solution 40% (cat #ML083), and Ampicillin sodium salt (PCT1101) were purchased from Himedia (Mumbai, India). Tris (cat #79420), Glycine (cat #64072), Sodium phosphate dibasic dodecahydrate (cat #57085), Isopropanol (cat #67800), Agar Powder (cat #19661), IPTG (cat #54110), PMSF (cat #87606), and DNAase (cat #14658) were purchased from SRL (Mumbai, India). Magnesium chloride hexahydrate (cat #DC0D693741), Sodium dihydrogen phosphate monohydrate (cat #DF0D701140), FITC labelled poly-L-lysine (cat #P3069) (MW = 30000-70000 Da), poly-DL-alanine (cat#P9003) (MW = 1000-5000 Da), poly (L-lysine hydrobromide) (cat#P2636) (MW = 30000-70000 Da), polyethylene glycol 20000 **(**cat #818897**)** and Amicon ultra-15 centrifugal filter (cat #UFC9003) were purchased from Merck. Qualigens Hydrochloric acid (cat #Q29505), Qiagen Ni-NTA agarose (cat #30210) Thermo Scientific NHS-Rhodamine (cat #46406) were used. All the enzymes BamHI-HF (Cat #R3136S), HindIII-HF (cat #R2104S), T4DNA ligase (cat #M0202S), and Q5 HF 2X Master mix (cat #M0492S) were purchased from NEB, USA.

## Methods

### Protein expression and purification

The Pup gene (a gift from Prof. Heran Darwin's lab, NYU, USA ) was inserted into the pET23b(+)(Novagen) vector containg $His_6$ site and a TEV protease cleavage site, a gift from Tania Baker's lab, MIT, USA, between BamHI and HindIII restriction sites to facilitate the protein purification using His-tag and for further removal of the His-tag. PCR for Pup was performed using the primers named BamHI_Pup_fwd(5'-ATAGGATCCATGGCGCAAGAGCAGACC-3') and Pup_HindIII_rev (5'-TATAAGCTTTCACTGTCCGCCCTTTTGGA-3') followed by restriction digestion using BamHI and HindIII enzymes. Double digested Pup gene was inserted into the pET23b(+) vector using DNA Ligase enzyme. The plasmid containing the Pup gene was transformed into the *E. coli* BL21(DE3) cells. The bacterial cells were grown at 37˚ C in the LB. The $His_6$-Pup protein expression was induced using 0.5 mM IPTG for 4 hours after the $OD_{600}$ reached 0.6. The cells were harvested by centrifuging at 4200 rpm for 20 minutes at $4^0$C. The cell pellet was resuspended using lysis buffer (10 mM imidazole, 20 mM sodium phosphate, 200 mM NaCl, pH 7.5 ) and was lysed using a probe sonicator. The lysed cells were incubated with 1mM PMSF and 300 U DNase, 2mM $MgCl_2$, at $4^0$C for 30 minutes. Further, it was centrifuged at 9000 rpm for 50 minutes. The supernatant was incubated with 2 ml Ni-NTA beads for 90 minutes at $4^0$C. The Ni-NTA beads bound to $His_6$-Pup protein were washed with 150 ml of lysis buffer followed by elution with buffer containing 300 mM imidazole (300 mM imidazole, 20 mM sodium phosphate, 200 mM NaCl, pH 7.5). Further, the $His_6$-Pup protein was purified by size exclusion chromatography (SEC) using cytiva HiLoad 16/600 superdex 75 prep grade column. The final SEC buffer composition was 20 mM Tris-HCl, 150 mM NaCl, pH 7.5. The purity of the Pup was checked on an 18% SDS- PAGE. The $His_6$ tag of the Pup protein was removed using TEV protease.

