Anomalous Dense Liquid Condensates Host the Nucleation of Tumor Suppressor p53 Fibrils

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SUMMARY

About half of human cancers are associated with mutations of the tumor suppressor p53. Gained oncogenic functions of the mutants have been related to aggregation behaviors of wild-type and mutant p53. The thermodynamic and kinetic mechanisms of p53 aggregation are poorly understood. Here we find that wild-type p53 forms an anomalous liquid phase. The liquid condensates exhibit several behaviors beyond the scope of classical phase transition theories: their size, ca. 100 nm, is independent of the p53 concentration and decoupled from the protein mass held in the liquid phase. Furthermore, the liquid phase lacks constant solubility. The nucleation of p53 fibrils deviates from the accepted mechanism of sequential association of single solute molecules. We find the liquid condensates serve as pre-assembled precursors of high p53 concentration that facilitate fibril assembly. Fibril nucleation hosted by precursors represents a novel biological pathway, which opens avenues to suppress protein fibrillation in aggregation diseases.

INTRODUCTION

Liquid-liquid phase separation constitutes the formation pathway of several common organelles such as nucleoli, Cajal bodies, and P granules, which are not delimited by membranes (Uversky, 2017; Wei et al., 2017). The condensates generated by phase separation are thought to concentrate biological reactants and to coordinate responses to environmental stress, among other functions (Shin and Brangwynne, 2017). A fundamental open question concerns the mechanism and consequences of the transition of the liquid condensates into ordered or disordered solids (Shin and Brangwynne, 2017) that may be associated with the growing class of aggregation diseases, such as sickle cell, Alzheimer's, and diabetes mellitus 2 (Aguzzi and O'Connor, 2010; Kato et al., 2018; Knowles et al., 2014).

The transcription factor p53, one of the most important tumor suppressors, transforms after mutation into a powerful cancer promoter that blocks the anti-cancer activity of wild-type p53, its paralogs p63 and p73, and other distinct anti-cancer pathways (Bieging et al., 2014; Joerger and Fersht, 2016). Several mechanisms of the gain of oncogenic function (GOF) of mutant p53 have been proposed (Muller and Vousden, 2013). Malignant mutations that destabilize the p53 conformation enhance its fibrillation (Ang et al., 2006; Olivier et al., 2002). The mutants' co-aggregation with wild-type p53 and its paralogs has been recognized as a potent GOF mechanism (Costa et al., 2016; Xu et al., 2011). Fibril suppression, for instance by stabilization of the mutant p53 conformation, has been identified as a general way to fight cancer (Joerger and Fersht, 2016; Soragni et al., 2016). Accordingly, cancer has been defined, in certain respect, as an aggregation-related disease (Costa et al., 2016; Xu et al., 2011).

The aggregation of mutant and wild-type p53, *in vitro* and *in vivo*, exhibits features typical of β-amyloid fibrillation. The amyloid domains bind to Thioflavin T (ThT), a fluorescent dye that recognizes β-aggregates, and the fluorescence intensity increases following a typical sigmoid curve (Costa et al., 2016; Wang and Fersht, 2017; Wilcken et al., 2012; Xu et al., 2011). Other characteristics of p53 fibrillation have provoked questions on the molecular mechanisms. Notably, the concentration independence of the parameters of the sigmoid curve and the short initial time period have suggested that a "non-classical mechanism" of fibril nucleation and growth operates (Wang and Fersht, 2017; Wilcken et al., 2012). Tests of whether mutant p53 fibrils cross seed wild-type aggregation have produced both positive and negative outcomes (Wang and Fersht, 2015; Xu et al., 2011). Furthermore, the significance of intracellular crowding for the kinetics of fibril nucleation and growth is incompletely understood. In cells, p53 localizes in the nucleus, in which DNA, protein complexes, RNAs, and multiple other types of biomolecules occupy up to 50% of the total volume and significantly modify protein folding, stability, and aggregation (van den Berg et al., 2017).

To address these questions, we explore the aggregation of wild-type p53 at near physiological conditions and in crowded environments. Besides serving as a reference for mutant p53 behaviors, aggregation of wild-type p53 is of interest because it may, under certain conditions, behave like mutant p53 (Muller and Vousden, 2013). Wild-type p53 has been detected in a mutant conformation in hypoxic cells (Gogna et al., 2012) and after binding to a p53 regulator protein, MDM2 (Sasaki et al., 2007). We demonstrate a non-classical nucleation mechanism of p53 fibrils, whereby anomalous p53 liquid condensates serve as pre-assembled precursors that host fibril assembly. We establish the kinetic and thermodynamic laws that govern the formation and decay of p53 condensates and demonstrate that they deviate from the classical laws of phase separation that also govern the formation of dense protein liquids (Shin and Brangwynne, 2017; Uversky, 2017; Wei et al., 2017); this disparity identifies the p53 condensate as a new class of liquid.

