Enzymes within the de novo purine biosynthetic pathway spatially organize into dynamic intracellular assemblies called purinosomes. The formation of purinosomes has been correlated with growth conditions resulting in high purine demand, and therefore, the cellular advantage of complexation has been hypothesized to enhance metabolite flux through the pathway. However, the properties of this cellular structure are unclear.

Here, we define the purinosome in a transient expression system as a biomolecular condensate using fluorescence microscopy. We show that purinosomes, as denoted by formylglycinamidine ribonucleotide synthase granules in purine-depleted HeLa cells, are spherical and appear to coalesce when two come into contact, all liquid-like characteristics that are consistent with previously reported condensates. We further explored the biophysical and biochemical means that drive the liquid–liquid phase separation of these structures. We found that the process of enzyme condensation into purinosomes is likely driven by the oligomeric state of the pathway enzymes and not a result of intrinsic disorder, the presence of low-complexity domains, the assistance of RNA scaffolds, or changes in intracellular pH. Finally, we demonstrate that the heat shock protein 90 KDa helps to regulate the physical changes in intracellular pH. Purinosomes, as denoted by for- mylglycinamidine ribonucleotide synthase (FGAMS). FGAMS transitions from a diffuse state into multienzyme assemblies called purinosomes upon changes in growth conditions that increase purine demand (5, 6). The purinosome is hypothesized to exist as a metabolon, a complex of sequential metabolic enzymes to enhance metabolite flux through a given pathway (7), and has been further substantiated by identifying intracellular hotspots of purine biosynthesis through mass spectral imaging (8). Components of the purinosome exist beyond the six enzymes in the biosynthetic pathway and include downstream enzymes and regulatory proteins such as molecular chaperones and kinases (6, 9, 10). The collective actions of these regulatory proteins are likely what governs the formation and, in part, the function of the complex. The function of the complex is also reliant on the availability of substrates and cofactors derived from mitochondrial processes (11), further supporting our conclusions that purinosomes and mitochondria have a symbiotic relationship (12, 13).

The compartmentalization of purine biosynthetic enzymes into these intracellular organizations has hinted that these bodies might be an example in the growing list of biomolecular condensates (e.g., nucleoli, P-bodies, stress granules, RNA transport granules). Biomolecular condensates are highly heterogeneous complexes that undergo a liquid–liquid phase separation (LLPS) driven by weak and transient interactions among their constitutive components, commonly referred to as multivalency (14–16). The multivalency can be achieved as a
product of interactions between folded protein domains, such as highly ordered oligomers, and/or self-associating intrinsically disordered regions (IDRs). With the advancements in fluorescence microscopy, we have greatly advanced our understanding of the biophysical properties of these membrane-less organelles. The process of LLPS has been observed within DNA repair (17–19), transmembrane signaling (20, 21), cytoskeleton formation (22, 23), ribonucleoprotein transport (24, 25), and more recently, metabolism (26). Changes in a cell’s microenvironment and intracellular needs often modify the attractive forces within condensates and impact their formation and stability. Therefore, identifying the mechanism that triggers and regulates the dynamic process might provide insights into their function and the overall cellular advantage of forming condensates over more traditional macromolecular complexes.

In this report, we build on our previous biophysical and biochemical characterizations of the purinosome in purine-depleted HeLa cells to demonstrate its ability to behave similar to other reported biomolecular condensates. Purine depletion of HeLa cells results in activation of the DNPB and has been correlated with purinosome formation (8, 10). We show that purinosomes exist as uniformly spherical intracellular assemblies and readily fuse with one another when they come in close contact. The mechanism that drives the LLPS is likely because of the oligomerization of pathway enzymes into higher ordered structures and not a consequence of intrinsic disorder. Last, we propose a mechanism by which regulation of the condensate’s liquid-like properties is achieved by the assistance of molecular chaperones. Together, these results help us further understand the nature of this assembly and provide further clues into how the purinosome is organized and regulated in cells.