The gene and protein sequence of the Pup protein along with His-tag and TEV recognition site.
cDNA sequence:
ATGGGCAGCAGCCATCACCATCACCATCACGATTACGATATCCCAACGACCGAGAATCTT
TATTTTCAGGGATCCATGGCGCAAGAGCAGACCAAGCGTGGCGGTGGCGGCGGCGATGA
TGACGACATCGCCGGCAGCACCGCCGCGGGCCAGGAGCGTCGCGAAAAGCTGACCGAG
GAGACCGACGATCTGCTCGACGAAATCGACGACGTCCTCGAGGAGAACGCCGAGGACTT
CGTCCGCGCATACGTCCAAAAGGGCGGACAG

Protein sequence:
MGSSHHHHHHDYDIPTTENLYFQGSMAQEQTKRGGGGGDDDDIAGSTAAGQERREKLTEET
DDLLDEIDDVLEENAEDFVRAYVQKGGQ

**Circular Dichroism**

Circular dichroism (CD) experiment was performed on a 60 μM Pup in 50 mM Ionic strength PBS in a 1-cm path-length quartz cuvette using a spectropolarimeter.

**Sample preparation for LLPS**

Poly-L-lysine (PLL) (30000-70000 Da) was dissolved in nuclease-free water to make it 350 mg/ml stock concentration, which was further diluted to 35 mg/ml and 3.5 mg/ml concentration. Pup protein was dissolved in the SEC buffer at pH 7.5 The PLL and Pup were mixed in the SEC buffer (20mM Tris-HCl, 150mM NaCl, pH 7.5) at different concentrations and incubated at $25^0$C for 5-10 minutes. A similar procedure was followed for FITC labelled PLL - Pup and PLL-NHS rhodamine labelled pup as well as the samples in which both were labelled.

**Micro-well plate reader: Turbidity measurement**

The Pup-PLL LLPS system's turbidity measurement was performed using a multi-mode plate reader (BioTek synergy H1M) on a 384-well plate. The experiment was conducted at $25^0$C, and the kinetics of LLPS formation were observed by measuring optical density (OD) at 600 nm for 20 minutes.

**Optical microscopy studies**

For imaging phase separated coacervates, 20X and 40X objective lenses of infinity microscope were used, with a custom-built flow cell setup. The ends were sealed with paraffin wax after adding the coacervate mixture to the flow cell.

**Protein labelling**

Pup protein was labelled with the NHS Rhodamine by following the standard protocol given by Thermo Scientific™. Briefly, the labelling is based on an amine-ester linkage with the protein's primary amine, either the free amino terminus or the lysine residues. NHS-Rhodamine was dissolved in DMSO to prepare a stock solution of 10 mg/ml and stored at $-20^0$C. The tagging was done at a 10-fold excess of the dye to achieve high tagging efficiency. The reaction was incubated for 1 hour at $25^0$C in PBS buffer (20 mM sodium phosphate, 200 mM NaCl, pH 7.5) under dark conditions. The free dye was removed by dialysis at $4^0$C under dark conditions, and the labelled protein was dialysed into the SEC buffer. The labelled Pup was stored at $4^0$C.

**pH dependent studies**

To maintain the same salt and buffer composition, we have taken the SEC buffer at pH 7.5 and obtained the pH range of 3-12, either by reducing or increasing the pH using 5N HCl or 5N NaOH. Further, both PLL and pup was buffer exchanged separately to the desired pH solution using 3kDa Amicon ultra centrifugal filter and the pH was confirmed using both pH meter and pH paper strips. PLL and Pup at the desired pH were then mixed and incubated at $25^0$C for 10 minutes before proceeding for the microscopy.

**Confocal microscopy studies**

A laser scanning confocal microscope (Leica TCS SP8) with 40x objective with custom built flow cell setup were used for fluorescent imaging. The fluorophore-labeled droplets (droplets formed by 2% labelled protein+ 98% unlabeled protein for FITC-PLL (0.175 mg/ml), 5% labelled protein + 95% unlabeled protein for NHS rhodamine-Pup (40 μM)) were observed using appropriate fluorescence channels (488 nm for FITC, 561 nm for rhodamine). All the images were taken at room temperature.