RESULTS

Detection and identification of p53 liquid condensates

Oblique illumination microscopy (OIM, Figure 1B and Video S1) (Vorontsova et al., 2015a) revealed that the aggregation of p53 strongly depends on temperature (Figure 1C). (For purification procedures and p53 identification, see Figures S1 and S2.) Solutions incubated at 15°C for 20 min contain no visible aggregates. Whereas solutions kept at 18 and 25°C contain a few aggregates, pronounced aggregation is observed in solutions heated to 37 and 42°C. The average radius R and number N of the aggregates both increase nonlinearly with temperature (Figure 1D and E). The remarkable growth in size and number of these aggregates at temperatures approaching 42°C (Figure 1D and E), the mid-denaturation point of p53 (Bell et al., 2002), suggests that unfolding of p53 may be an essential trigger for aggregation.

To elucidate the mechanism of the aggregates detected in Figure 1C, we started with solutions of unaggregated protein. We removed possible aggregates by filtration and

characterized unaggregated p53 at 15°C by dynamic light scattering. The intensity-intensity correlation functions of the filtrate revealed a broad shoulder (Figure 1F), indicating the presence of distinct scatterers. The characteristic decorrelation time of that shoulder, 90 μs, corresponds to an average diffusivity D_m =32 μm²s⁻¹ (Figure 1G), which relates (by the Stokes-Einstein law $D_m = k_B T/6\pi\eta R_m$, where $k_B T$ is the thermal energy, η = 1.5 mPa·s is independently measured solvent viscosity, and R_m is the molecular radius) to an average R_m = 6 nm, higher than the ca. 4 nm radius expected for a p53 tetramer of molecular weight 175 kg mol⁻¹ (Chalkey et al., 1994). Consonantly with the exaggerated radius, the considerable polydispersity μτ² ≈ 0.35 (Figure 1G) indicates the presence of high-order oligomers (Rajagopalan et al., 2011; Xu et al., 2011). Both D_m and μτ² remained steady over time at 15°C.

To determine whether the aggregates detected in Figure 1C are droplets of dense p53 liquid, we measured the concentration of p53 in equilibrium with the aggregate phase, C_f. We incubated solutions at 15 °C and 37 °C for 20 min and removed the aggregates by filtration. After incubation at 15°C, C_f was nearly identical to the initial concentration C_o (Figure 1H), confirming the lack of aggregates at this temperature (Figure 1C). In solutions incubated at 37°C, however, C_f was sharply lower than C_o, with aggregates capturing up to 80% of the total p53 (Figure 1I). Surprisingly, C_f was not constant, but instead increased exponentially with C_o (inset to Figure 1I). The finding of increasing terminal concentration is in striking contrast with examples of dense protein liquids, which equilibrate with solutions of constant concentration (Broide et al., 1991; Chen et al., 2004; Galkin et al., 2002; Muschol and Rosenberger, 1997; Uversky, 2017; Wei et al., 2017).

Earlier studies reported the formation of p53 amyloid structures (Ang et al., 2006; Costa et al., 2016; Wang and Fersht, 2017; Wilcken et al., 2012; Xu et al., 2011) at 37°C. Similar to dense liquids but in contrast to the aggregates characterized in Figure 1C-E and I, amyloid fibrils exhibit solubility, which is independent of the initial solution concentration (Qiang et al., 2013). To

further assess whether the p53 aggregates are amyloid fibrils, we employed the 1-anilino-8-naphthalenesulfonate (ANS) assay. ANS emits fluorescence at 500 nm when it associates to exposed hydrophobic sites of partially unfolded protein segments and amyloid fibrils (Hawe et al., 2008). The pronounced ANS fluorescence intensity immediately after introduction in the solution (Figure 1J) indicates the presence of misfolded segments that may correspond to the intrinsically disordered TAD and PRR domains of p53 (Figure 1A). The fluorescence intensity was steady for ca. 40 min at 37°C (Figure 1J) and ca. 200 min at 15°C and then ascended, suggesting inception of amyloid fibrillation likely due to misfolding of the structured DBD and OD regions, Figure 1A (Wang and Fersht, 2017; Wilcken et al., 2012). The observed fibrillation delay is likely due to slow nucleation (Wang and Fersht, 2017; Wilcken et al., 2012); importantly, it indicates that the aggregates observed in Figure 1C after 20 min incubation at temperatures in the range 18 – 37°C are not amyloids.

The reversibility of these aggregates, demonstrated by the light scattering results in Figure 1K and L, indicates that they are not disordered agglomerates either. Indeed, the intensity-intensity correlation functions g_2 (Figure 1K) reveal two shoulders, corresponding to the diffusion of two scatterers with characteristic times ca. 100 μs and 10 ms. The fast scatterers are likely the p53 oligomers detected in the filtered solutions at 15°C (Figure 1K). The average radius of the large scatterers, 145 nm, is similar to the aggregate radius revealed by OIM at this temperature, 37°C (Figure 1D). The relative volume of the condensate population ϕ_2, evaluated from g_2 (Pan et al., 2007), is a sensitive function of the p53 concentration (Figure 1L): 1.7-fold dilution of C_0 induces ca. 8× reduction of ϕ_2. The strong dependence of ϕ_2 on C_0 is a manifestation of the condensate reversibility, rarely observed for disordered agglomerates.

