PHASE SEPARATION

Phase separation provides a mechanism to reduce noise in cells

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Expression of proteins inside cells is noisy, causing variability in protein concentration among identical cells. A central problem in cellular control is how cells cope with this inherent noise. Compartmentalization of proteins through phase separation has been suggested as a potential mechanism to reduce noise, but systematic studies to support this idea have been missing. In this study, we used a physical model that links noise in protein concentration to theory of phase separation to show that liquid droplets can effectively reduce noise. We provide experimental support for noise reduction by phase separation using engineered proteins that form liquid-like compartments in mammalian cells. Thus, phase separation can play an important role in biological signal processing and control.

Stochasticity in gene expression causes substantial noise in protein concentration, even in genetically identical cell populations grown in the same environmental conditions (*1*–*4*). Despite this noise, living organisms display an extraordinary degree of robustness and exhibit precise spatial and temporal organization.

Liquid-liquid phase separation provides a potential mechanism to reduce noise in protein concentration (*5*–*7*). This is because in a phase-separating system, the concentrations inside and outside the droplets are constrained by thermodynamic laws. When the total concentration of protein changes, the droplets will change in number and size, but the concentration outside of the droplets may be insensitive to these changes (Fig. 1, A and B).

The thermodynamic constraints on coexisting concentrations are well established for macroscopic phase-separating systems at equilibrium. However, phase-separating systems inside cells exhibit mesoscopic noise and are, in general, out of equilibrium. Therefore, whether cells can effectively use phase separation to control protein concentration levels and potentially reduce noise is unclear.

To study under which conditions noise reduction can be effective, we developed a mesoscopic theory that links protein concentration fluctuations to the physics of liquid-liquid phase separation (*8*–*11*). The theory is based on the thermodynamics of a binary mixture segregating into a dilute and droplet phase when the total protein concentration exceeds a threshold value (Fig. 1C and supplementary text 1.1). In our model, we account for nonequilibrium fluctuations due to stochastic synthesis and turnover of protein (Fig. 1D). This system can be described by a master equation, which captures the statistics of the dilute-phase and total protein concentrations, ϕ_+ and $\bar{\phi}$, which can be characterized by their mean $\langle\phi\rangle$ and noise strength $CV^2[\phi] = \sigma^2[\phi]/\langle\phi^2\rangle$, where $\sigma^2[\phi]$ is the variance.

We first considered a situation in which the exchange of protein between phases is much faster than protein synthesis and degradation, which we refer to as quasi-equilibrium. As the mean of the total protein concentration $\langle\bar{\phi}\rangle$ increases and approaches the threshold value ϕ^*, droplets begin to form while the noise strength $CV^2[\phi_+]$ of the dilute-phase concentration ϕ_+ starts to decline (Fig. 1E). For larger mean total concentration, the noise strength settles at a minimum with approximately Poissonian noise (supplementary text 1.1.3). We further show that around the minimal noise strength, concentration fluctuations due to protein expression are suppressed, and the remaining fluctuations are predominantly thermal fluctuations of the phase-separating system (supplementary text 1.2.2). Therefore, as long as phase separation is much faster than protein synthesis and degradation, droplets can reduce noise in protein concentration down to the Poisson limit.

We next considered situations in which the time scales of protein expression and phase separation approach each other. Figure 1F shows the noise strength for three different time-scale ratios of protein diffusion and turnover (τ_D/τ_p) versus mean total concentration. As before, the noise strength in the dilute phase first approaches a minimum as the mean total concentration increases. However, beyond this minimum, the noise strength starts to increase. This is because for highly expressing cells, the rate of protein synthesis is fast compared with the time it takes for a protein to diffuse into a droplet. As a consequence, proteins can accumulate in the dilute phase, which hampers the system's ability to reduce noise at high protein-synthesis rates. The minimal noise strength depends on the time scales of protein diffusion and turnover and is generally above the Poisson limit (Fig. 1G). Thus, noise reduction by phase separation is predicted to be most effective for long-lived and fast-diffusing proteins.

