QUANTIFICATION AND STATISTICAL ANALYSIS

Fluorescence correlation spectroscopy

GFP and mCherry fluorescence values were converted to absolute concentrations using fluorescence correlation spectroscopy (FCS), performed as described previously (Bracha et al., 2018) with minor modifications. Data for diffusion and concentration of indicated fluorescent fusion proteins were obtained with 30-second FCS measurement time. The measurements were performed on U2OS G3BP1/2 2KO ("G3BP KO") cell populations expressing iLID-mGFP or mCherry-sspB, fusion protein conditions that were chosen based on the assumption that such non-native fusion proteins would be monomeric and feature no major endogenous binding partners. Images were taken using a Nikon A1 laser scanning confocal microscope with an oil immersion objective (Plan Apo 60X/1.4 numerical aperture, Nikon). All measurements and data analysis were performed using the SymPhoTime Software (PicoQuant). The autocorrelation function for simple diffusion is:

$$G(\tau) = G(0)\left(1+\left(\frac{\tau}{\tau_D}\right)\right)^{-1}\left(1+\left(\frac{\tau}{\kappa^2\tau_D}\right)\right)^{-0.5}$$

The variables in the above equation are defined as follows:
$G(0)$ is magnitude at short time scales; τ is the lag time; τ_D is the half decay time; and κ is the ratio of axial to radial of measurement volume ($\kappa = (\omega_z/\omega_{xy})$. Here, ω_{xy} = 0.19 μm and κ = 5.1, which is determined by the fluorophore dye Alexa488 in water. The parameters τ_D and $G(0)$ are optimized in the fit and are used to determine the diffusion coefficient ($D = \omega_{xy}^2/4\tau_D$) and molecule concentration ($C = (\pi^{\frac{3}{2}}\omega_{xy}^2\omega_z G(0))^{-1}$).

The fluorescence to concentration calibration curves displayed in **Figure S1C** were used for all experiments that quantitatively assess the concentrations of mCherry- and mGFP-tagged fusion proteins in WT and G3BP KO U2OS cells. Such FCS calibration curves

yielded several findings that support the precision of such estimates. These are detailed below.

First, independently performed mCherry FCS experiments yielded concentration estimates that were <5% different from previous measurements (Bracha et al., 2018). Further, the aforementioned study used an autocatalytic P2A system to co-express mGFP and mCherry at equimolar ratios, with GFP concentrations extrapolated from the FCS calibration curves determined for mCherry. This indirectly extrapolated calibration curve predicted GFP concentrations that differed by <20% from the independently obtained calibrations and estimations used in this study.

Second, the slope determined in **Figure 3B**, which quantifies stoichiometry between USP10 and G3BP required to differentiate cells that form stress granules from those that are unable to, is remarkably close to 1 (~0.98). A slope of 1 is predicted for such a competitive inhibitor ("cap") expressed at concentrations far greater than its K_d and is further confirmed by nearly equivalent slopes for other strong inhibitors ("caps" e.g. USP10 NIM and CAPRIN1 NIM).

Third, we estimated the concentration of G3BP1/2 in U2OS cell cytoplasm by adding the G3BP concentration for rescue (620 nM) **(Figure 3B)** and USP10 concentration for SG inhibition (1560 nM) **(Figure S3B)** to extrapolate a concentration of ~2180 nM. This value is approximately equal to independently obtained mass spectrometry values in HeLa cells (1808 nM in cytoplasm, a value extrapolated from the reported estimate of 1446 nM in whole cells, based on the assumption that the nucleus accounts for 20% of total volume and all G3BP is located to the cytosol (Hein et al., 2015)). Importantly, Western blot confirms similar levels of both G3BP1 and G3BP2 in HeLa and U2OS cells **(Figure S1B)**.

Fourth, we determined that mGFP-G3BP1 and G3BP1-mCherry feature identical SG rescue concentration thresholds (**Figure S1D**, within 50 nM of each other), despite different fluorescent protein tags. Taken together, these observations give confidence

that our FCS calibration curves are highly accurate for estimating fluorescent protein concentration in living cells.