The independence of the aggregate size R of C_o and its decoupling from the volume of the aggregate phase ϕ_2 distinguish the observed structures from common protein condensate phases, such as crystals, amyloid fibrils, dense liquids, and amorphous precipitates. In further divergence

from classical phase behaviors, these condensates did not display constant solution concentration at equilibrium. For all common protein assemblies, the equilibrium protein concentration in the supernatant is constant and independent of the initial concentration (Asherie et al., 1998; Chernov and Komatsu, 1995; Fabry et al., 2001; Safari et al., 2017; Thomson et al., 1987). Furthermore, the size of these assemblies after equilibration, measured as fibril length, crystal width, or droplet radius, grows concurrently with the initial solution concentration as an implementation of the law of mass action (Chaikin and Lubensky, 1995; van Raaij et al., 2008).

Unlike these typical aggregates, the size of the observed p53 condensates was independent of p53 concentration and the volume of the condensate phase increased with C_o (Figure 1L). These unusual behaviors have been previously reported for abnormal liquid condensates of several proteins (Gliko et al., 2005; Li et al., 2012; Schubert et al., 2017; Sleutel and Van Driessche, 2014; Yamazaki et al., 2017). We conclude that the anomalous condensates detected in Figure 1 represent clusters of abnormal p53 liquid.

The anomalous p53 liquid condensates in crowded solutions

For further insight into the properties and mechanisms of the anomalous p53 liquid condensates, we examined their behaviors upon addition of a crowding agent. We chose Ficoll 70 kDa, a cross-linked compact polysaccharide with radius 4.7 nm (Georgalis et al., 2012) and concentrations up to 90 mg ml^{-1}, which occupies ca. 35% of the solution volume, to mimic the conditions inside cells. OIM revealed that p53 condensation was boosted when Ficoll was added to solutions (Figure 2A and Video S2). Condensation was enhanced likely because crowding amplified the driving force for reactions that reduce the reactant volume, such as aggregation (Ellis and Minton, 2003). The average size of the condensates, 80 – 90 nm, was independent of the p53 concentration (Figure 2B), analogously to the behavior in uncrowded solutions (Figure 1L), and also of the Ficoll concentration (Figure 2C). By contrast, the amount of p53 in the condensates increased at higher

initial concentration of the solution (Figure 2D). The decoupled p53 amount in the condensates and the condensate size highlights the similarity to the behavior of anomalous p53 liquid condensates existing in uncrowded p53 solutions (Figure 1).

The current theory of anomalous protein liquid condensates suggests that transient protein complexes such as dimers, for single chain proteins, or misassembled oligomers, for oligomeric proteins, accumulate in the condensate core (Chan et al., 2012; Pan et al., 2010; Vorontsova et al., 2015a). In this model, the condensate size is determined by the balance between the lifetime of the complexes and their rate of outward diffusion from the condensate and is, hence, independent of the protein concentration and the other thermodynamic parameters of the solution (Pan et al., 2010; Safari et al., 2017). By contrast, the amount of protein captured in the condensates increases exponentially with the protein concentration as a consequence of a thermodynamic equilibrium between the condensates and the bulk solution (Li et al., 2012; Pan et al., 2010).

The presence of misassembled oligomers in p53 solutions is attested by the exaggerated size and significant polydispersity of unaggregated p53 (Figure 1G). It is feasible that one or more of the misassembled oligomers (distinct from the long-lived dimers and tetramers (Rajagopalan et al., 2011)) would have lifetimes of order 100 μs that underlie condensates of size ca. 100 nm (Chan et al., 2012). To test the applicability of two tenets of the anomalous protein liquid model to the p53 condensates, that the anomalous liquid is enriched in p53 oligomers distinct from the majority tetramers and is in equilibrium with the solution, we examined the striking quasi-exponential dependence of the equilibrium solution concentration C_f on its initial value C_o in both crowded and uncrowded solutions (Figures 2D and 1I).

Thermodynamic analysis discussed in the Supplemental Information accounts for the distinctive composition of the condensates by positing that the modified standard chemical potential of p53 captured in them, ψ_2, differs from that in the solution μ_1^o. In defining μ_1^o, we ignore the polydispersity of the p53 oligomers. We account for the solution nonideality due to

interactions between the p53 solute molecules by introducing an activity coefficient γ as $\ln\gamma = 2B_2M_wC_f$, where B_2 is the second osmotic virial coefficient and M_w is the p53 molecular weight. Assuming equilibrium, $\mu_1 = \mu_2$, the concentration of p53 captured in the condensates is given by $C_2 = C_f \exp\left(-\frac{\psi_2-\mu_1^0}{RT}\right)\exp(2B_2M_wC_f)$. This relation predicts that C_f increases quasi-exponentially with $C_0 = C_2 + C_f$ (Figure S5) and C_2/C_f should be an exponential function of C_f with exponent related to B_2.