To test the concept of noise reduction inside cells, we used a recombinant 2NT-DDX4^{YFP} protein that phase-separates in vitro (fig. S21). We expressed 2NT-DDX4^{YFP} inside HeLa cells and examined protein concentration and spatial distribution using live-cell microscopy (Fig. 2A). Transient transfection generated a broad range of protein expression levels because of large variability in plasmid transfection efficiency. Similar to the previously described (*12*) DDX4^{YFP}, the 2NT-DDX4^{YFP} variant formed heterologous compartments inside nuclei of transfected HeLa cells (fig. S22). The 2NT-DDX4^{YFP} droplets fused with each other, coarsened over time, and showed high internal recovery after photobleaching, which together confirmed their liquid-like behavior (*13*) (fig. S23). Pixel fluctuation analysis (*14*) showed no evidence for small clusters below the diffraction limit, which could lead to an overestimation of dilute-phase concentration (materials and methods and fig. S24). We used statistical methods (*15*) to estimate the parameters of our nonequilibrium model from time-lapse 2NT-DDX4^{YFP} expression data (Fig. 2B, materials and methods, and supplementary text 2.2).

We next determined the mean concentration and noise strength of 2NT-DDX4^{YFP} in more than 10,000 cells 24 hours after transfection (Fig. 2C) and performed a comparison with our theory. To achieve this, we randomly selected subpopulations from the total pool of cells with prescribed mean total protein concentration and noise strength. For each subpopulation, we then quantified the mean and noise strength of dilute-phase 2NT-DDX4^{YFP} concentration. The results show that protein concentration noise is indeed reduced in the dilute phase as soon as the mean 2NT-DDX4^{YFP} concentration approaches the threshold concentration (Fig. 2D). To compare noise reduction with theory, we used subpopulations of cells with mean total concentration and noise strength that correspond to the statistics of total protein concentration in our model (supplementary text 2.3). The data reveal the features of noise reduction as predicted by our nonequilibrium theory—in particular, a minimum of noise strength at a particular mean total concentration (Fig. 2D). In the experimental data, noise reduction is less pronounced than in the theory (compare green dotted and solid lines), possibly because of additional cell-to-cell differences within

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subpopulations that are not captured by our model (supplementary text 2.4).

The condensates formed by 2NT-DDX4^{YFP} dissolve during mitosis (Fig. 3A), similar to other membraneless organelles (*16*). To test whether dissolving the condensates leads to an increase in noise strength, we followed 141 individual droplet-containing cells through mitosis (fig. S25) and quantified the fluorescence intensity in the dilute phase before, during, and after mitosis. We found that droplet dissolution in mitosis is associated with a more than twofold increase of noise strength in the dilute phase (Fig. 3, B to D, and movie S1). In most postmitotic cells, droplets reform, which again leads to a reduction of noise strength in the dilute nucleoplasmic phase. These data strongly suggest that the lower noise levels in the dilute phase observed during interphase are indeed governed by phase separation and, furthermore, that concentration