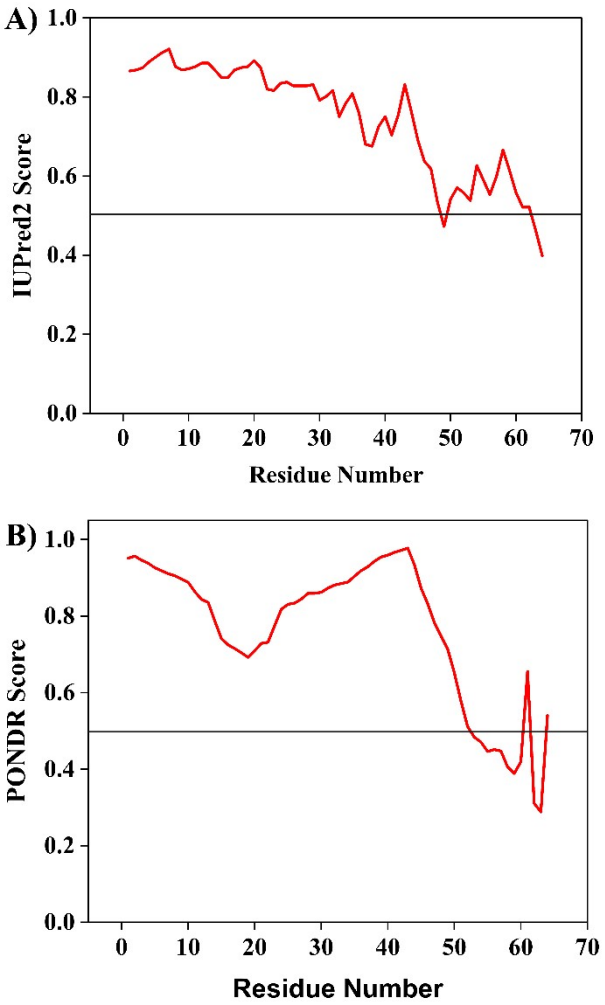


**Figure SI-1.** Prediction of disordered regions and using (A) IUPred2 (B) PONDR

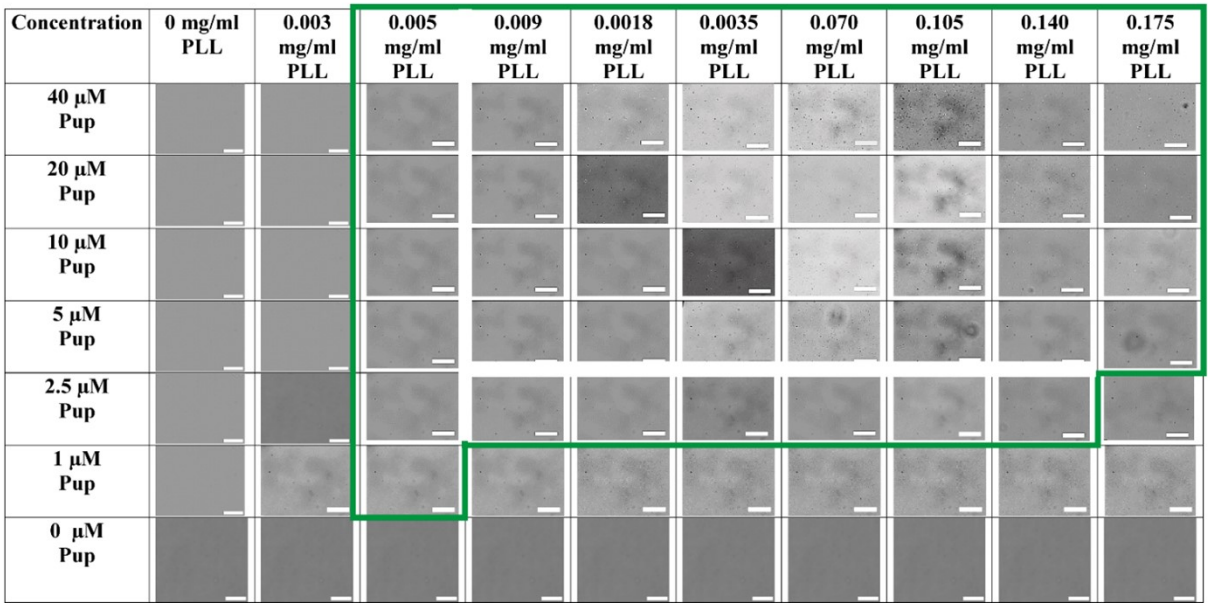


**Figure S2.** Bright field optical micrographs of Pup protein and PLL coacervates at different concentrations in SEC buffer, pH 7.5 at 25°C; scale bar: 50µm