To test this prediction, we determined B_2 of the p53 solution by static light scattering (Berne and Pecora, 2000). This analysis yielded average molecular weight M_w =303 kg mol^{-1} and B_2 = -1.1×10^{-3} mol m^3 kg^{-2} (Figure 2E). The value of M_w, about two-fold that of the p53 tetramer, indicates the presence of higher order misassembled oligomers in the solution, consistent with the high polydispersity of p53 solutions and the slow diffusion of unaggregated p53 observed with DLS (Figure 1G). The considerable negative value of B_2 indicates strong effective attraction between p53 molecules. Correspondingly, the slopes of $\ln(C_2/C_f)$ as a function of C_f (Figure 2F) were negative in both crowded and uncrowded solutions. The slopes of the correlations between $\ln(C_2/C_f)$ and C_f, 10.0 and 14.0 m^3 kg^{-1} (Figure 2F), are significantly greater than the product $2B_2M_w$ = 0.66 m^3 kg^{-1}. This discrepancy is likely due to the lower magnitude of B_2, determined at 15^{o}C in uncrowded solutions, than at 37^{o}C or in the presence of Ficoll, the conditions at which the behaviors depicted in Figure 2F were recorded. Higher temperature destabilizes the conformation of the ordered p53 domains, which increases the protein hydrophobicity and its contribution to intermolecular attraction. Similarly, the enhanced aggregation propensity in crowded solutions is equivalent to effective attraction. Both factors lead to significantly greater magnitude of B_2. Determinations of B_2 in the presence of Ficoll or at 37^{o}C were obstructed by the aggregation of p53 (Figures 1C and 2A). The intercepts of the straight lines in Figure 2F are 2.33 and 1.85, suggesting that ψ_2 is lower than μ_1^0 by ca. 5 kJ mol^{-1}. The higher stability of the anomalous liquid is consistent with the high fraction of protein captured in it.

To test whether Ficoll infiltrates the anomalous liquid condensates, we examined by OIM solutions of fluorescently-labeled Ficoll with and without p53. We observed that p53 condensates induced by crowders also occur in solutions containing fluorescently-labeled Ficoll (Figure 2H). Moreover, some of these p53 condensates exhibit localized fluorescence (Figure 2I). Because solutions of Ficoll free of p53 are uniformly bright (Figure 2G), this result affirms that Ficoll is captured in some of the p53 condensates.

The behaviors of the p53 condensates in both crowded and uncrowded solutions conform to the predictions of the model of anomalous protein liquid. In application to p53, this model implies that the condensates represent p53 species diffusing towards the condensate core, where they associate into transient misassembled oligomers. In turn, the misassembled oligomers diffuse out of the condensates and decay into stable p53 species. These dynamics are illustrated in Figure 2J.

Maturation of liquid condensates by Ostwald ripening

The surface between two phases accrues excess free energy due to the disparate coordination numbers and degrees of freedom of the molecules in the respective phases (Gibbs, 1876; Gibbs, 1993). The surface free energy may dominate the behaviors of phases consisting of multiple nanoscale domains, such as the anomalous liquid revealed in Figures 1 and 2, in which the molecules at the interface represent a significant fraction of the total phase mass. To establish whether the surface free energy of the condensate regulates its characteristics, we monitored the evolution of the condensate population during incubation at 15°C in solutions crowded with Ficoll (Figure 3A). We observed that the condensate radius R increased as a power law of time, $t^{0.30\pm0.10}$ (Figure 3B), whereas the number of condensates N decreased roughly proportionally to the elapsed time (Figure 3C). To exclude that the evolutions of R and N are due to condensate transformation into amyloid fibrils, we characterized the structural integrity of the protein in the anomalous liquid using the ANS assay. The ANS fluorescence of p53 solutions without or with Ficoll exhibited constant intensity over four hours (Figure 3C inset) and the average value

(0.1–0.12) was less than 15% of that of fibrillar aggregates. This finding confirms that p53 is stable at low temperatures, in accord with the stability observed using DLS (Figure 1F and G), and that Ficoll does not destabilize the p53 conformation.

The evolutions of the p53 condensates size and number (Figure 3B and C) are a signature of Ostwald ripening of the domains of a disperse phase close to equilibrium with its environment (Lifshitz and Slyozov, 1961). Ostwald ripening is driven by the higher stability of larger domains due to the lower relative contribution of the surface free energy. The less stable smaller domains discharge molecules that diffuse and associate to larger condensates (Figure 3D).

p53 fibrils nucleate within p53 liquid condensates

Formation of amyloid fibrils of wild-type and mutant p53 is a distinguishing behavior of this protein *in vivo* and *in vitro* (Ano Bom et al., 2012; Wang and Fersht, 2015; Xu et al., 2011). To probe p53 fibrillation, we incubated p53 solutions at 37°C without and with Ficoll and tested the fibrillar nature of the assembled aggregates using Thioflavin T (ThT). The fluorescence emission of ThT at 488 nm considerably increases upon binding to β-sheet stacks common in amyloid fibrils. Micrographs of p53 aggregates without (Figure 4A) or with (Figure 4B) Ficoll revealed that p53 forms fibrillar structures at 37 °C independent of presence of crowder.