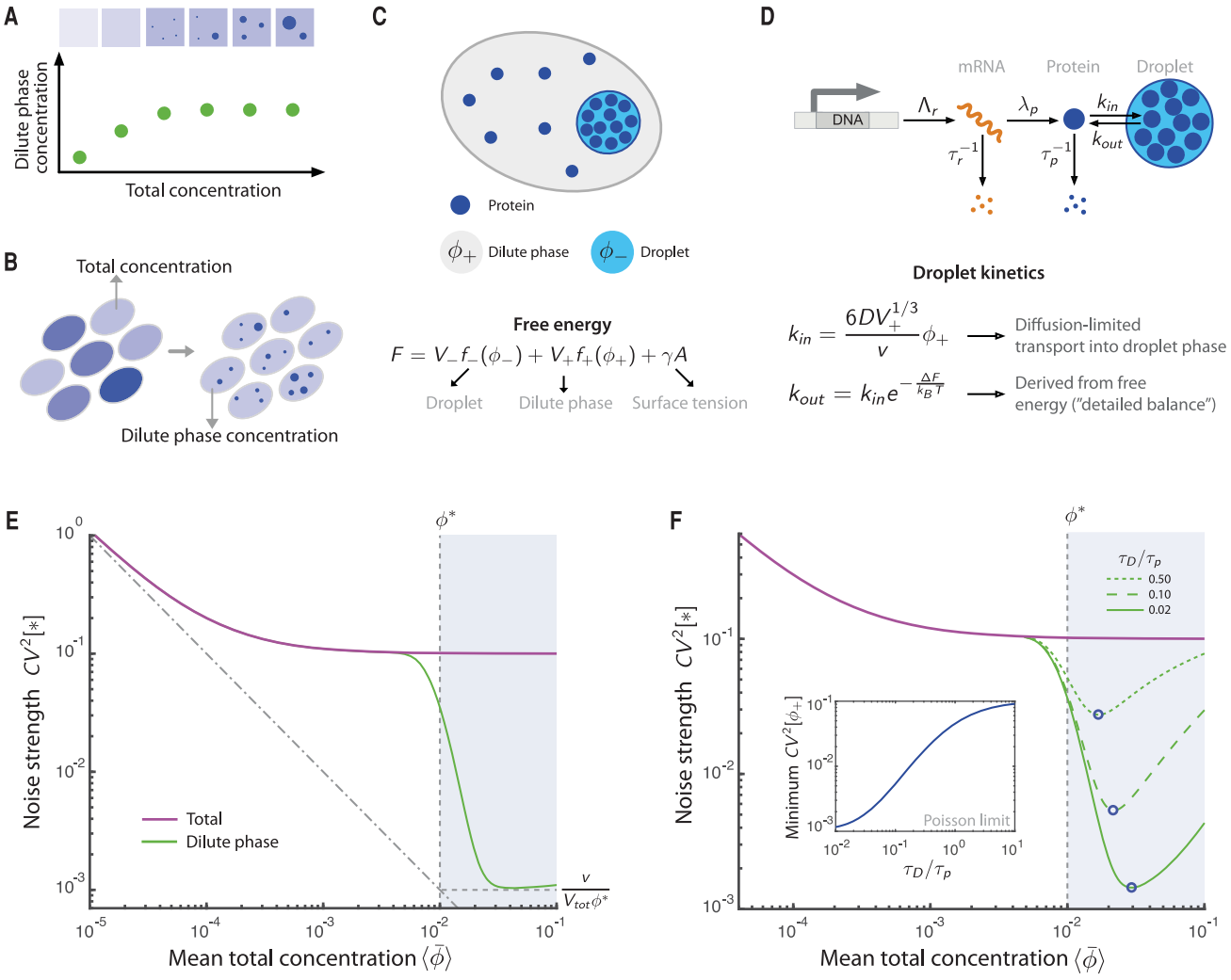

Fig. 1. Concentration fluctuations in a mesoscopic model of phase separation. (**A**) In a simple phase-separating system, the dilute-phase concentration saturates when the total concentration exceeds a threshold. In this regime, droplets can vary in size and number, but the dilute-phase concentration is insensitive to changes in total concentration. (**B**) Phase separation could reduce variability in protein concentration (represented by color intensity) by forming liquid compartments of variable number and size. (**C**) Mesoscopic droplet model of a binary mixture at equilibrium. The mixture consists of a solvent and a protein, which can phase-separate into a dilute and a dense droplet phase. We normalize concentrations c by molecular volume v of the protein and use normalized concentrations (volume fractions) $\phi = vc$. We denote the normalized concentrations in the dilute and droplet phase and the total system as ϕ_+, ϕ_-, and $\bar{\phi}$, respectively. Phase separation is governed by a free energy, which has contributions from both coexisting phases as well as the separating interface, which has a surface tension (supplementary text 1.1.1). (**D**) Droplet model in the presence of protein production and turnover. Fluctuations in total protein concentration are described by a gene expression model accounting for stochastic production and degradation of mRNA and protein (*27*). Additionally, cell-to-cell variability in the transcription rate is taken into account. We capture the dynamics of protein phase separation by stochastic exchange of protein between the dilute and droplet phases. We consider the transport of molecules into the droplet phase (k_{in}) to be diffusion limited. The rate of the corresponding reverse transition (k_{out}) follows from detailed balance (supplementary text 1.1.5). We consider the average protein lifetime τ_p to be the same in both phases. (**E**) Noise strength of total and dilute-phase concentration as a function