Image analysis

All images were analyzed using a combination of manual image segmentation (ImageJ), custom semi-automated workflows in ImageJ, and automated analysis in MATLAB 2018b. In all experiments, regions of interest were selected in ImageJ and average cytoplasmic intensities were calculated using the aforementioned FCS calibration curves. The presence of stress granules was, in cases other than the cycling experiments, determined by manual scoring based upon co-localization with a protein marker of stress granules that features diffuse distribution in the cytoplasm in the absence of stress (and further, is diffuse in the cytoplasm of stressed G3BP KO cells without ectopic expression of a protein that rescues stress granule defects).

Manual image segmentation

The average fluorescence intensity for mCherry and mGFP in an individual cell was used to approximate the concentration of associated fusion proteins. This was determined by using manual image segmentation (ImageJ) to draw 4.5 x 4.5 μm square ROIs in cytoplasmic regions featuring homogenous distribution of fluorescence (i.e. regions with low density of membrane-bound organelles like the Golgi). The aforementioned FCS calibration curves were then used to determine the protein's concentration. Presence or absence of stress granules was manually annotated. For Corelet phase diagrams, phase separation was assessed based on whether visible "puncta" formed following a 5-minute activation time course (6-second intervals between images). Only fully activated cells were considered to avoid confounding effects related to diffusion-based capture (Bracha et al., 2018).

Light-dark cycling experiments

Individual regions of interest, which remained in the field of view throughout the time course, were manually selected. Standard deviations were calculated from the measured mCherry intensity and were normalized by the standard deviation at the first frame taken.

G3BP rescue competition data analysis in G3BP KO U2OS cells

The concentration of each cell was determined via manual image segmentation as previously described, and absence or presence of stress granules was annotated. To determine a boundary from the data, a support vector machine (SVM) trained using the concentrations of the two components as explanatory variables and the categorical stress granule state as a response variable by applying the *fitcsvm()* function in the MATLAB Statistics and Machine Learning package using the default solver. Briefly, a support vector machine constructs a linear decision surface based on boundary points ("support vectors"), with the assumption that the data is linearly separable. In this two-dimensional case, the parameters of slope and intercept were extracted to calculate the minimal G3BP concentration for stress granule formation as well as the stoichiometry of interactions with proteins of interest (i.e. the slope of the corresponding line).

Phase diagrams and calculation of threshold valence

For each phase diagram, mean concentrations of both iLID-GFP-Fe core and mCherry-sspB-tagged proteins were calculated and assigned to the category of having or not having stress granules. To determine phase threshold boundaries in an automated and unbiased fashion, an SVM regressor was again used, using the core concentration and log2-transformed valence as explanatory variables with the presence of phase separated structures as a categorical response variable. However, because the data was not linearly separable, a polynomial kernel with degree=2 was used to account for the curvature of the phase threshold. Then, to calculate the decision surface, the score of the SVM was calculated at all points in a 50-by-50 grid in the phase diagram, and a contour line representing the phase threshold was drawn connecting points with a score of 0 using MATLAB's *contour*() function. Specific values for threshold valence at specified core concentrations were then calculated by linearly interpolating the zero-score contour line.

Quantification of threshold concentration for inhibition of stress granule assembly (WT cells) or rescue (G3BP KO cells)

For each experiment, the concentration of the protein of interest was determined for each cell, and the presence (or absence) of stress granules was categorized. The threshold concentration of inhibition (or rescue) was defined as the concentration of protein of interest at which cells had a 50 percent chance of having stress granules. Specifically, the probability density was calculated by binning the concentration distribution using a square root number rule. Within each bin, the probability of having stress granules was calculated as the number of cells with stress granules over the total number of cells in that bin. This results in a monotonic function; its value at a probability of 0.5 was then interpolated to determine the threshold concentration of inhibition or rescue. This was repeated for each replicate and standard error of the mean between replicates was used to determine error bars. The bin size was used as the error if it was greater than that calculated for the SEM or in experiments with a single replicate.