To test whether the fibrillar structures incorporate Ficoll, we incubated fluorescently-labeled Ficoll with p53 at 37 °C for ca. 6 hours, until aggregation was complete. The micrograph of p53-free solution (Figure 4C) displayed homogeneous fluorescence, whereas Ficoll/p53 mixtures exhibited significant heterogeneity of the fluorescent label (Figure 4D) demonstrating that, similar to anomalous p53 liquid condensates, these fibrillar aggregates contain Ficoll. To test the coexistence of anomalous liquid and aggregates in the fibrillar structures, we probed whether the p53 fibrils display constant solubility (Figure 4E). Unlike amyloid fibrils, which exhibit a constant concentration in equilibrium with a fibril polymorph (Qiang et al., 2013), the final p53

concentration after fibrillation increased monotonically with the initial concentration (Figure 4E). A quasi-exponential increase of the captured p53 amount with higher p53 concentration (Figures 1I and 2D) is an intrinsic property of the anomalous p53 liquid. Thus, the variable solution concentration at equilibrium with the fibrillar structures implies that they represent a co-condensate of amyloid fibrils and anomalous liquid.

The coexistence of anomalous liquid and fibrils suggests that p53 fibrils may nucleate within p53 liquid condensates. To test this hypothesis, we investigated the effect of Ficoll on the kinetics of fibrillation of p53. Time-dependent ANS fluorescence revealed that p53 solution lacking crowders exhibited a lag phase, corresponding to fibril nucleation, of ca. 40 min (Figure 4F), somewhat longer than previously reported (Ano Bom et al., 2012; Wang and Fersht, 2015; Wilcken et al., 2012). We tentatively attribute this extension to higher pH, employed here, and the avoidance of Zn^{2+}-binding buffer components, which stabilizes the structure and delays fibrillation. Surprisingly, p53 solutions containing Ficoll evinced significantly longer lag phases (Figure 4F). This observation is counterintuitive since the excluded volume effects of Ficoll and the associated boost of the p53 chemical potential μ_1 would spur fibril nucleation (Ellis and Minton, 2003). The faster fibril growth in crowded solutions, manifest as steeper gain of ANS fluorescence after the lag phase (Figure 4F), concurs with crowding-induced μ_1 increase. Suppressed nucleation in the presence of Ficoll coheres with nuclei assembly hosted within the anomalous liquid condensates. The Ficoll entrapped in the anomalous liquid (Figure 2H – J) may hinder the motions of the p53 molecules *en route* to a nucleus. Notably, the observed delay in fibril nucleation due to the segregation of Ficoll in the liquid may be specific to the selected crowder. Within this scenario, the accelerated fibril growth in the presence of Ficoll suggests that after nucleation, the fibrils emerge from the liquid condensates and grow in the p53 solution. The mechanism of non-classical fibril nucleation assisted by anomalous p53 liquid, followed by classical growth, is illustrated in Figure 4G.

DISCUSSION

The anomalous p53 liquid condensates identified here share several characteristics with the dense liquids observed with other proteins (Shin and Brangwynne, 2017; Uversky, 2017; Wei et al., 2017). Their formation is reversible and they are in equilibrium with the solution. They form at solution concentrations as low as 1 μM and capture up to 80% of the protein mass; both parameters are lower by ca. 1000-fold than with anomalous liquid condensates of folded proteins (Gliko et al., 2005; Schubert et al., 2017; Sleutel and Van Driessche, 2014; Vorontsova et al., 2015a; Yamazaki et al., 2017). The presence of large disordered regions in the structure is viewed as a prerequisite for conventional liquid condensation at micromolar concentrations (Feric et al., 2016; Shin and Brangwynne, 2017; Uversky, 2017; Wei et al., 2017). In analogy, we conjecture that the low p53 concentration driving anomalous condensation and the large p53 fraction captured in the anomalous condensates are due to the disordered transactivation and proline-rich regions of p53.

Importantly, the anomalous liquid condensates of p53 are distinct from dense liquid droplets. The defining features of the anomalous condensates are the independence of their size on the solution thermodynamic parameters, including the p53 concentration, and the variable solution concentration at equilibrium.

During maturation, the size of the anomalous liquid condensates evolves following a ca. 1/3 exponent with time, a signature of Ostwald ripening (Lifshitz and Slyozov, 1961). Maturation by Ostwald ripening has been demonstrated for both anomalous and regular protein dense liquid condensates (Li et al., 2012; Shah et al., 2004). The condensate growth law following a ca. 1/3 exponent with time signifies that the fractal dimension of the p53 dense liquid is close to three (Streitenberger and Förster, 1991), i.e., the condensates are compact objects and not loose molecular assemblies. Furthermore, the finding of condensate maturation by Ostwald ripening highlights the significance of surface free energy for the condensate behaviors.

The fast formation of anomalous liquid condensates and their reversibility may underlie a potential role of the anomalous condensates as loci for storage and protection of p53 (Saad et al., 2017). The condensates may represent a fibril-independent pathway to oncogenicity: unidentified pre-nuclear aggregates of two p53 mutants (R282W and R100P) were shown to accumulate the tumor suppressors p63 and p73 (Xu et al., 2011). In this respect, the p53 liquid condensates may be akin to other protofibrillar assemblies known to trigger disease (Lansbury and Lashuel, 2006).