of mean total concentration in the quasi-equilibrium situation. The minimum of the noise strength in the dilute phase is approximately given by $CV^2[\phi_+] \approx v/(\phi^* V_{tot})$, corresponding to Poissonian noise (dashed horizontal line). The dashed vertical line indicates the threshold concentration ϕ^*. Parameter values are given in table S1. (**F**) Influence of time scales on noise reduction. Noise strength is shown as a function of mean total concentration for three different ratios of the protein diffusion time τ_D and protein lifetime τ_p. The blue circles indicate the minima of the noise strength. (Inset) Minimal noise strength shown as a function of τ_D/τ_p. Parameter values are given in table S2.

noise in cells can be controlled by regulating phase separation.

To test whether noise reduction occurs in an endogenous system, we examined the nucleolus. The nucleolus consists of three coexisting phases that vary in their material properties (*17*). The outermost phase, known as the granular component (GC), exhibits liquid-like properties and is enriched in a protein called nucleophosmin (NPM1), which forms liquid-like droplets in vitro (*17*). Using CRISPR-Cas9, we tagged native NPM1 with mNeonGreen inside HCT116 cells and measured fluorescence intensities in the dilute nucleoplasmic phase of individual cells, which coexists with the GC phase of the nucleolus (Fig. 3, E to H). Similar to the 2NT-DDX4^{YFP} experiments, we found that the dissolution of the GC phase in mitosis is associated with an approximate twofold increase in noise strength in the dilute phase (Fig. 3H and movie S2). Our data suggest that for NPM1 at native expression levels, concentration noise is reduced in the presence of phase-separated condensates.

We used experiment and theory to show that the formation of liquid condensates can reduce concentration noise of proteins participating in phase separation. Many liquid compartments have been identified recently, but their biological function is often