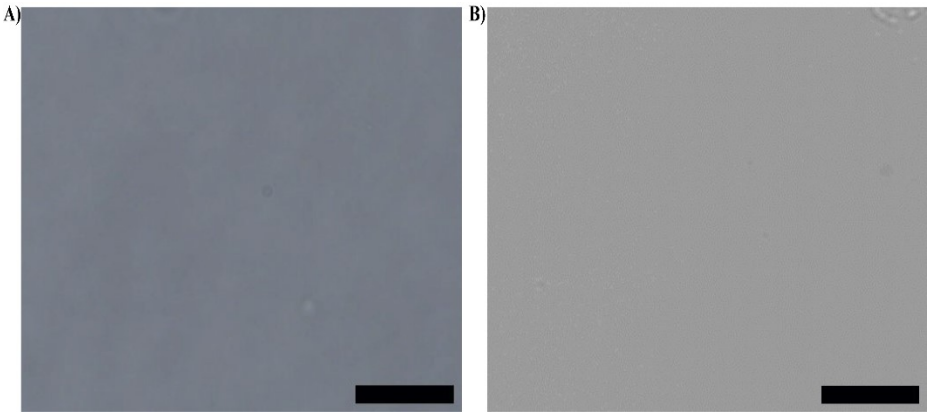


**Figure S3.** Bright field optical micrographs of only Pup protein, 40 µM (A) and only Poly-L-Lysine, 0.175 mg/ml (B) in SEC buffer, pH 7.5 at 25°C. No self-coacervation was observed. Scale bar: 10 µm.

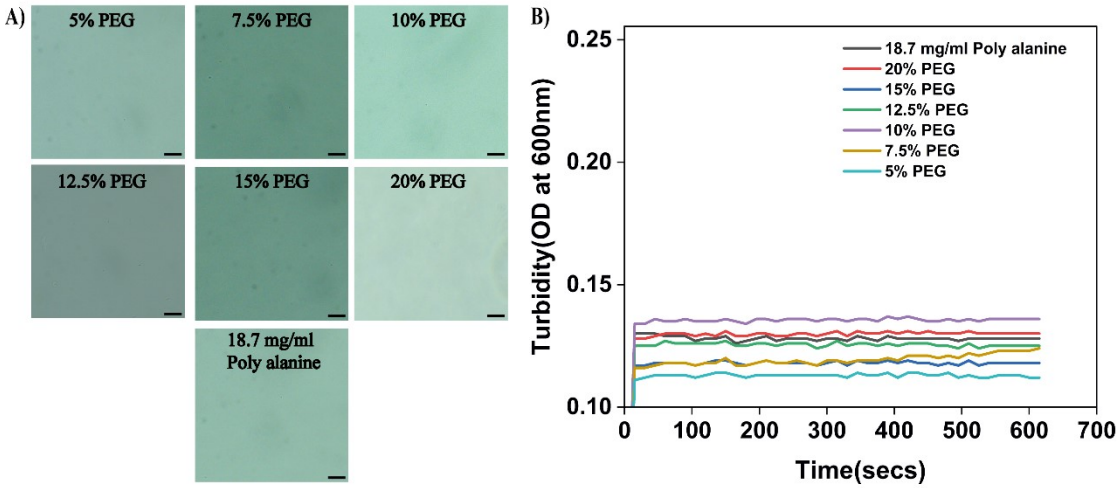


**Figure S4.** (A) Bright field optical micrographs of Pup protein, 40 µM in the presence of various concentrations of PEG-20000 and polyalanine (1000-5000 M.Wt) do not show any coacervate formation. Scale bar: 20 µm. (B) Turbidity plots of Pup mixed with different concentrations of PEG and polyalanine do not indicate formation of any coacervates.