Similar to other amyloid structures, the formation of p53 fibrils starts with nucleation, whereby local fluctuations of the p53 concentration result in a region of concentrated and ordered p53 molecules with sufficiently unfolded DNA-binding domains to allow the assembly of a stacked β-sheet array; this array serves as nucleus for the growth of a fibril (Kashchiev et al., 2013). The creation of a nucleus encounters significant free energy barriers due to the emergence of a surface dividing the fibril from the solution (Gibbs, 1876, 1878; Gibbs, 1993; Kashchiev, 2000). Hence, successful nucleation events are extremely rare. Recent advances in understanding protein phase transitions have demonstrated that nucleation is dramatically faster if the formation of a concentrated protein region precedes ordering (ten Wolde and Frenkel, 1997); this conclusion has been cast as the two-step nucleation mechanism (Kashchiev et al., 2005; Pan et al., 2005; Vekilov, 2010; Vorontsova et al., 2015b). The anomalous protein liquid condensates have been identified as regions of high protein concentration that serve as precursors and facilitators of ordering into structured nuclei of crystals and sickle hemoglobin polymers (Galkin et al., 2007; Sleutel and Van Driessche, 2014; Uzunova et al., 2010; Yamazaki et al., 2017). Theoretical analyses predict that a similar mechanism would apply to the nucleation of amyloid fibrils (Saric et al., 2014); experimental evidence is scarce (Krishnan and Lindquist, 2005; Lansbury and Lashuel, 2006). Careful tests should examine the validity of the two-step nucleation scenario for each protein forming amyloid structures of interest (Auer et al., 2012).

Nucleation of p53 amyloid fibrils within anomalous p53 liquid suggests resolutions to several mysteries of p53 aggregation kinetics. The uncommon independence of the nucleation rate constants of p53 concentration (Wang and Fersht, 2017; Wilcken et al., 2012) may be due to nucleation housed in anomalous liquid, in which the p53 concentration may be constant. The contradictory results on cross-seeding of p63 and p73 aggregation with p53 fibrils (Ano Bom et al., 2012; Wang and Fersht, 2015; Xu et al., 2011) may be due to coexistence of fibrils and anomalous liquid and the distinct proportions that each aggregate class occupies in independently prepared seeds.

The strong dependence of the volume of the anomalous liquid phase on the p53 concentration suggests that the anomalous condensates could be sensitively controlled by modifying the concentration of free p53, for instance, by the negative regulators MDM2 and MDMX. MDM2 and MDMX cooperate to ubiquitinate p53, resulting in nuclear export and proteasomal degradation in the cytosol (Wade et al., 2013). MDMX inhibits p53 directly, by binding to its transactivation domain of p53 (Popowicz et al., 2008). The consequences of ubiquitination and nuclear export on liquid condensation are modeled by lowering the concentration of p53. In addition, MDMX binding to the disordered TAD region induces folding (Joerger and Fersht, 2016). Since the presence of large disordered regions in the p53 structure may be a prerequisite for anomalous liquid condensation at micromolar concentrations, it is feasible that that MDMX may strongly suppress anomalous liquid condensation.

Cancer-associated p53 mutations are concentrated in the DNA-binding domain (Joerger and Fersht, 2008). They are broadly subdivided into contact mutations that remove essential DNA interaction sites and structural mutations that perturb the structure of the ordered DNA-binding domain (DBD), thereby reducing its kinetic and thermodynamic stability (Joerger and Fersht, 2008, 2016). Whereas the enhanced aggregation of the structurally destabilized mutants and the associated oncogenic GOF has been supported by numerous experiments *in vitro* and in

cell culture(Costa et al., 2016; Freed-Pastor et al., 2012; Joerger and Fersht, 2008, 2016; Rajagopalan et al., 2011; Wang and Fersht, 2017; Wilcken et al., 2012; Xu et al., 2011), the contact mutations R273H, R273C, R248W, which preserve the protein conformation, raise additional unsettled questions. The dominant-negative effects of R273H and R248W, where arginine at sites 273 and 248 is replaced with, respectively, histidine and tryptophan (Ano Bom et al., 2012; Joerger and Fersht, 2016), have been attributed to the formation of hybrid p53 tetramers with the wild-type allele (Xu et al., 2011), which lowers the concentration of active p53. No mechanism, however, has been proposed for the suppression of the p63 and p73 function and this void is hard to reconcile with the severity of cancers associated with the binding mutations (Seagle et al., 2015). The finding of anomalous liquid condensates that host fibril nucleation suggests that the enhanced aggregation of missense p53 mutants with preserved conformational stability may be due to enhanced condensate formation. Indeed, the amount of the anomalous liquid phase is governed by the intermolecular interactions in the solution, quantified as the second virial coefficient B_2. In turn, altering the charge of the protein by substituting a positive side chains with a neutral one significantly enhances intermolecular attraction and the magnitude of B_2.

