Supporting information for

"Amino Acids Modulate Liquid-Liquid Phase Separation in vitro and in vivo by Regulating Protein-Protein Interactions"

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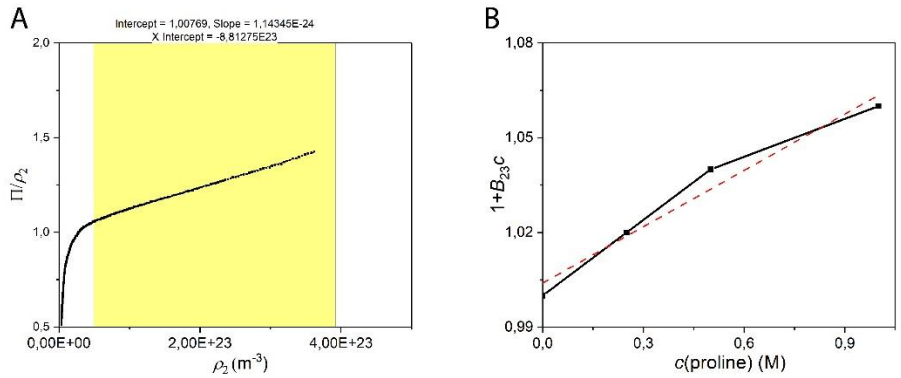

Figure S1: A. An example of the linear fitting of the curve $\Pi/\rho 2$ versus $\rho 2$ for BSA-BSA interaction in the presence of proline. The noisy part of low protein concentrations close to the meniscus is omitted from the linear fitting; B. Change of $1+B_{23}c$ for BSA–proline interaction versus proline concentrations (c). The curve is linearly fitted and the slope (B_{23}) equals 0.06 ± 0.01 M^{-1}.