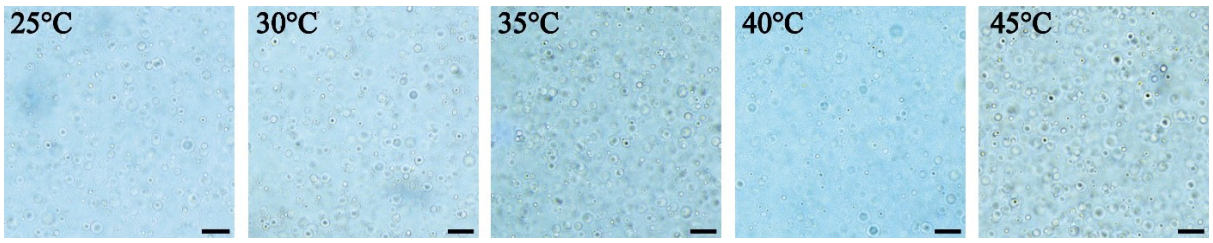


**Figure S5.** Bright field optical micrographs of Pup (40 µM) + PLL (0.175 mg/ml) coacervates at different temperatures ranging from 25°C to 45°C indicating their thermal stability.

Table -1 Circular Dichroism data corresponding to figure 1C

| **Wavelength (nm)** | **Ellipticity (mdeg)** |
|---|---|
| 250 | 0.42539 |
| 249 | 0.21242 |
| 248 | 0.21601 |
| 247 | 0.04269 |
| 246 | 0.04984 |
| 245 | -0.1355 |
| 244 | -0.3464 |
| 243 | -0.4748 |
| 242 | -0.7132 |
| 241 | -0.8767 |
| 240 | -1.133 |
| 239 | -1.4349 |
| 238 | -1.6643 |
| 237 | -1.9163 |
| 236 | -2.4251 |
| 235 | -2.8508 |
| 234 | -3.2607 |
| 233 | -3.6273 |
| 232 | -4.0198 |
| 231 | -4.5947 |
| 230 | -4.9549 |
| 229 | -5.3229 |
| 228 | -5.6002 |
| 227 | -5.8608 |
| 226 | -6.2333 |
| 225 | -6.2755 |
| 224 | -6.6458 |
| 223 | -6.5371 |
| 222 | -6.7372 |
| 221 | -6.5949 |
| 220 | -6.8131 |
| 219 | -6.7858 |
| 218 | -6.7027 |
| 217 | -6.9704 |
| 216 | -7.0988 |
| 215 | -7.4003 |
| 214 | -7.8443 |
| 213 | -8.2501 |
| 212 | -9.1759 |
| 211 | -10.217 |

| | |
|---|---|
| 210 | -11.512 |
| 209 | -12.921 |
| 208 | -14.661 |
| 207 | -16.091 |
| 206 | -17.595 |
| 205 | -19.586 |
| 204 | -20.772 |
| 203 | -22.194 |
| 202 | -23.022 |
| 201 | -23.608 |
| 200 | -23.272 |
| 199 | -22.324 |
| 198 | -21.089 |
| 197 | -18.975 |
| 196 | -17.364 |
| 195 | -15.835 |
| 194 | -12.661 |
| 193 | -8.0513 |
| 192 | -1.1164 |