In cells, p53 localizes in the nucleus, in which DNA, protein complexes, RNAs and multiple other types of biomolecules occupy up to 50% of the total volume. To mimic crowding, we employ Ficoll, a cross-linked polysaccharide. Whereas the fraction of the solution volume occupied by the crowder is representative of the intracellular environment, the distribution of the charges on the surface of the Ficoll molecules may diverge from that of densely charged molecules like DNA, RNA, and proteins. Hence, the observed delay in fibril nucleation due to the segregation of Ficoll in the condensates may be specific to the selected crowder. It helped to elucidate a crucial aspect of the fibril nucleation mechanism, but it may be immaterial to intracellular p53 aggregation.

We demonstrate a dramatic boost in anomalous liquid volume induced by crowding, which may signify a strong correlation between macromolecular crowding and the rates and degree of

p53 fibrillation *in vivo*. Such correlation provides a mechanistic link between the increased cancer frequently and the decreases in cellular and tissue hydration in the aged (Hatters et al., 2002; Popkin et al., 2010). As the loss of cellular water is equivalent to larger fraction of cellular volume occupied by macromolecules, our results suggest accelerated p53 aggregation with advancing age is due to the increased macromolecular crowding.

LIMITATIONS OF THE STUDY

Our study clearly demonstrates the presence of dense liquid condensates in solutions of p53. These condensates exhibit several anomalous properties that distinguish them from the typical protein condensates, such as crystals, amyloid fibrils, and dense liquids. The anomalous properties of the condensates observed in p53 solutions highlight their similarity to the mesoscopic clusters observed in solutions of several proteins and studied in depth in the last ten years owing to their role as precursors for the nucleation of ordered protein solids. Two behaviors distinguish the anomalous p53 condensates from the mesoscopic clusters: they form at concentrations lower by about 1000× and they capture a significant fraction of the protein in the solution. We tentatively attributed these behaviors to the large disordered region of p53, but this correlation needs to be theoretically and experimentally examined.

The evidence that the anomalous p53 condensates host the nucleation of p53 amyloid fibrils, while solid, is indirect. Direct imaging, perhaps by liquid-phase electron microscopy, could provide definitive data.

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AUTHOR CONTRIBUTIONS

A.B.K. and P.G.V conceived this work. J.C.C. and P.G.V. directed the research. Z.W. prepared the plasmid and expressed the protein. K.T and M.S.S. purified the protein. M.S.S. characterized p53 aggregation. M.S.S., P.G.V., and J.C.C. analyzed the data. A.B.K. and P.G.V. developed theory. P.G.V., M.S.S., and J.C.C. wrote the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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FIGURE TITLES AND LEGENDS

Figure 1. Anomalous p53 liquid condensates.

A. The structure of p53. The transactivation domain (TAD, 1-61), proline-rich region (PRR, 61-94), DNA binding domain (DBD, 94-292), and oligomerization domain (OD, 325-356) are colored as red, gold, purple, and green, respectively. TAD and PRR are intrinsically disordered; DBD and OD are structured. p53 is a homotetramer that readily decays to dimers.

B. Schematic of oblique illumination microscopy (OIM). A green laser illuminates a thin solution layer at an oblique angle. The light scattered by particles in the solution is collected by a microscope lens.

C. Representative OIM micrographs collected from a p53 solution with concentration C_0 = 2.2 μM after incubation for 20 minutes at each temperature. The observed volume is 5 × 80 × 120 μm^3 depth × height × width. Aggregates appear as cyan speckles.

D, E. The average radius R and the total number N, respectively, of the aggregates, determined by OIM from images as in **C**. The average of five determinations in distinct solution volumes is shown. Error bars indicate standard deviations. Temperature range where aggregation is not observed is shaded.

F. Intensity correlation functions g_2 of the light scattered by a filtered p53 solution with C_0 = 6 μM at 15°C acquired at different times.

G. Evolution of diffusivity D_m and dispersity $\mu\tau^2$ of unaggregated p53 evaluated from the g_2s in **F**. The error bars are generated from four distinct g_2s and are smaller than the symbols.

H, I. Concentration of p53 after incubation for 20 min at, respectively, 15°C and 37°C, as a function of the initial solution concentration C_0. △, the concentration of the p53 solution C_f after filtration to remove aggregates. ■, concentration C_2 of p53 incorporated into aggregates, evaluated as $C_2 = C_0 - C_f$.

J. Evolution of the intensity of fluorescence at 490 nm of 1-anilino-8-naphthalenesulfonate (ANS) in the presence of p53 at concentrations listed on the plot at 37°C. Green color denotes signal attributed to fibrils.

K. Intensity correlation functions g_2 of unfiltered p53 solutions. Solution at 1.5 μM was incubated at 37°C for 4 hours and serially diluted to 1.3 and 0.9 μM. Ten g_2s were averaged at each concentration and the error bars represent the standard deviation.

L. The dependence of anomalous liquid volume ϕ_2 and the average radius R of the condensates on the protein concentration evaluated from g_2s in **K**. The error bars are generated from four distinct g_2s and are smaller than the symbols for R. Horizontal line designates the average R = 145 nm.