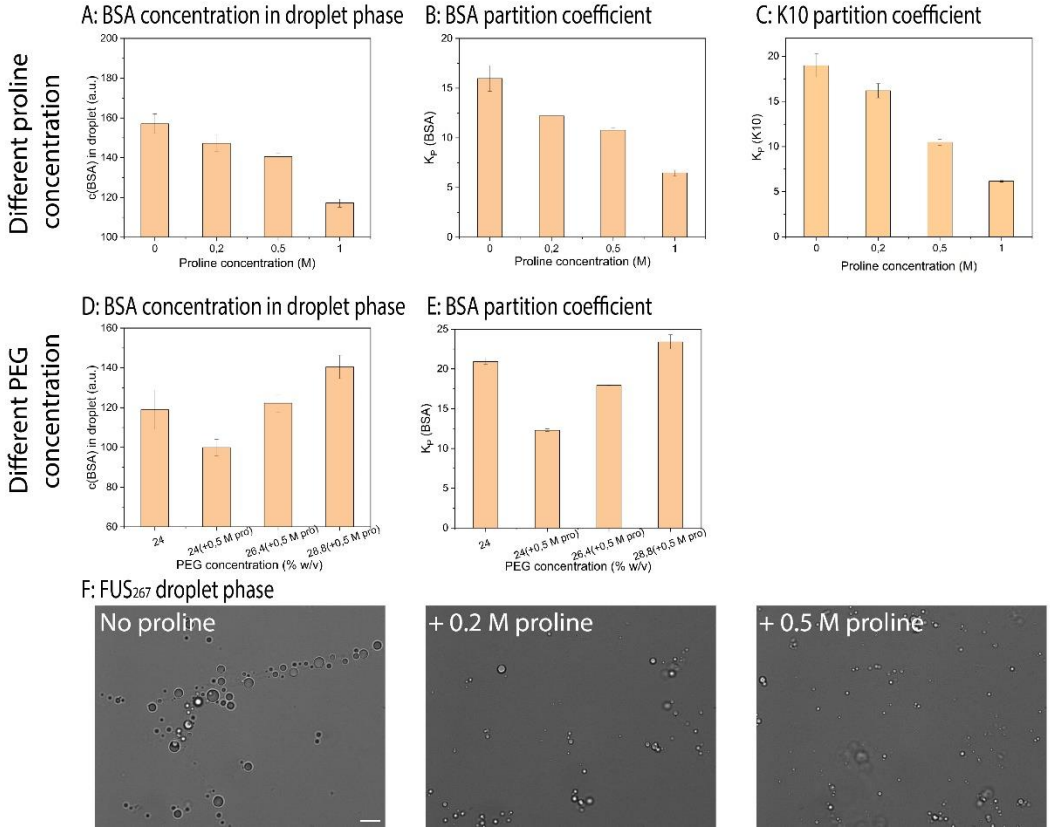

Figure S2: A. Fluorescence intensity of rhodamine-labelled BSA in the droplet phase with different proline concentrations after 60 mins incubation; B. Partition coefficient (K_p) of rhodamine-labeled BSA in the droplet phase by measuring the fluorescence intensity with different proline concentrations after 60 mins incubation; C. Partition coefficient (K_p) of FITC-labelled poly-lysine (K10) in the BSA droplet phase by measuring the fluorescence intensity with different proline concentrations; D. Fluorescence intensity of rhodamine-labelled BSA in the droplet phase after the addition of 0.5 M proline with different PEG concentrations, compared to the case of no proline addition (1st bar); E. Partition coefficient (K_p) of rhodamine-labelled BSA in the droplet phase by measuring the fluorescence intensity after the addition of 0.5 M proline

with different PEG concentrations, compared to the case of no proline addition (1st bar). F. Widefield optical microscopy images of FUS_{267} droplets in the presence of different proline concentrations (0, 0.2 and 0.5 M). Scale bar: 10 μm.

SI1: amino acid extraction and LC-MS analysis

The protocol was adjusted from the literature[1]:

Step 1: AAs extraction

The cells in a 6-well culture plate were washed twice with 1 ml of PBS on ice and collected using a cold plastic cell scraper in 1 ml of 80% (v/v) LC-MS–grade methanol (−80 °C). Then the cell suspension was gently transferred into a pre-cooled Eppendorf tube. The samples were immediately freeze-thawed in liquid nitrogen three times with 30 s vigorous vortex between each freeze cycle. Lysed samples were stored at −80 °C overnight for protein precipitation. The lysate was centrifuged at 20,000 g for 10 min at 4 °C to remove protein precipitates and dried by lyophilization. The samples were finally dispersed in 50% acetonitrile in analytical-grade water.

Step 2: AAs analysis

Samples are further diluted for analysis by ultrahigh-performance liquid chromatography high-resolution mass spectrometry, and tandem mass spectrometry. The analysis was conducted on a 6470B Triple Quadrupole Mass Spectrometer coupled to the 1260 series HPLC system (Agilent Technologies). 1 μl aliquots of the diluted samples were injected onto a 2.1 × 100 mm, 2.7 μm Agilent InfinityLab Poroshell 120 HILIC-Z column heated at 35° C. A binary gradient system consisting of A (10/90 v/v 10 mM Ammonium Formate in Formic acid-water solution, pH 3: water), and B (10/90 v/v 10 mM Ammonium Formate in Formic acid-water solution, pH 3: acetonitrile) was used. Sample separation was carried out at 0.8 ml/min over a 23 min total run time. The initial condition was 1/99 v/v A:B. The proportion of the solvent B was linearly decreased from 1/99 v/v A:B to 18/82 v/v A:B, from 0 min to 15 min, where the separation occurred. The rest of the run was meant to ensure proper washing of the system and avoid carryover. From 15 min to 16 min the composition went from 18/82 v/v A:B to 40/60 v/v A:B. Finally, the gradient was brought back to initial conditions linearly from minute 16 to minute 19, and let re-equilibrate until minute 23, where the pressure was back to the initial value. Detection was operated in positive ionization mode using the AJS Jet stream ESI source. AJS settings were as follows: Gas flow: 5 L/min; gas temperature: 300 °C; nebulizer pressure, 45 psi; sheath gas temperature: 350 ºC; sheath gas flow: 11 L/min; capillary voltage: 3500 V; Nozzle voltage: 500 V. The mass analyzer was used in the Multiple Reaction Monitoring (MRM) mode, with fragmentor voltage 88 V and cell acceleration voltage of 4 V. Two transitions were monitored, 116.1 to 0, with collision energy of 0 V, and 116.1 to 28.3 with collision energy of 40 V. Finally, the obtained data were

analyzed by using MassHunter Qualitative Analysis (Agilent Technologies. Inc.) and manual integration of the chromatograms for each transition (**Figure S3)**. The proline concentration after the pre-treatment (80,000 units) is ~130 times larger than the control (6000 units) and estimated to be ~130 mM as the proline concentration in the normal condition (no proline pre-treatment) is ~1 mM[2,3].