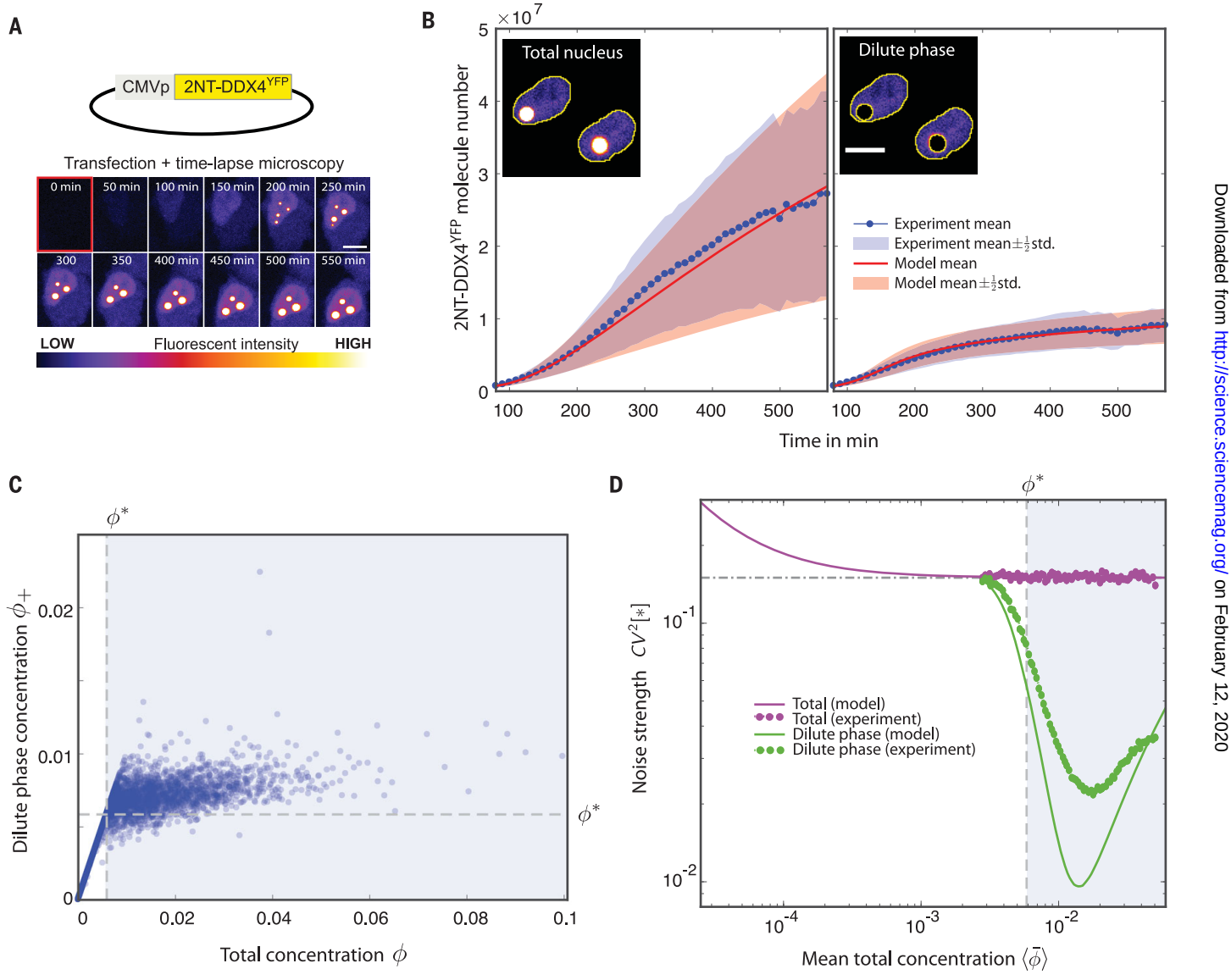

Fig. 2. Reduction of 2NT-DDX4^{YFP} concentration noise in HeLa cells. (**A**) Time-lapse microscopy of a typical cell initiating expression of 2NT-DDX4^{YFP} and phase separation after transfection and plasmid incorporation. CMVp, cytomegalovirus promoter. (**B**) Mean and variance of 2NT-DDX4^{YFP} total (left) and dilute-phase (right) molecule numbers as a function of time. Model parameters were estimated from the time-lapse data (blue) by using statistical methods (supplementary text 2.2). Corresponding model behaviors are shown in red. Shaded areas correspond to half a standard deviation (std.) above and below the mean. The estimated model parameters are given in tables S4 and S5. (**C**) Scatterplot of total versus dilute-phase 2NT-DDX4^{YFP} concentration quantified in more than 10^4 cells 24 hours after transfection. The threshold concentration ϕ^* was estimated as the average of the dilute-phase concentration of the 5% cells with smallest droplets (dashed lines) (supplementary text 2.3). (**D**) Noise strength in dilute-phase (green) 2NT-DDX4^{YFP} concentration as a function of the mean total 2NT-DDX4^{YFP} concentration determined by generating subpopulations with imposed statistics of total concentration (purple). The experimental data are compared with the noise strength predicted by the model by using the parameters estimated from the time-lapse data shown in (B) (green solid line) (supplementary text 2.3).

unclear (*5*, *18*, *19*). Our results suggest that some of them could serve to maintain protein levels within narrow ranges of concentration. For instance, splicing speckles could stabilize splicing activity by controlling the dilute-phase concentration of splicing factors (*20*).