Figure 2. p53 condensates in crowded solutions.

A. Representative OIM micrographs collected immediately after addition of crowders to a p53 solution with C_0 = 1.2 μM held for 20 min at T = 15°C. Ficoll concentration is listed in each image. The observed volume is 5 × 80 × 120 μm^3. Condensates appear as red spots.

B, C. The average radius of the condensates R as a function of Ficoll and p53 concentrations, respectively determined by OIM. The average of five determinations in distinct solution volumes is shown. Error bars indicate standard deviations. The high number of condensates prevents their accurate count by OIM.

D. Concentration of p53 after incubation for 20 min at 15°C in the presence of 56 mg ml^{-1} Ficoll as a function of the initial solution concentration C_0. △, the concentration of p53 solution C_f after filtration to remove condensates. ■, concentration of p53 incorporated into the anomalous liquid, evaluated as $C_2 = C_0 - C_f$.

E. The osmotic compressibility of the solution KC_0/R_θ (K, instrument constant; R_θ, Rayleigh ratio of the intensity of light scattered at angle $\theta = 90^o$ to that of incident light) as a function of C_0 at 15°C. The value of the second osmotic virial coefficient B_2 evaluated from the slope of the data is displayed.

F. The correlation between $\ln(C_2/C_f)$ and C_f in crowded solutions at 15°C (red) and uncrowded solutions at 37°C (blue) from data in, respectively, Figures 2D and 1I.

G. Fluorescence OIM image of 15 mg ml^{-1} BODIPY-labeled Ficoll. **H.** OIM micrograph of same solution after addition of 1.2 μM p53. **I.** Fluorescence OIM micrograph of same solution volume as in **H** taken within 1 min of **H**. Red circles in H and I highlight condensates containing BODIPY-labeled Ficoll.

J. Schematic of formation of anomalous p53 liquid condensates owing to the dynamics of formation, decay, and diffusion of transient misassembled oligomers, tentatively represented as pentamers that are hypothesized to form in the solution in addition to the long-lived monomers, dimers, and tetramers.

Figure 3. Maturation of the liquid condensates.

A. Representative OIM images collected after incubation times indicated in the images. The observed volume is 5 × 80 × 120 μm^3. Condensates appear as red spots. T = 15°C. C_0 = 1.2 μM. C_{Ficoll} = 56 mg ml^{-1}.

B, C. The average radius R and number density N, respectively, of the condensates as a function of time, corresponding to the images in **a**. The average of five determinations in distinct solution volumes is shown. Error bars indicate standard deviations. Inset: Normalized fluorescence emission at 500 nm of 7.7 μM p53 solution in the presence of 100 μM ANS and the indicated concentrations of Ficoll. T = 15°C. The emission intensity is normalized to the maximum value observed in presence of fibrillar aggregates.

D. Schematic of Ostwald ripening, whereby smaller condensates dissolve while larger condensates grow.

Figure 4. p53 fibrillation.

A, B. Representative fluorescence micrograph of p53 fibrils formed by incubation at 37°C over 12 hours decorated with Thioflavin T (ThT); **A,** with no Ficoll, and **B,** in the presence of 56 mg ml^{-1} Ficoll. The ThT and p53 concentrations are 200 μM and 7.7 μM, respectively.

C, D. Representative fluorescence micrographs of 56 mg ml^{-1} fluorescently-labeled Ficoll with no protein in **c**, and in presence of 7.7 μM p53 after incubation at 37°C over 12 hours in **D**.

E. Concentration of p53 after incubation at 37°C in the presence of 56 mg ml^{-1} Ficoll as a function of the initial solution concentration C_0. △, the concentration of p53 solution C_f after filtration to remove aggregates. ■, concentration C_2 of p53 incorporated into aggregates, evaluated as $C_2 = C_0 - C_f$.

F. Evolution of the intensity of fluorescence at 500 nm of ANS in the presence of p53 at Ficoll concentrations listed on the plot at 37°C. The p53 and ANS concentrations are 6.5 ± 0.5 and 200 μM, respectively.

G. Schematic of fibril nucleation within anomalous p53 liquid condensates, followed by growth via sequential association of monomers from the solution.

SUPPLEMENTAL VIDEOS

Video S1. Oblique illumination microscopy (OIM) monitoring of the condensates in a p53 solution with concentration C_0 = 2.2 μM after incubation for 20 minutes at 37^{o}C. Related to Figure 1. The size of a light spot is governed by by the deviation of the respective condensate from the image focal plane and is not related to the condensate size. A dedicated image processing algorithm traces the trajectories of the individual condensates. The mean squared displacement in the image plane is correlated to the elapsed time between images and the diffusion coefficient of each condensate is computed from the slope of the correlation. The size of each condensate is evaluated using its diffusion coefficient and the Stokes Einstein relation.

Video S2. Oblique illumination microscopy (OIM) monitoring of the condensates in a p53 solution with concentration C_0 = 1.2 μM after incubation for 20 minutes at 15^{o}C in the presence of 100 mg ml^{-1} Ficoll. Related to Figure 2.Note that solutions without crowder exhibit no condensates at this temperature.