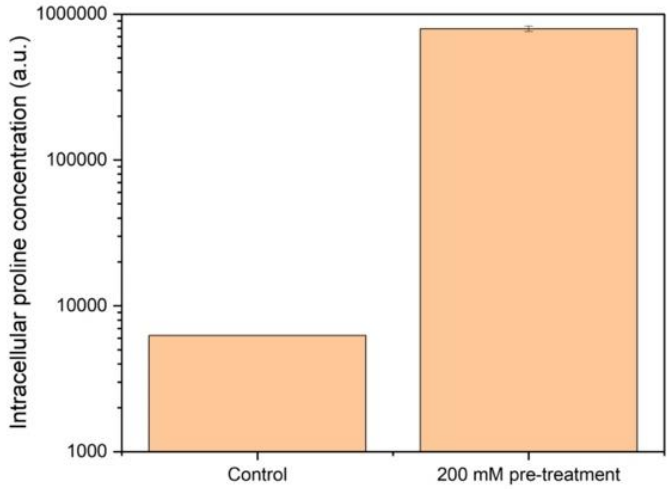

Figure S3: LC-MS result. The intracellular proline concentration measured by LC-MS was ~6000 (a.u.) for U2OS cells without any proline pre-treatment and ~80,000 (a.u.) for U2OS cells with 200 mM proline pre-treatment for 2 h. The intracellular proline concentration after the proline pre-treatment is ~130 mM as the intracellular proline concentration without any proline pre-treatment is ~1 mM[2,3].

SI2: cell proliferation assay (MTT assay[4])

The cells were seeded in a 96-well plate (Greiner Ref655891) and incubated overnight at 37°C, 5% CO_2. The culture media was aspirated and AAs supplemented culture media was added to incubate for 2 hrs at 37°C, 5% CO_2. An appropriate amount of CellTiter 96® AQueous One Solution Reagent was added to each 96-well plate containing the samples. The well plates were then incubated at 37°C, 5% CO_2 for 1 hr. The absorbance at 490 nm in each well was finally recorded using a 96-well plate reader, **Figure S4**.

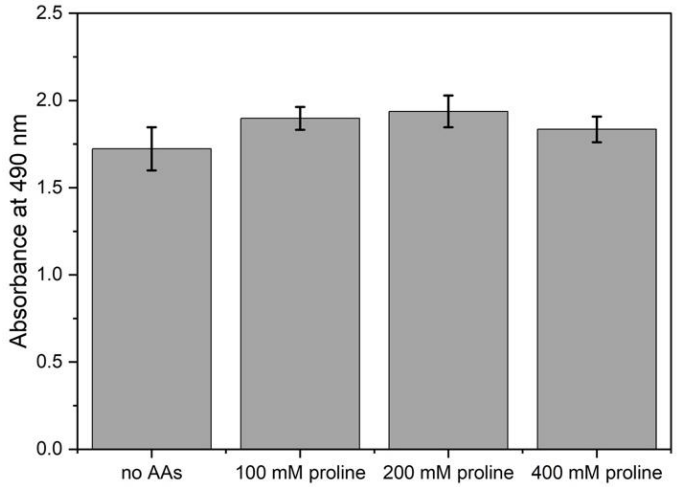

Figure S4: Cell proliferation assay result. The cell viability was evaluated by reading the absorbance at 490 nm after the pre-treatment of proline at 100, 200, and 400 mM for 2 hrs.