Results

The purinosome demonstrates liquid-like properties in cells

To probe whether the purinosome exhibits liquid-like properties, we first sought out whether an enzyme concentration–dependent threshold exists that might promote an LLPS. For this, we used a modified HeLa Tet-Off cell line (HeTOFLI) that stably expresses LacI-NLS to allow for the IPTG induction of protein expression (27). These cells were transfected with an inducible construct encoding FGAMS-enhanced YFP (EYFP), and the induced expression was monitored over time by fluorescence microscopy (Figs. 1 and S1). Previously, we have used FGAMS as a marker to study the intracellular dynamics of the purinosome, and we infer that most assemblies of FGAMS under purine-depleted growth conditions likely also contain other enzymes in the DNPB pathway (5, 12, 13). At early time points, purine-depleted HeTOFLI cells expressing FGAMS-EYFP displayed a diffuse phenotype that transitioned into discrete granules as time progressed. Figure 1 shows the condensation of FGAMS-EYFP in a representative cell at 12 h post-IPTG induction. Intensity values across a defined region within the same cell before and after triggering condensation verified that the FGAMS-EYFP within the cell was being concentrated into these assemblies (Fig. 1B) further implying that the purinosome is capable of undergoing an LLPS.

Three properties are widely shared amongst commonly reported liquid-like condensates: the free exchange of molecules in and out of the condensate, the spherical nature of the protein assembly, and the ability of condensates to fuse when in close contact (15). We asked whether the purinosome exhibits these characteristics. Previously, enzymes within the DNPB pathway have been shown to freely exchange between the bulk solvent and the purinosome by fluorescence recovery after photobleaching (5, 28). The calculated diffusion coefficients for the various enzymes as they assemble into purinosomes suggest that the complex is formed through several multivalent interactions in a step-wise manner (28). We continued our investigation by exploring the spherical nature of purinosomes in purine-depleted HeLa cells by lattice light sheet microscopy (29). A characterization of 41 purinosomes demonstrated that those segmented FGAMS-mCherry assemblies were spherical with an average sphericity of 0.97 ± 0.03 (Figs. 2, A and B and S2). Last, we monitored the movement of purinosomes over a 5 min time course (one image every 5 s) using instantaneous structured illumination high-resolution fluorescence microscopy. In several instances, purinosomes, as denoted by FGAMS-enhanced GFP puncta, appear to merge with one another to form larger punctate structures. Figure 2 shows one such event in which purinosome 1 fuses with purinosome 2 at 365 s (Figs. 2, C–E and S3A, Movie S1). The area of the coalesced purinosome was roughly the sum of the two purinosomes combined (Fig. 2F). Together, these data provide overwhelming support that the purinosome is an example of a liquid condensate, and the process of enzyme condensation is likely a regulatory mechanism leveraged in purine metabolism.

The oligomeric state of the purinosome contributes to its condensation

The multivalency observed in biomolecular condensates can be induced through a variety of mechanisms including protein oligomerization and self-assembly via IDRs or nucleic acid scaffolds. Physicochemical features, as identified by amino acid sequence, within proteins shown to phase transition have given insight into other proteins within the human proteome that might also drive biomolecular condensate formation by self-assembly. A classical characteristic that differentiates a protein that phase transitions from one that does not is the presence of long IDRs and/or low-complexity domains (15, 30–32). Therefore, bioinformatic analyses were conducted to predict the likelihood that any of the enzymes in the DNPB pathway have such propensity to drive the observed LLPS. Using the IUPred2A predictor (33, 34) and the Predictor of Natural Disordered Regions (PONDR-VSL2) (35) algorithms, all enzymes did not display long stretches (>30 amino acids) of intrinsic disorder (Fig. S4). In addition, we also queried whether any of these enzymes had stretches of low complexity using the SEG algorithm (36).
Low-complexity domains were identified in all enzymes except for phosphoribosylaminoimidazole succinocarboxamide synthetase (PAICS) and aminoimidazole carboxamide ribonucleotide transformylase (ATIC) (Fig. S4); however, the length in any of these regions is relatively very short compared with reported phase-separated proteins (37, 38).