Our work discusses the effects of phase separation in the context of active molecular turnover and provides insights into the interplay between nonequilibrium fluctuations and phase separation. Noise reduction predicted by our simple model was similar to the experimentally measured values for the 2NT-DDX4^{YFP} system. In our theory, we have not considered systems with multiple phase-separating components, which provide more complex thermodynamic constraints on concentrations (*21*, *22*). Extending this theory to multicomponent situations will, therefore, be an interesting subject for future research. Moreover, active

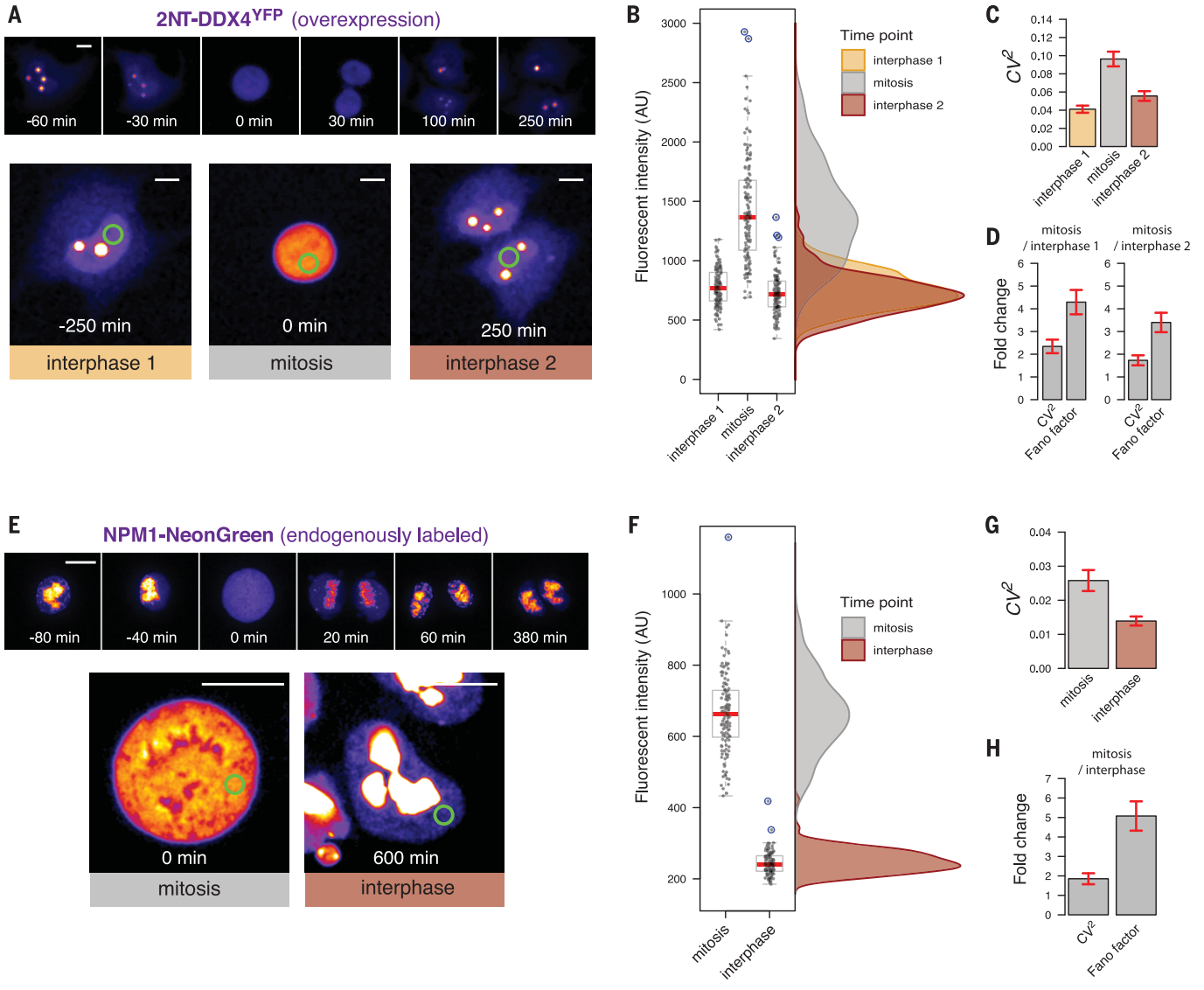

Fig. 3. Droplet dissolution during mitosis results in increased noise in protein concentration. (**A**) HeLa cell expressing 2NT-DDX4^{YFP} from a transiently transfected plasmid imaged during mitosis. (**B**) Quantification of mean 2NT-DDX4^{YFP} fluorescence intensity in the dilute phase [green circle in (A)] of the same 141 cells before, during, and after mitosis. Distribution of the measured values is displayed as a density histogram. AU, arbitrary units. (**C**) Noise in the protein concentration of the dilute phase increased from 0.038 (±0.004) in interphase to 0.081 (±0.008) in mitosis and reduced down to 0.046 (±0.005) in subsequent interphase. (**D**) Fold increase in the noise of 2NT-DDX4^{YFP} dilute-phase concentration ϕ_+ during mitosis compared with preceding and subsequent interphase. Bar plots represent ratios of the noise strength $CV^2[\phi_+]$ for each group. For comparison, the increase in noise has been quantified also in terms of the Fano factor ($FF[\phi_+] = \sigma^2[\phi_+]/\langle\phi_+\rangle$), another common measure to quantify fluctuations. (**E**) HCT116 cell expressing NPM1 labeled endogenously with NeonGreen imaged during mitosis. (**F**) Quantification of mean NPM1-NeonGreen fluorescence intensity in the dilute phase of 127 cells imaged during and after mitosis [green circle in (E)]. Distribution of the measured values is displayed as a density histogram. (**G**) Noise in the protein concentration of the dilute phase decreased from 0.026 (±0.003) in mitosis to 0.014 (±0.001) in subsequent interphase. (**H**) Noise of NPM1-NeonGreen dilute-phase concentration during mitosis and subsequent interphase. Bar