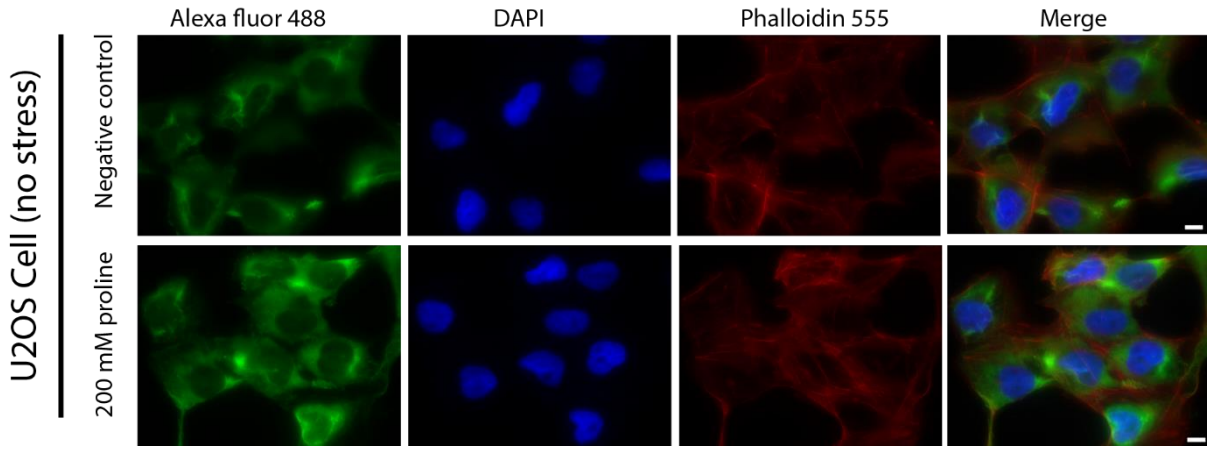

Figure S5: Immunofluorescence microscopy showing normal U2OS cells without any stress after the incubation for 2 hrs in culture media without proline (negative control) and with 200 mM proline.

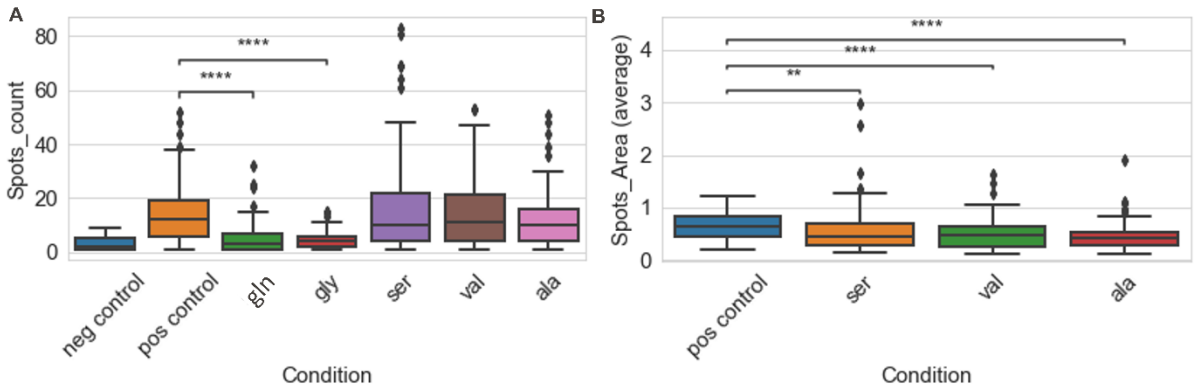

Figure S6: The effect of serine, valine, and alanine (400 mM) on the number (A) and size (B) of SGs by the pre-treatment for 2 hrs.