We also investigated the tendency for the enzymes in the DNPB pathway to aggregate using the TANGO (39) and Waltz algorithms (40). The TANGO algorithm accounts for the general physicochemical determinants of protein β-aggregation, whereas Waltz algorithm identifies sequences that promote amyloid structures over amorphous aggregates. Results from the TANGO algorithm predicted that all the pathway enzymes have some degree of aggregation as noted by more than five consecutive amino acids with an aggregation tendency greater than 5% (Fig. S5). The purinosome core proteins (phosphoribosyl pyrophosphate amidotransferase [PPAT], glycaminide ribonucleotide transformylase [GART], and FGAMS) were more aggregation prone, and several of these stretches also predicted to contribute to the formation of amyloid-like assemblies by the Waltz algorithm (Fig. S5). These results are consistent with the overall tendency of globular proteins to have a high degree of β-aggregation nucleating regions when compared with intrinsically disordered proteins (41); however, this does necessarily suggest that these proteins form aggregates. These identified stretches likely are critical for the overall proper folding, stability, and activity of the protein.

Extended nutrient starvation of human cells has been known to induce a stress response to inhibit protein translation and trigger the phase separation of proteins into stress granules (42). We have long prescribed that this is not the case with the purinosome because of its lack of association with the stress granule–nucleating protein G3BP1 (Ras-GAP SH3 binding protein 1) and to differences in their defining characteristics with the purinosome (6, 9, 11, 43). Recent work has suggested that a stress granule might not be comprised of all the traditional protein markers and that the composition is dependent on the type and duration of the stress (44). Therefore, we asked whether poly(A)^+ mRNA is present...
within the purinosome suggesting that these condensates are part of the RNA granule family void of G3BP1. Using fluorescence in situ hybridization, the colocalization of poly(A)$^+$ mRNAs with purinosomes was assessed. Consistent with this body being distinct from stress granules and P-bodies, no colocalization was observed (Fig. S6A). We also investigated...
whether the purinosome might be a result of nutrient-deprived stalled translational initiation complexes, a known phenomenon giving rise to early stage stress granule formation (42). Purine-depleted HeLa cells were grown in the presence or the absence of purines, and the repression of translation analyzed by the induction of Ser51 phosphorylation on eukaryotic translation initiation factor 2α. Using a 1 h arsenite treatment as a positive control, we show that purine-depleted HeLa cells do not have repressed translation and are consistent with HeLa cells grown in the presence of purines (Fig. S6B). We also investigated whether overexpression of FGAMS by transient transfection might repress translation. Similar to the untransfected HeLa cells, no increase in the phosphorylation of eukaryotic translation initiation factor 2α was observed (Fig. S6B). Together, all these data would suggest that despite the chronic starvation of HeLa cells for purines, the purinosome is not an example of a stress granule and that condensate formation is not driven by the presence of an RNA scaffold.

Nutrient starvation can also lead to changes in intracellular pH previously shown to promote an LLPS in eukaryotes (45–47). To assess whether the pH is different in HeLa cells under purinosome-forming (purine-depleted) growth conditions, we used the pH-sensitive fluorescent dye, cSNARF-1 (48). HeLa cells co-stained with cSNARF-1 and Hoechst 33342 were imaged by confocal microscopy. Background-subtracted ratios of non-nuclear cSNARF-1 emissions between 561 to 598 nm and 620 to 753 nm were converted to pH values after establishment of a calibration curve (Fig. S7, A and B) using the nigericin method (49, 50). Intracellular pH values for normal and purine-depleted HeLa cells were not significantly different at 7.61 ± 0.10 and 7.66 ± 0.07, respectively (Fig. S7, C and D). Therefore, we conclude that pH is likely not a factor in the formation of the purinosome condensate.

Our investigations to date do not point to a self-assembly mechanism that drives purinosome formation. Therefore, we hypothesize that the multivalency could be achieved through the oligomerization of the given pathway enzymes. Within the DNPB pathway, PAICS is an octamer, PPAT and adenylo-succinate lyase (ADSL) exist between dimeric and tetrameric states, and GART and ATIC are functional dimers (6). A recent study showing the purinosome in neurons suggested that PAICS might act as a scaffold for the purinosome (51). Other studies have suggested that perturbing the oligomeric structure and/or composition of the purinosome impacts its formation. First, purine-depleted HeLa cells deficient in the expression of one pathway enzyme showed a reduction (FGAMS and PAICS) or a complete loss in purinosome content compared with wildtype cells (52). Second, disruption of the active ATIC homodimer with a cyclic peptide-derived inhibitor, compound 14, resulted in a loss of hypoxia-induced purinosome formation (11). Therefore, the increased weak and transient interactions between enzymes in the pathway could drive a thermodynamically favorable process of an LLPS and promote a unique composition. Given these findings, it is plausible that higher order macromolecular structures, such as metabolons, can emerge solely between pathway enzymes that induce the phase transition.