Partitioning coefficient image analysis

To determine partition coefficients (PCs) of fluorescently tagged proteins of interest into stress granules (marker = GFP-CAPRIN1 or mCherry-CAPRIN1), confocal microscopy images were taken at three different settings to prevent oversaturation of the images in both fluorescent channels (488, GFP; 546, mCherry). For each set of images, the image with the highest intensities yet lacking saturated pixels was analyzed. Stress granules (SGs) were first segmented in the CAPRIN1 channel by applying a Laplacian of Gaussians (LoG) filter with a kernel size of 6-pixels to the image. The resulting image was then thresholded and a mask from pixels with a LoG intensity of greater than 1.5 standard deviations was generated. Pixels near the border of the image or SGs containing fewer than 9-pixels were removed from the analysis. To determine the intensity inside SGs while avoiding intensity gradients near the edge of the SGs, masks were thinned. Likewise, to calculate the background intensity near but outside the SGs, an annulus was constructed by subtracting a mask thickened 8-times from that thickened 4-times from the original thresholded image (bwmorph, MATLAB2018b). Then, for each identified SG, an average intensity inside and outside the SG was calculated by background subtracting and averaging the intensities of the corresponding pixels in the fluorescently tagged protein of interest channel based on the aforementioned

segmentation of the CAPRIN1 channel. The partition coefficient (PC) was calculated by solving the following linear system: $PC*I_{in}=I_{out}$ (mldivide, MATLAB2018b). Error was calculated from the standard error of the mean of intensity I_{in} and I_{out} and then propagated by combining in quadrature.

Model of PPI network phase separation

We adapted the SAFT formalism (CHAPMAN et al., 1989) to model a mixture of colloidal particles with associative interactions, in which each binding site can engage in at most one bond at a time. A colloid in this model refers either to a protein monomer or complex, or to a substrate monomer. Denoting the number of binding sites of type A on a colloid of type i by f_{iA}, we used a prescribed PPI network to specify which binding site pairs (iA, jB) are allowed to interact. Our SAFT-based approach requires two key approximations (Jacobs et al., 2014): First, we used a mixture of colloids with no attractive interactions as the reference state, which means that spatial correlations due to associative interactions are not taken into account. Second, the functional form of the free energy assumes that correlations among binding site availabilities can be ignored.

Defining the volume fraction occupied by colloids of type i as ϕ_i, the total dimensionless Helmholtz free energy density, F/kT, is (Jacobs et al., 2014; Michelsen and Hendriks, 2001)

$$\frac{F}{kT} = \frac{F_{\text{ref}}}{kT} + \sum_i \left(\frac{\mu_{\text{assoc},i}}{kT} \right) \phi_i - \frac{P_{\text{assoc}}}{kT},$$

$$\frac{F_{\text{ref}}}{kT} = \sum_i \phi_i \log \phi_i + \left(1 - \sum_j \phi_j\right) \log\left(1 - \sum_j \phi_j\right),$$

$$\frac{\mu_{\text{assoc},i}}{kT} = \sum_A f_{iA} \log X_{iA},$$

$$\frac{P_{\text{assoc}}}{kT} = -\frac{1}{2} \sum_i \phi_i \sum_A f_{iA} (1 - X_{iA}),$$

where F_{ref} is the free energy of a reference system of colloids with no attractive interactions; $\mu_{\text{assoc},i}$ and P_{assoc} are the associative contributions to the chemical potential of colloid type i and the pressure, respectively; and X_{iA} denotes the fraction of binding sites of type A on a colloid of type i that are unbound at equilibrium. The expression for X_{iA} is given by the chemical equilibrium equations

$$X_{iA} = \left[1 + \sum_j \phi_j \sum_B f_{jB} X_{jB} \Delta_{iA,jB}\right]^{-1},$$

which must be solved self-consistently at fixed $\{\phi_i\}$. The interaction parameters $\{\Delta_{iA,jB}\}$ are non-zero only for binding site pairs that are connected in the prescribed PPI network. We choose to work in the strong-binding regime, taking $\Delta_{iA,jB} = 10^4$ for all interacting binding site pairs, so that X_{iA} is determined primarily by the topology of the PPI network.

Phase coexistence and free-energy landscape calculations

We identified the conditions for phase coexistence in two steps. First, we calculated the convex hull of a grid of points $(\{\phi_i\}, F[\{\phi_i\}]/kT)$ (Mao et al., 2019; Wolff et al., 2011). We identified facets of the hull that correspond to coexistence regions by comparing the distances between the vertices of the facets to the minimum distance between adjacent points on the grid. In this way, we concluded that at most three phases can simultaneously coexist given the networks in **Figure 7A,B**, and that at most two phases can coexist given the networks in **Figure 7C,D**. The values of $\{\phi_i\}$ at the vertices of a facet approximate the coexistence concentrations at the chemical potential vector, $\{\mu_i/kT \equiv \partial(F/kT)/\phi_i\}$, determined from the facet normal vector.