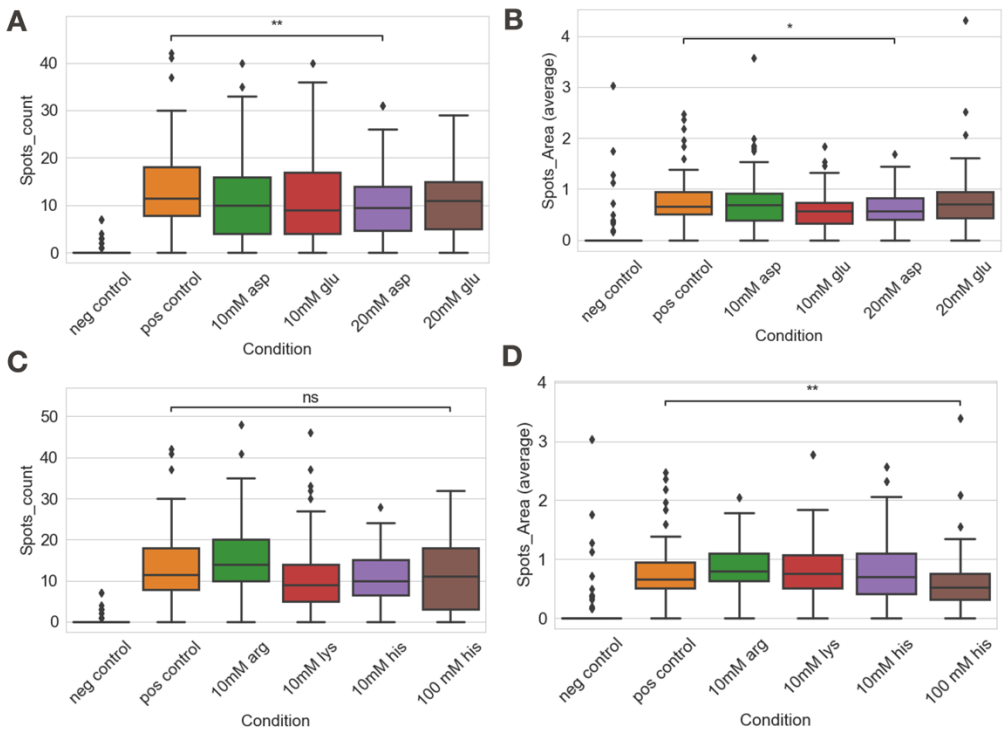

Figure S7: The effect of negatively charged proteinogenic AAs including asparagine, and glutamic acid on the number (A) and size (B) of SGs; The effect of positively charged proteinogenic AAs including arginine, lysine, and histidine on the number (C) and size (D) of SGs. Note: the concentration of charged AAs used here is the maximum concentration without affecting buffer pH significantly (6.6 – 8.6).

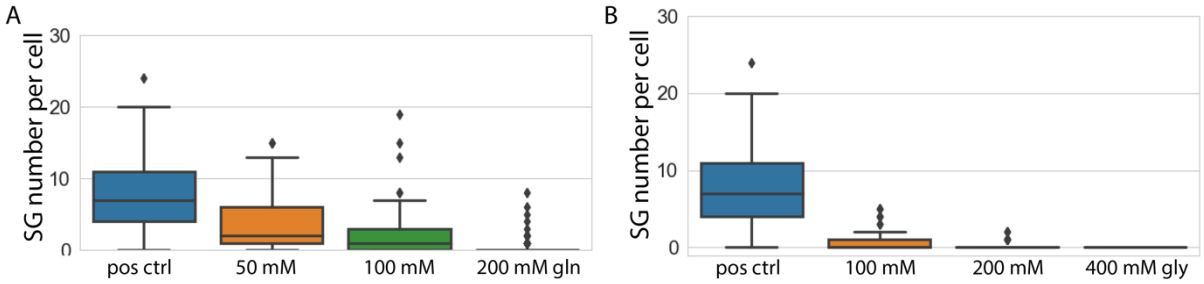

Figure S8: (A) The number of SGs per cell after the pre-treatment with 50, 100, and 200 mM glutamine in response to heat shock stress; (B) The number of SGs per cell after the pre-treatment with 100, 200, and 400 mM glycine in response to heat shock stress.