Molecular chaperones help regulate the purinosomes’ biophysical properties

The process of condensation proceeds along a continuum ranging from LLPS driven by weak, transient, and reversible interactions to stronger and irreversible interactions such as those observed in insoluble aggregates and polymeric structures. The regulation of condensate maturation has been proposed to be controlled through the inclusion of modifying enzymes (53), such as kinases (54, 55) and heat shock proteins (HSPs) (56–59).

Previously, we have demonstrated a role for molecular chaperones in the formation of the purinosome (9, 60). Molecular chaperones, HSP 90 KDa (HSP90) and HSP70, colocalized with purinosomes in purine-depleted HeLa cells (9), and the biochemical interactions between HSP90 and pathway enzymes, PPAT and FGAMS, were no longer detected upon the inhibition of HSP90 activity (60). Given this, we explored whether HSP90 might help preserve the liquid-like state of purinosomes by acting as a modifying enzyme for the condensate. We first asked whether HSP90 inhibition can artificially induce the formation of intracellular FGAMS granules in the absence of purinosome-forming growth conditions. For these studies, we used STA9090 (ganetesib), an inhibitor of HSP90 activity, at a concentration not shown to significantly perturb HeLa cell viability (Fig. S8). HeLa cells transiently expressing FGAMS-mCherry were treated with STA9090, and the number of cells displaying intracellular FGAMS granules was monitored over a 2 h period. After 2 h of exposure, a majority of all transfected cells showed the emergence of FGAMS granules compared with transfected cells treated with dimethyl sulfoxide (Fig. 3, A and B). The emergence of granular FGAMS structures was also observed in purine-depleted HeLa cells suggesting that the impact that HSP90 inhibition has on FGAMS is independent of purine levels (Fig. S9). Using instantaneous structured illumination high-resolution fluorescence microscopy, we were able to show that roughly half of the FGAMS clusters in purine-depleted HeLa cells became less circular after a 2 h treatment with STA9090. This emergent subpopulation of FGAMS containing assemblies was distinct from the highly circular purinosomes (Fig. 3C).

We also investigated whether ADSL, another pathway enzyme not previously shown to be dependent on HSP90 (60), also formed granules upon HSP90 inhibition. During the 2 h study, HeLa cells transiently expressing mTurquoise2-ADSL did not have a tendency to form granular structures upon STA9090 treatment (Figs. 3, D and E and S9). We next asked whether the STA9090-induced FGAMS granules would result in the formation of purinosome-like structures as evident in the coclustering of ADSL granules. In this study, we transiently overexpressed FGAMS-mCherry with mTurquoise2-ADSL in purine-depleted HeLa cells and investigated whether upon HSP90 inhibition, the association of FGAMS and ADSL seen
in purinosomes was ablated. Consistent with previous observations, HSP90 inhibition resulted in the formation of FGAMS clusters; however, the diffuse ADSL phenotype persisted (Fig. 3F). These results demonstrate that inhibiting HSP90 activity results in the formation of FGAMS-only assemblies with a morphology distinct from that of purinosome condensates and suggests that HSP90 likely assists in regulating its liquid-like properties.

Discussion

Emerging evidence across many metabolic processes suggests that enzymes within a given pathway condense and undergo a phase transition to maintain cellular homeostasis (26). With this study, we present further evidence supporting the notion that the purinosome, as denoted by discrete FGAMS assemblies under purine-depleted growth conditions, behaves like a classically defined biomolecular condensate with liquid-like properties in a transient expression system.