Table -2 Turbidity data corresponding to figure 2A

| **Time (s)** | **Only Pup** | **Only 0.175 mg/ml PLL** | **80µM Pup + 0.175 mg/ml PLL** | **40µM Pup + 0.175 mg/ml PLL** | **20µM Pup + 0.175 mg/ml PLL** | **10µM Pup + 0.175 mg/ml PLL** | **5µM Pup + 0.175 mg/ml PLL** |
|---|---|---|---|---|---|---|---|
| 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 30 | 0.04 | 0.09 | 0.267 | 0.126 | 0.053 | 0.052 | 0.042 |
| 60 | 0.04 | 0.09 | 0.323 | 0.149 | 0.052 | 0.052 | 0.042 |
| 90 | 0.039 | 0.088 | 0.346 | 0.161 | 0.052 | 0.053 | 0.042 |
| 120 | 0.039 | 0.088 | 0.358 | 0.169 | 0.053 | 0.054 | 0.042 |
| 150 | 0.039 | 0.088 | 0.361 | 0.177 | 0.053 | 0.056 | 0.042 |
| 180 | 0.039 | 0.088 | 0.364 | 0.183 | 0.053 | 0.058 | 0.042 |
| 210 | 0.039 | 0.087 | 0.368 | 0.188 | 0.053 | 0.061 | 0.042 |
| 240 | 0.039 | 0.087 | 0.371 | 0.193 | 0.053 | 0.064 | 0.042 |
| 270 | 0.039 | 0.087 | 0.375 | 0.196 | 0.054 | 0.067 | 0.042 |
| 300 | 0.039 | 0.086 | 0.379 | 0.199 | 0.054 | 0.068 | 0.042 |
| 330 | 0.039 | 0.085 | 0.381 | 0.201 | 0.054 | 0.069 | 0.042 |
| 360 | 0.039 | 0.086 | 0.381 | 0.203 | 0.054 | 0.069 | 0.042 |
| 390 | 0.039 | 0.086 | 0.383 | 0.205 | 0.054 | 0.069 | 0.042 |
| 420 | 0.039 | 0.085 | 0.381 | 0.207 | 0.054 | 0.07 | 0.042 |
| 450 | 0.039 | 0.086 | 0.382 | 0.208 | 0.054 | 0.069 | 0.042 |
| 480 | 0.039 | 0.085 | 0.382 | 0.209 | 0.054 | 0.069 | 0.042 |
| 510 | 0.039 | 0.085 | 0.381 | 0.21 | 0.055 | 0.067 | 0.042 |
| 540 | 0.039 | 0.085 | 0.382 | 0.211 | 0.055 | 0.066 | 0.042 |
| 570 | 0.039 | 0.084 | 0.382 | 0.212 | 0.055 | 0.066 | 0.042 |
| 600 | 0.039 | 0.084 | 0.383 | 0.212 | 0.056 | 0.063 | 0.042 |
| 630 | 0.039 | 0.084 | 0.383 | 0.213 | 0.056 | 0.062 | 0.043 |
| 660 | 0.04 | 0.09 | 0.383 | 0.214 | 0.056 | 0.06 | 0.043 |
| 690 | 0.04 | 0.09 | 0.383 | 0.214 | 0.056 | 0.058 | 0.043 |
| 720 | 0.039 | 0.088 | 0.383 | 0.214 | 0.057 | 0.056 | 0.042 |
| 750 | 0.039 | 0.088 | 0.383 | 0.214 | 0.056 | 0.055 | 0.042 |
| 780 | 0.039 | 0.088 | 0.383 | 0.215 | 0.057 | 0.054 | 0.043 |
| 810 | 0.039 | 0.088 | 0.383 | 0.215 | 0.057 | 0.054 | 0.043 |
| 840 | 0.039 | 0.087 | 0.383 | 0.215 | 0.057 | 0.053 | 0.043 |
| 870 | 0.039 | 0.087 | 0.383 | 0.215 | 0.057 | 0.053 | 0.043 |
| 900 | 0.039 | 0.087 | 0.383 | 0.215 | 0.058 | 0.054 | 0.043 |
| 930 | 0.039 | 0.086 | 0.383 | 0.214 | 0.058 | 0.053 | 0.043 |
| 960 | 0.039 | 0.085 | 0.383 | 0.214 | 0.058 | 0.053 | 0.043 |
| 990 | 0.039 | 0.086 | 0.383 | 0.214 | 0.058 | 0.054 | 0.043 |
| 1020 | 0.039 | 0.086 | 0.383 | 0.214 | 0.058 | 0.053 | 0.043 |
| 1050 | 0.039 | 0.085 | 0.383 | 0.214 | 0.058 | 0.053 | 0.043 |
| 1080 | 0.039 | 0.086 | 0.383 | 0.214 | 0.059 | 0.054 | 0.043 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 1110 | 0.039 | 0.085 | 0.383 | 0.213 | 0.059 | 0.054 | 0.043 |
| 1140 | 0.039 | 0.085 | 0.383 | 0.213 | 0.059 | 0.054 | 0.043 |
| 1170 | 0.039 | 0.085 | 0.383 | 0.212 | 0.059 | 0.054 | 0.043 |
| 1200 | 0.039 | 0.084 | 0.383 | 0.212 | 0.059 | 0.054 | 0.043 |