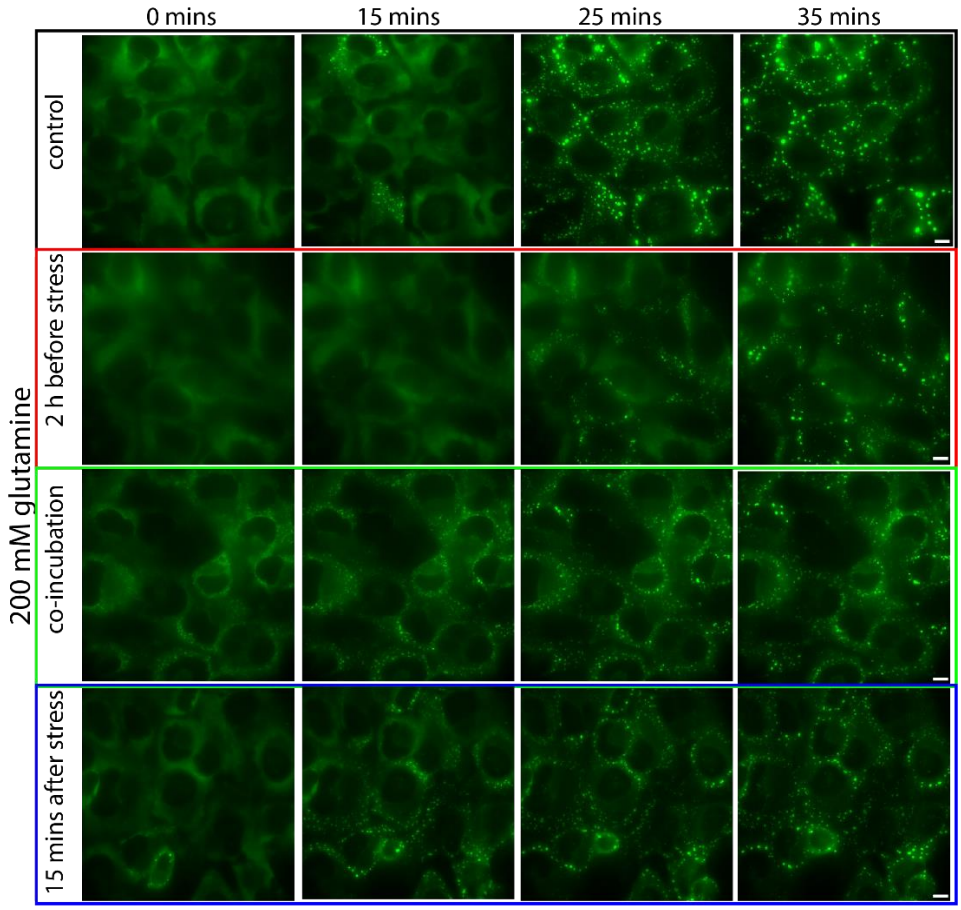

Figure S9: The effect of glutamine (200 mM) on the formation process of stress granules (SGs) studied by live cell microscopy. The pre-treatment for 2 hours, the co-treatment, and the post-treatment (15 mins after the addition of NaAs) with 200 mM glutamine all suppressed the formation of SGs compared to positive control (no exogenous AAs were added) by live cell microscopy of U2OS cells with GFP tagged G3BP1. Scale bar: 10 µm.

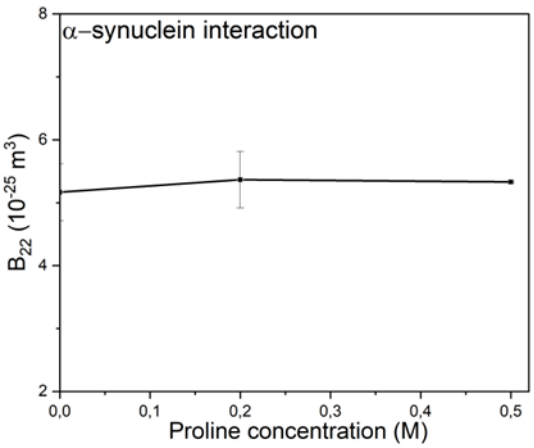

Figure S10: B_{22} for α-synuclein interaction with different proline concentrations (0.2 and 0.5 M).

Reference:

1. Peng, W., Schröder, L. F., Song, P., Wong, Y. C. & Krainc, D. Parkin regulates amino acid homeostasis at mitochondria-lysosome (M/L) contact sites in Parkinson's disease. *Science Advances* **9**, eadh3347 (2023).
2. Hutson, R. G. *et al.* Amino acid control of asparagine synthetase: relation to asparaginase resistance in human leukemia cells. *American Journal of Physiology-Cell Physiology* **272**, C1691–C1699 (1997).
3. Bergström, J., Fürst, P., Norée, L. O. & Vinnars, E. Intracellular free amino acid concentration in human muscle tissue. *Journal of Applied Physiology* **36**, 693–697 (1974).
4. Ghasemi, M., Turnbull, T., Sebastian, S. & Kempson, I. The MTT Assay: Utility, Limitations, Pitfalls, and Interpretation in Bulk and Single-Cell Analysis. *Int J Mol Sci* **22**, 12827 (2021).