plots represent ratios of $CV^2[\phi_+]$ and $FF[\phi_+]$. Scale bar is 10 μm. Bottom panels in (A) and (E) were set to high contrast to demonstrate the change of intensity in the dilute, nucleoplasmic phase. Boxplots indicate median (red bar) and first and third quartiles. Lower and upper whiskers extend to 1.5 times the interquartile range from the first and third quartile, respectively. Outliers that fell 2.5 standard deviations away from the mean (blue circles in boxplots) were excluded from the analysis. Error bars in bar plots represent standard errors of the measurements calculated with bootstrapping.

chemical processes such as posttranslational modifications influence endogenous phase-separated compartments (*23*) and could contribute to noise in notable ways.

Previous studies have proposed that spatial compartmentalization of molecules can be a potential mechanism to enhance the robustness of biological systems (*24*–*26*). For instance, delayed nuclear export of transcripts can lead to reduced variability in cytoplasmic RNA (*24*), and protein clustering can enhance the robustness of biological switches (*25*) and subcellular gradient formation (*26*). Understanding how membrane- and non–membrane-bound compartments affect noise in living systems remains a substantial challenge.

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SUPPLEMENTARY MATERIALS

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Materials and Methods
Supplementary Text
Figs. S1 to S26
Tables S1 to S5
References (*28*–*35*)
Movies S1 and S2

View/request a protocol for this paper from *Bio-protocol*.

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Keeping the noise down

Protein concentrations in a cell fluctuate considerably because of stochasticity in gene expression and variations in the cell's microenvironment. How cells cope with concentration fluctuations when precision is important is unclear. Klosin *et al.* used a combination of theoretical and experimental work to demonstrate that phase-separated compartments can effectively reduce protein concentration noise in cells (see the Perspective by Riback and Brangwynne). The results suggest that phase separation provides a mechanism to enhance the robustness of biological systems.

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