We showed that the condensation of FGAMS was concentration dependent; however, we do not fully understand whether there are size restrictions to retain the liquid-like properties of the purinosome. We can hypothesize that the size of a functional purinosome is limited based on the expression of pathway enzymes, their stoichiometric makeup, and their subcellular localization. Calculations representing an equimolar distribution of pathway enzymes suggest that purinosome sizes likely will not exceed 300 nm in diameter (61). In addition, the subcellular localization might restrict its overall size. In this study, we observed the directed motion of purinosomes (consistent with their movement along microtubules) until they merge with a stationary purinosome (Fig. S3, B–F). Based on our previous characterizations of purinosome motion and subcellular localization (12), we prescribe those stationary purinosomes are at the mitochondria–microtubule interface. This interface might limit the size a purinosome can be in order to retain its function. Investigations into the size requirements and composition of the purinosome are ongoing.

We have only started to understand the biochemical mechanisms that govern purinosome formation and its stability. This study highlighted HSP90 as a way to sustain the properties of the condensate. Inhibition of HSP90 activity altered the properties of the purinosome driven likely by an increase in misfolded FGAMS protein within the condensate.
This accumulation of the misfolded protein created complexes that diminished the available contacts needed with other enzymes in the DNPB pathway. Therefore, the inclusion of this regulatory protein in the condensate could limit the number of possible transient interactions and influence the overall stoichiometry of the purinosome simply by modulating the folded state of FGAMS. Recently, a chemical affinity strategy using immobilized PU-H71, an inhibitor of HSP90, was shown to coisolate a majority of the enzymes within the DNPB pathway with HSP90 (62). This study is consistent with our findings and suggests that HSP90 might serve as a core scaffold (Fig. 4).

This study does not rule out the possibility that the purinosome is a multiphasic condensate as shown in ATPase-modulated stress granules (63). We can conceive that chaperoning is imperative and isolated to a substructure within the purinosome (Fig. 4). Based on the diffusion coefficients and protein proximity studies, the first three enzymes in the pathway (PPAT, GART, and FGAMS) have been assigned to act as the core of the purinosome (28, 64). Interestingly, two of these three proteins are also under regulation by HSP90 (60). The proposed stepwise assembly of the purinosome supports this hypothesis as once the HSP90 clients adopt a conformation for purinosome formation, they are able to move to a second compartment where the other enzymes are recruited to adopt a unique stoichiometry and facilitate substrate channeling.

This study begs to ask if the purinosome exists as a condensate outside a transient overexpression system. Recent platforms studying the purinosome with endogenous proteins indicate that the number of functional purinosomes per purine-depleted HeLa cell is approximately 10-fold lower than those inferred from overexpressed systems (8, 11). This discrepancy points to the overexpression of pathway enzymes promoting subcellular organizations void of specific components, and therefore, not contributing fully to the observed substrate channeling and enhanced purine production. Yet, at endogenous levels, not currently amendable to live cell fluorescence imaging, the enzymes still appear to cluster into purinosome metabolons suggesting that a condensate is still likely present (8, 11, 52). The focus for subsequent studies is to characterize how the activity of the purinosome condensate is regulated on the endogenous level, possibly through its post-translational modifications, after various pathway enzymes assembly within the condensate.

**Experimental procedures**

Full experimental procedures including cell culture, fluorescence microscopy, data analysis (Figs. S1–S9) are provided in the supporting information.

**Data availability**

The data supporting the findings of this study are available within the article and supporting information files. Additional data files underpinning this study are available upon request.

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**Supporting information**—This article contains supporting information (5, 12, 27, 29, 33–36, 39, 40, 43, 49, 50, 60, 65–68).

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Abbreviations—The abbreviations used are: ADSL, adenosuccinate lyase; ATIC, aminomimidazole carboxamide ribonucleotide transformylase; DNBP, de novo purine biosynthetic; ETTP, enhanced YFP; FGAMS, formylglycinamidine ribonucleotide synthetase; GART, glycinamide ribonucleotide transformylase; HeTO-FLI, HeLa Tet-Off cell line; HSP, heat shock protein; IDR, intrinsically disordered region; LLPS, liquid–liquid phase separation; PAICS, phosphoribosylaminoimidazole succinocarboxamide synthetase; PPAT, phosphoribosyl pyrophosphate amidotransferase.

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