We then used the discretized convex hull results as a starting point for higher-precision phase-coexistence calculations. These off-grid calculations were used to tune $\{\phi_i\}$ and $\{\mu_i\}$ to ensure equal chemical potentials and pressures among all phases, as required for coexistence at equilibrium (Rubinstein, 2003). However, specifying three-phase (two-phase) coexistence in a mixture of four colloid types leaves two (three) other degrees of

freedom undetermined. We therefore needed to specify the chemical potential differences among three components in **Figure 7A,B** and among four components in **Figure 7C,D**. In **Figure 7A**, we started from the coexistence facet with $\{\mu_i\}$ closest in chemical potential space to the centroid of all three-phase coexistence facets, and then fixed the chemical potential differences $\mu_{\text{Node1}} - \mu_{\text{Substrate}} = 0.85kT$ and $\mu_{\text{Bridge}} - \mu_{\text{Node1}} = 3.5kT$. For the sake of comparison, we chose the same fixed chemical potential differences in **Figure 7B-D**. In **Figure 7C,D**, we chose the additional chemical potential difference $\mu_{\text{Node2}} - \mu_{\text{Bridge}}$ to be equal to the value obtained from the three-phase coexistence calculation in **Figure 7A**.

To generate the free-energy plots shown in **Figure 7**, we calculated

$$\frac{\Delta F\left(\{\phi_i\}, \{\mu_i^{(\text{coex})}\}\right)}{kT} = \frac{F(\{\phi_i\})}{kT} - \sum_i \left(\frac{\mu_i^{(\text{coex})}}{kT}\right)\phi_i,$$

at the coexistence chemical potential vector $\{\mu_i^{(\text{coex})}\}$. We then plotted $\Delta F / kT$ along a linear path through concentration space, $\{\Delta\phi_i\}$, between each pair of coexisting phases. In **Figure 7C,D**, where there is no stable α phase, we calculated the free energy along a linear path to the location of the α phase in panel A. The compositions reported on the free-energy plots are the volume fractions of the components present in each phase, normalized by the total colloid volume fraction in that phase and rounded to the nearest 5%.

We note in the main text that the increased free-energy barrier height between the α and β phases in **Figure 7B** tends to disfavor wetting of these phases. Strictly speaking, the three-phase junction (α, β, and dilute) pictured in the cartoon in **Figure 7A** is mechanically stable when the surface tension between the α and β phases, $\gamma_{\alpha\beta}$, is less than the sum of the surface tensions between the other pairs of phases, $\gamma_{\alpha\text{Dil}} + \gamma_{\beta\text{Dil}}$ (Gennes, 2004). According to the Cahn--Hilliard theory of planar interfaces (CAHN and

HILLIARD, 1958) $\gamma_{\alpha\beta} \propto \int_{\alpha}^{\beta} d(\Delta\phi_{\alpha\beta})\,[\Delta F(\Delta\phi_{\alpha\beta})/kT]^{1/2}$, where $\Delta\phi_{\alpha\beta}$ is the distance along a linear path in concentration space between the phases α and β. Assuming that the constant of proportionality is roughly the same for all pairs of phases, we find that $\gamma_{\alpha\beta} \lesssim \gamma_{\alpha\mathrm{Dil}} + \gamma_{\beta\mathrm{Dil}}$ in **Figure 7A**, while $\gamma_{\alpha\beta} > \gamma_{\alpha\mathrm{Dil}} + \gamma_{\beta\mathrm{Dil}}$ in **Figure 7B**. Nevertheless, the true morphologies depend on the exact values of these proportionality constants, the minimum-free-energy paths through concentration space that connect the phases, the curvature of the physical interfaces, and other details that are beyond the scope of this minimal model.

Importantly, the qualitative features of these plots, including the number of phases in the coexistence region and the relative heights of the barriers, are relatively insensitive to the choice of $\Delta\mu$ values. We also verified that these qualitative features are not sensitive to variations in the relative binding interaction parameters.

Data and Code Availability

The raw imaging datasets and associated custom MATLAB code supporting the current study have not been deposited in a public repository because of their large size (~1 TB) but are available from the corresponding author on request.